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Prem L. Bhalla
Mohan B. Singh
Editors

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Edited by

Prem L. Bhalla and Mohan B. Singh

Plant Molecular Biology and Biotechnology Laboratory, School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne Parkville, VIC, Australia

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Editors
Prem L. Bhalla
Plant Molecular Biology and Biotechnology
Laboratory, School of Agriculture
and Food, Faculty of Veterinary
and Agricultural Sciences
The University of Melbourne
Parkville, VIC, Australia

Mohan B. Singh
Plant Molecular Biology and Biotechnology
Laboratory, School of Agriculture
and Food, Faculty of Veterinary
and Agricultural Sciences
The University of Melbourne
Parkville, VIC, Australia

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Preface

Wheat has long been integral to human civilization. Humans have consumed wheat for thousands of years. The present-day bread wheat is a polyploid crop consisting of three genomes believed to have originated around 10,000 BC. Wheat is one of the major staple food crops grown globally; it provides a significant part (around 50%) of calories for humans. Wheat grains are rich in carbohydrates, proteins, essential vitamins, calcium, iron, fiber, and other micronutrients important for general good health. Moreover, the wheat grain protein, gluten, is also used in the pharmaceutical and paper-making industries. The carbohydrate-rich vegetative parts of a wheat plant can also be used for bio-fuel production. China, India, and the USA are currently the leading wheat-producing countries, followed by the former USSR, France, and Canada.

The green revolution that resulted from high-yielding dwarf wheat and rice varieties turned countries like India from the brink of mass famine to a net exporter of wheat. Wheat is the primary crop grown globally, occupying the dominant position in world grain trade.

Our society is facing multidimensional challenges from climate change and population growth. To meet our future demands, we need to grow more food on the same area of arable land; however, the land available for cultivation is decreasing due to urbanization and other nonagricultural uses. Moreover, food production and distribution are severely affected by unforeseen climatic conditions. Limited water for crop irrigation and high energy costs put an extra strain on food production.

Global wheat production has been declining, thus raising concerns about food security. Worldwide wheat production in 2017 has been forecasted to reach 744.5 million tons by FAO, indicating a reduction from 2016. Moreover, there is a variation in wheat production from year to year, which adds uncertainty. Therefore, a major goal is to develop knowledge and technology in order to breed wheat varieties that can show yield stability in varying environmental conditions.

Conventional plant breeding is limited by the available gene pool, which cannot sustain the growth in wheat production and meet the growing demand. Recent progress in molecular biology, including recombinant methods, has the potential to breed future high-yielding wheat varieties that require less fertilizer and herbicides and are disease and stress tolerant.

The aim of *Wheat Biotechnology: Methods and Protocols* is to provide a background on recent new technology developments highlighting the potential of the genomic era in wheat breeding with detailed instruction on the methodology, which is complemented by overview chapters on the status of new technology application in major wheat producing countries. The protocol chapters contain an introduction to bring the reader up to speed on the literature, followed by detailed step-by-step instructions for applying these techniques and methods, and notes on troubleshooting. The topics cover methods underpinning the latest developments in the field of wheat biotechnology. The chapters are written by internationally renowned scientists who are actively working on cutting-edge technology for

wheat. This book is directed at individuals in academia and industry alike and forms a valuable resource for educators and beginners in the area of applying biotechnological techniques in wheat breeding.

Parkville, VIC, Australia *Prem L. Bhalla*
Mohan B. Singh

Contents

Contributors

Alexandra M. Allen • *School of Biological Sciences, University of Bristol, Bristol, UK*

Nii O. Ankrah • *Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA; School of Biological Sciences, Washington State University, Pullman, WA, USA*

Khalid Anwar • *Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, Delhi, India*

Naveen Arora • *Allergy and Immunology Section, CSIR—Institute of Genomics and Integrative Biology, Delhi, India*

Gary L. A. Barker • *School of Biological Sciences, University of Bristol, Bristol, UK*

Pierre Barret • *INRA, UMR GDEC, Genetic, Diversity and Ecophysiology of Cereals, Clermont Ferrand, France*

Prem L. Bhalla • *Plant Molecular Biology and Biotechnology Laboratory, School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC, Australia*

Antonio Blanco • *Department of Soil, Plant and Food Sciences, University of Bari "Aldo Moro", Bari, Italy*

Rhoda A. T. Brew-Appiah • *Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA*

Amanda J. Burridge • *School of Biological Sciences, University of Bristol, Bristol, UK*

Tomáš Čermák • *Department of Genetics, Cell Biology and Development and Center for Genome Engineering, University of Minnesota, Minneapolis, MN, USA*

Chon-Kit Kenneth Chan • *School of Plant Biology and Institute of Agriculture, University of Western Australia, Crawley, WA, Australia*

Junli Chang • *The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China*

Harsh Chauhan • *Indian Institute of Technology Roorkee, Roorkee, India*

Jane Coghill • *School of Biological Sciences, University of Bristol, Bristol, UK*

Shaun J. Curtin • *Cereal Disease Laboratory, Agricultural Research Service, United States Department of Agriculture, St Paul, MN, USA*

Priyanka Das • *Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, Delhi, India*

David Edwards • *School of Plant Biology and Institute of Agriculture, University of Western Australia, Crawley, WA, Australia*

Keith J. Edwards • *School of Biological Sciences, University of Bristol, Bristol, UK*

John Fellers • *Department of Plant Pathology, USDA-ARS-HWWGRU, Manhattan, KS, USA*

Agata Gadaleta • *Department of Environmental and Territorial Sciences, University of Bari "Aldo Moro", Bari, Italy*

Caixia Gao • *State Key Laboratory of Plant Cell and Chromosome Engineering, and Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*

Angelica Giancaspro • *Department of Environmental and Territorial Sciences, University of Bari "Aldo Moro", Bari, Italy*
Sonia Goel • *National Research Centre on Plant Biotechnology, Indian Council of Agricultural Research, New Delhi, India*
Scott Haley • *Department of Soil and Crop Science, Colorado State University, Fort Collins, CO, USA*
Jiapeng Han • *The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China*
Guangyuan He • *The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China*
Goetz Hensel • *Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Seeland/OT Gatersleben, Germany*
Rohit Joshi • *Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, Delhi, India*
Jagdeep Kaur • *Donald Danforth Plant Science Center, Saint Louis, MO, USA*
Paramjit Khurana • *Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi, India*
Jochen Kumlehn • *Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Seeland/OT Gatersleben, Germany*
Weiguo Liu • *Syngenta, Research Triangle Park, Cary, NC, USA*
Bill Malcolm • *School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC, Australia*
Cornelia Marthe • *Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Seeland/OT Gatersleben, Germany*
Ankita Mishra • *Allergy and Immunology Section, CSIR—Institute of Genomics and Integrative Biology, Delhi, India*
Ashwani Pareek • *Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, Delhi, India*
Sheng Quan • *National Center for Molecular Characterization of Genetically Modified Organisms, SJTU-Bor Luh Food Safety Center, School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai, China*
Agnès E. Ricroch • *Evolutionary Genetics and Plant Breeding, AgroParisTech, Paris, Cedex 05, France; Durability, Innovation, Resources and Ethics, University Paris-Sud, Paris, France; College of Interdisciplinary Studies, Université Paris-Saclay, Paris, France*
Sachin Rustgi • *Department of Plant and Environmental Sciences, Clemson University Pee Dee Research and Education Center, Florence, SC, USA; Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA*
Meenakshi Santra • *Department of Soil and Crop Science, Colorado State University, Fort Collins, CO, USA*
Armin Scheben • *School of Plant Biology and Institute of Agriculture, University of Western Australia, Crawley, WA, Australia*
Scott Seifert • *Department of Soil and Crop Science, Colorado State University, Fort Collins, CO, USA*
Dilip Shah • *Donald Danforth Plant Science Center, Saint Louis, MO, USA*

Akanksha Sharma • *Plant Molecular Biology and Biotechnology Laboratory, School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC, Australia*

Mohan B. Singh • *Plant Molecular Biology and Biotechnology Laboratory, School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC, Australia*

Kalpana Singh • *National Research Centre on Plant Biotechnology, Indian Council of Agricultural Research, New Delhi, India*

N. K. Singh • *National Research Centre on Plant Biotechnology, Indian Council of Agricultural Research, New Delhi, India*

Sneh L. Singla-Pareek • *Plant Stress Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, Delhi, India*

Yue Sun • *Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA*

Caroline Tassy • *INRA, UMR GDEC, Genetic, Diversity and Ecophysiology of Cereals, Clermont Ferrand, France*

Yanpeng Wang • *State Key Laboratory of Plant Cell and Chromosome Engineering, and Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*

Hong Wang • *Department of Soil and Crop Science, Colorado State University, Fort Collins, CO, USA*

Christy Waterfall • *School of Biological Sciences, University of Bristol, Bristol, UK*

Diter von Wettstein • *Department of Crop and Soil Sciences, Washington State University, Pullman, WA, USA*

Paul A. Wilkinson • *School of Biological Sciences, University of Bristol, Bristol, UK*

Mark O. Winfield • *School of Biological Sciences, University of Bristol, Bristol, UK*

Guangxiao Yang • *The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China*

Litao Yang • *National Center for Molecular Characterization of Genetically Modified Organisms, SJTU-Bor Luh Food Safety Center, School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai, China*

Xiaofen Yu • *The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China*

Yuxuan Yuan • *School of Plant Biology and Institute of Agriculture, University of Western Australia, Crawley, WA, Australia*

Dabing Zhang • *National Center for Molecular Characterization of Genetically Modified Organisms, SJTU-Bor Luh Food Safety Center, School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai, China*

Yuan Zong • *State Key Laboratory of Plant Cell and Chromosome Engineering, and Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China; University of Chinese Academy of Sciences, Beijing, China*

Part I

Background and Overview

Chapter 1

Enabling Molecular Technologies for Trait Improvement in Wheat

Prem L. Bhalla, Akanksha Sharma, and Mohan B. Singh

Abstract

Wheat is the major staple food crop and a source of calories for humans worldwide. A steady increase in the wheat production is essential to meet the demands of an ever-increasing global population and to achieve food security. The large size and structurally intricate genome of polyploid wheat had hindered the genomic analysis. However, with the advent of new genomic technologies such as next generation sequencing has led to genome drafts for bread wheat and its progenitors and has paved the way to design new strategies for crop improvement. Here we provide an overview of the advancements made in wheat genomics together with the available "omics approaches" and bioinformatics resources developed for wheat research. Advances in genomic, transcriptomic, and metabolomic technologies are highlighted as options to circumvent existing bottlenecks in the phenotypic and genomic selection and gene transfer. The contemporary reverse genetics approaches, including the novel genome editing techniques to inform targeted manipulation of a single/multiple genes and strategies for generating marker-free transgenic wheat plants, emphasize potential to revolutionize wheat improvement shortly.

Key words Wheat, Next generation sequencing (NGS), "Omics", Transcriptomics, Metabolomics, TILLING, RNAi, Genome editing, CRISPR/Cas, Marker-free transgenic wheat

1 Introduction

During the past two decades, significant progress has been made in developing and deploying wheat genomics platforms for the identification of agronomically valuable loci and to enhance crop performance [1–6]. The development of omics-based research coupled with next generation sequencing (NGS) technologies and improved bioinformatics approaches have provided key resources to unravel the genetic architecture of complex crop species such as wheat [7, 8]. In parallel, comparative genetics approach that is used for evolutionary studies and transferring information from model species to crop plants has further revolutionized the strategies for trait improvement in wheat [9–12].

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The recent advent of genome editing techniques such as targeted gene modification using transcription activator-like effector nucleases (TALENs) [13, 14] or zinc finger nucleases (ZFNs) [15] or clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas9) [16, 17], collectively referred to as genome editing with engineered nucleases (GEEN), has revitalized approaches in plant functional genomics [18, 19]. As a consequence, investigations initiated in wheat utilizing TALENs and CRISPR/Cas9 [20–24] have opened the door to crop improvement approaches. Correspondingly, a variety of novel transgenomics technologies such as antisense, RNA interference (RNAi), artificial microRNA expression (amiR), and virus-induced gene silencing (VIGS) have been developed to complement the traditional transgenesis-based genetic engineering and plant molecular biotechnology [25]. Thus, the combination of existing knowledge and resources within the wheat biotechnology and wheat functional genomics is providing the opportunity to decipher the genetic, biochemical and physiological basis of complex traits such as stress tolerance, disease resistance, and grain improvement that can be enhanced for generating improved wheat varieties.

Considering the advancements made in enabling technologies of plant functional genomics and plant biotechnology, here we provide an update on the promising techniques, and scope of genetics- and genomics-based approaches for improving wheat. We first overview the recent progress made in wheat genomics that has been spurred using NGS platforms. Next, we discuss the available online resources in wheat research and contributions of "omics" technologies in wheat improvement. Furthermore, we summarize how wheat functional genomics is on the verge of a revolution with the advent of novel transgenomics and genome-editing technologies and outline the strategies to generate marker-free transgenic wheat plants.

2 Wheat Genome and Its Potential

The large size of the bread wheat genome (~17 Gb), extensive stretches of repetitive DNA (>80%) as well as ploidy in wheat ($2n = 6\times = 42$, AABBDD allohexaploid) have challenged the wheat genome sequence to be decoded and thus impeded the progress in wheat genomics and its application toward crop improvement. Nevertheless, the recent progress in sequencing technology [26] have overcome these challenges, and thus genome drafts, as well as high-resolution transcriptomes for bread wheat and its progenitors, have been deciphered successfully [5, 27]. The first sequence of a model hexaploid cultivar Chinese Spring (CS42) was published in 2012, based on whole-genome shotgun sequencing using orthologous sequences from multiple kinds of grasses (Brachypodium, sorghum,

rice, and barley) as a guide for the assembly [28]. Although the assembly was highly fragmented and equivalent to only fivefold (5×) coverage of the estimated whole wheat genome (17 Gb), it was marked as a remarkable contribution in the field of wheat genomics. The draft genome cataloged 132,000 single nucleotide polymorphisms (SNPs) distributed over A, B, and D genomes and the number of genes was estimated at 94,000–96,000. Subsequently, the genome sequences of three diploid ancestors of wheat (*T. urartu*, *Ae. spletoides*, and *Ae. tauschii*) have also been decoded. The draft sequences for the A and D genome progenitors, *T. urartu* [29] and *Ae. tauschii* [30] have been acquired using a shotgun sequencing approach followed by de novo assembly and a 4 Gb physical map of *Ae. tauschii* has been generated using the SNaPshot BAC fingerprinting technology [31]. During 2014, The International Wheat Genome Sequencing Consortium (IWGSC) aimed at constructing a chromosome-based physical sequence of the wheat genome [32] using double ditelosomic wheat lines of the cultivar, "Chinese Spring." Gene annotation of the wheat genome sequence was made by (1) gene comparisons in related grass species such as Brachypodium, sorghum, rice, and barley, (2) available full-length wheat cDNAs, and (3) available RNA-seq data from "Chinese Spring" tissues at various stages of development. An estimation of about 106,000 protein coding genes was made in the chromosome-based sequence of the wheat genome, slightly greater than that estimated in the first draft sequence in 2012 [28]. Correspondingly, the wheat chromosome 3B has been fully sequenced [33]. So far, the survey sequence is complete for all the chromosomes, and the reference sequence of 3B and physical maps of 16 chromosomes are accessible (http://www.wheatgenome.org/). The assembly of physical maps of the remaining five chromosomes and the acquisition of the complete reference sequence of bread wheat are currently undergoing. Recently, a draft pangenome of hexaploid bread wheat has been deciphered (http://appliedbioinformatics.com.au/cgi-bin/gb2/gbrowse/WheatPan/) using a draft Chinese Spring wheat genome reference and whole genome sequencing data from 18 wheat cultivars [34]. In this study, the draft Chinese Spring wheat genome reference was reassembled and used as the basis for pangenome study while core and variable genes were identified across 18 different wheat cultivars. The reassembly produced a reference with larger assembly size and greatly reduced frequency of duplicated regions compared to the previously published draft genome. The whole genome sequence reads from 18 wheat cultivars were then mapped to the new reference assembly. The pangenome was estimated to contain 128,656 predicted genes of which 64.3% were identified as core, that is, present in all cultivars, while the remainder were identified as variable and displayed presence/absence variation [34].

3 Omics Spectrum in Wheat Research

In the recent years, the integrated use of omics technologies and analysis of omics-based data sets have facilitated numerous studies in a plant system. The omics methodologies have been applied first for DNA analysis (genomics), then extended for RNA (transcriptomics), protein (proteomics), and small metabolites (metabolomics). These technologies significantly contribute toward the identification of important genes and their manipulation for cereal improvement.

3.1 Genomics

In the last few years, the application of next-generation sequencing (NGS) techniques has been improved resulting in accelerated plant breeding. One of the most significant aspects of NGS is the ever reducing cost, enabling high-throughput research in very large and complex genomes, such as wheat. Correspondingly, comparative genomics has facilitated the mining of biological information from a genome sequence, through the uncovering of similarities and differences with genomes of closely and more distantly related species. Hence, genomics-based crop improvement has been potentially viewed as an extension of wheat genetics. In the Poaceae family of grasses, comparative genomics research has provided comprehensive datasets that demonstrate a high degree of synteny or collinearity or among genomes at the chromosome (macro) and the gene (micro) levels [35]. The phylogenetic relatedness and gene order conservation allow the species with small genome sizes such as *Brachypodium* and rice to be used as model systems for studying similar gene contents in complex genomes such as wheat and other crops (Fig. 1) [9, 10, 36, 37]. For example, a study of wheat root architecture led to an identification of potential genes targeting wheat root improvement through translational research from *Brachypodium* to wheat [9]. Similarly, the genome zipper approach has been applied for decoding the sequence of wheat chromosome 4A [38]. Genome zipper is an integrated approach that includes chromosome sorting, NGS technology, array hybridization, and systematic exploitation of conserved synteny with model grasses. In this report, mitotic chromosome flow sorting was first used to separately purify a pair of telocentric chromosomes that together form chromosome 4A of wheat. The chromosomes were then shotgun-sequenced followed by gene sequence comparisons with reference grass genomes (Rice, Brachypodium, and Sorghum) for detecting syntenic regions.

Genome-wide association studies (GWAS) and Quantitative trait loci (QTLs) mapping have enabled identification of critical genes in wheat that can be exploited in breeding wheat with enhanced traits such as stress tolerance and grain improvement [39, 40]. For example, in a recent report, genome-wide association

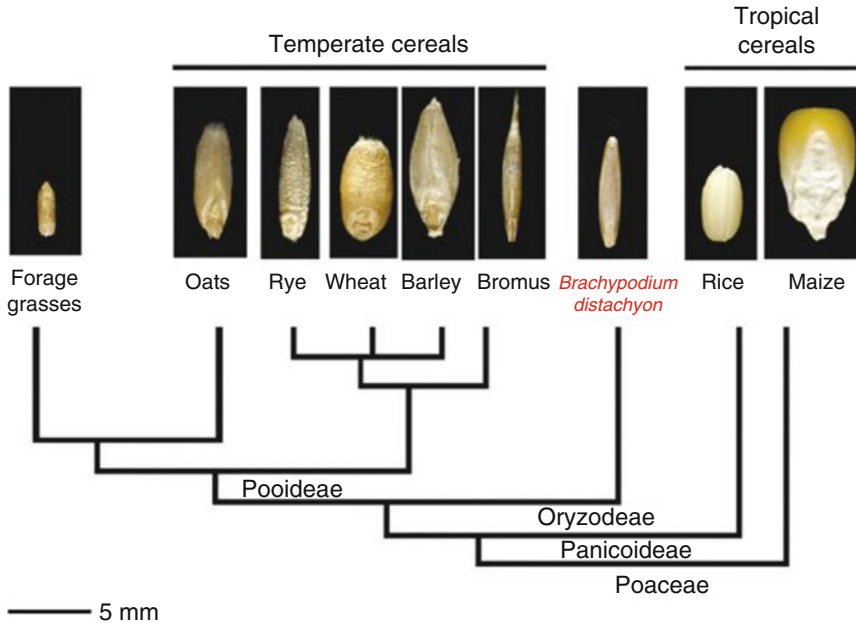

Fig. 1 Phylogenetic relationship between wheat and other grasses belonging to family Poaceae. The Poaceae family includes economically important cereal crops such as maize, rice, barley, oats, and forage grasses. Grasses are categorized based on type of caryopsis or grain (a single-seeded starch-rich fruit) these plants produce (scale, bar = 5 mm). Reprinted from Opanowicz et al. (2008, Trends in Plant Sci) [36], with permission from Elsevier

mapping in wheat has identified single nucleotide polymorphisms (SNPs) that were associated with salt tolerance traits [41]. In this study, the genetic variation for salt tolerance was exploited using GWAS for identifying QTLs conferring salt tolerance in 150 winter wheat cultivars. This report has highlighted 187 SNPs, represented by 37 QTLs, which were linked with the salt tolerance traits. Such polymorphisms can be exploited in breeding for salt tolerance in wheat. Similarly, a large number of genes and QTLs that are valuable for wheat improvement against drought stress have been identified using genome-based approaches [42] (Table 1). Merchuk-Ovnat et al. [42] selected QTL alleles conferring drought tolerance from wild emmur wheat (*Triticum turgidum* ssp. *dicoccoides*) and introgressed via marker-assisted selection, to enhance drought resistance in durum (*T. turgidum* ssp. *durum*) and bread (*T. aestivum*) wheat cultivars. The results indicated that introgression of wild emmur QTLs enhanced grain yield and productivity in domesticated wheat cultivars across drought environmental conditions. Similarly, a recent report has revealed new allelic variation distribution for 13 yield-related traits (thousand kernel weight, kernel length, kernel width, plant height, spike length, kernel weight per spike, kernel number per spike, fertile spikelet number per spike, sterile spikelet number per spike, total spikelet number per spike,

Table 1
Summary of recent GWAS and QTL mapping studies on agronomic traits in wheat

Trait exploited	Technique employed	Results	Reference
Salt tolerance	Genome-wide association mapping (GWAS). Genetic variatian was exploited at germination, seedling hydroponics and adult-field stages	187 SNPs representing 37 QTLs were identified to be significantly associated with salt tolerance traits	Oyiga et al. [41]
Drought and salt tolerance	Genome-wide phylogenetic and expression analysis of NAC superfamily in durum wheat	168 NAC genes were identified in durum wheat and phylogenetic analysis identified five wheat NAC genes as rice NAC homologs. In silico expression analysis revealed tissue-specific and drought and salt responsive wheat NAC genes	Saidi et al. [43]
Freezing and snow mold tolerance	QTL mapping was employed to identify molecular markers in winter wheat (*Triticum aestivum*) against snow mold fungus and freezing temperatures	One QTL associated with both freezing and snow mold tolerance was identified on chromosome 5A QTLs associated with only freezing tolerance were identified on 5A and 4B QTL associated with snow mold tolerance was identified on chromosome 6B	Kruse et al. [44]
Yield traits such as thousand kernel weight, kernel length, kernel width, plant height, spike length, kernel weight per spike, kernel number per spike, fertile spikelet number per spike, sterile spikelet number per spike, total spikelet number per spike, peduncle length, flag leaf base angle, and flag leaf direction	Genome-wide association studies (GWAS). Genetic variatian was exploited in 163 different wheat cultivars and agronomic traits were investigated in 14 environments at three locations over 3 years	Association studies were performed with kernel-related traits, panicle-related traits and plant architecture-related traits and 1769 significant SNPs were identified for 13 agronomic traits in 14 environments	Sun et al. [45]
Resistance to *Septoria tritici* blotch (STB), caused by the ascomycete fungus *Zymoseptoria tritici*	Genome-wide association study in a worldwide collection of 96 wheat accessions. The collection was evaluated following artificial infection at seedling and adult plant stages for STB resistance, heading date (Hd) and plant height (Ph) in field trials	In total, 73 marker–trait associations involving STB resistance were detected, the chromosomal locations of some of them were similar to known *Stb* genes or QTLs; while others were detected in new genomic regions. Field trials showed genetic association between STB resistance and Hd for few genotypes	Gerard et al. [46]

peduncle length, flag leaf base angle, and flag leaf direction) in a series of genome-wide association studies in 163 different cultivars of bread wheat [45]. Such QTLs can be dissected further to find their superior alleles for breeding and wheat improvement programs. Correspondingly, QTLs and SNPs associated with disease tolerance were also identified in wheat populations using GWAS (Table 1). For instance, in a recent study GWAS in a worldwide collection of 96 wheat accessions identified new genomic regions associated with Septoria tritici blotch (STB) resistance [46]. STB is one of the most devastating foliar diseases of wheat caused by the fungus *Zymoseptoria tritici*. In total, 73 marker–trait associations involving STB resistance were detected, the chromosomal locations of some of which were similar to known *Stb* genes or QTLs; while others were detected in new genomic regions. A summary of recent GWAS and QTL mapping events associated with agronomic traits is given in Table 1.

3.2 Transcriptomics

Gene expression analysis via high-throughput RNA-Seq and de novo transcriptome assembly has enabled the identification of candidate genes, prediction of gene functions, and characterization of transcriptional regulatory networks, in a wide variety of crop plants including wheat [47–50]. RNA-Seq has further enabled the detection of SNPs and the development of genome-specific markers for genetic mapping in wheat [51, 52]. For example, in a recent study, Illumina (Solexa)-based transcriptomes of bread wheat cultivar Yunong 201 and its mutant line, Yunong 3114 generated by EMS were analyzed for detecting single nucleotide variants (SNVs) to uncover the genetic basis of bread wheat kernel size [53]. While RNA-seq analysis has been a promising approach for identifying allele-specific and/or homoeolog-specific transcripts based on nucleotide polymorphisms in the sequenced read assembly, it has provided a comprehensive set of homoeolog-specific gene models in the tetraploid durum wheat transcriptome [54]. For example, a recent report has identified 168 NAC genes in durum wheat, involved in salt and drought stresses [43]. The NAC proteins are one of the largest plant-specific transcription factor (TF) families. They play important roles in plant development, response to biotic and abiotic cues and hormone signaling [55]. Both microarray- and RNA-Seq-based transcriptomic analyses have become the pillar for gene discovery and analyzing stress responses in wheat. This has been highlighted in a recent report where microarray based expression profiling was used to identify important genes, signaling proteins and transcription factors that are involved in drought-resistance response in wheat spike [56]. In this study of Liu et al. [56], gene expression was evaluated in the wheat spikes at the early grain-filling stage (6 days after anthesis). About 477 genes were identified to be upregulated under water deficit conditions and were mostly involved in signal transduction, metabolism, and

transcription. Transcription factors, signaling proteins, and abiotic stress-related genes were the main among the upregulated genes category. In the past few years, wheat transcriptome profiling has been extensively investigated in response to individual stresses, such as heat, drought or soil alkalinity [57–59]. For example, transcriptomes of wheat seedlings exposed to soil alkalinity stress (cultivars used in the study are SR4 and JN177) have been recently analyzed, and the results indicated that cultivar SR4 exhibits higher tolerance to alkaline stress [59]. Correspondingly, analysis of wheat transcriptome has also deciphered a wealth of candidate genes and pathways involved in susceptibility responses to different pathogens and pests [60–63]. A genome-wide gene expression profiling was conducted in a wheat cultivar susceptible to fusarium head blight infection for identifying wheat grain candidate genes and pathways involved in susceptibility responses to fusarium head blight. Fusarium head blight is one of the most devastating diseases of wheat that is caused by the fungal pathogen *Fusarium graminearum*. Expression profiling identified 1309 differentially expressed genes at five grain developmental stages, out of which, 536 candidate genes were impacted by the fungal pathogen effect [63]. Hence, the combination of genomics-based synteny analysis and transcriptome-based tissue- or stress condition-specific expression scanning can uncover novel agronomically important genes that can be exploited to generate abiotic or biotic stress tolerant wheat varieties.

3.3 Metabolomics

High-throughput metabolomics is employed form identifying and quantifying small-molecules (metabolites) in a given biological sample [64]. The data generated from metabolomics complements genomic, transcriptomic and proteomic studies, and has profound advantages in bridging the gap between genotype and phenotype [65]. The utility of metabolomics in relation to crop improvement has been reviewed in detail [66–68]. Metabolome profiling aims to provide a snapshot of the metabolites in response to various conditions such as environments, developmental stages, and genotypes. For instance, metabolomic analysis of developing grain in a wheat cultivar, Zhongmai 175 identified 74 different metabolites (amino acids, carbohydrates, organic acids, and lipids/fatty acids) over different grain developmental stages [69]. Similarly, metabolomic analysis of the response to high nitrogen fertilizer during grain development in wheat resulted in the identification of metabolites (amino acids, carbohydrates, organic acids, and lipids), which are primarily involved in carbon and nitrogen metabolism [70]. However, recently, metabolic and physiological analysis of wheat grain has revealed that excessive nitrogen application decrease the antioxidant capacity and grain filling in wheat [71]. In the study of Kong et al. [71], field grown wheat was treated with normal and excessive levels of nitogen fertilizer and the results of

physiological and metabolic profiling indicated that with the application of excessive nitrogen, oxidative stress increased while disturbing nitrogen, lipid, and secondary metabolisms, which resulted in acceleration of leaf senesence and grain filling rates in wheat, thereby leading to reduced grain yields. Correspondingly, many studies have used metabolome profiling for evaluating stress responses in wheat [72–74]. An integrated metabolomic–proteomic approach has been used to elucidate the mechanism by which wheat QTL (the Fusarium head blight resistance locus, *Fhb1*) contributes to resistance against *Fusarium graminearum* [75]. The results indicated that resistance in the *Fhb1* locus was mainly associated with the increased cell wall thickenings due to the deposition of hydroxycinnamic acid amides, phenolic glucosides and flavonoids. Recently, the integrated application of metabolome analysis and NGS-based genome or transcriptome analysis has become an effective systems-based approach to reveal gene functions as well as to explore gene regulatory networks and metabolic pathways in wheat [76]. Further, Francki et al. [77] employed an integrated metabolomics–genomics strategy to ascertain interconnecting biological networks and compare underlying genes regulating metabolite and trait variation in wheat grain from aneuploid and ditelosomic lines [77]. In this study, metabolite profile of mature wheat grain from a Chinese Spring cultivar (with a full complement of chromosomes) was compared with ditelosomic wheat lines (where genes on the short and long arms of A, B and D genomes of homoeologous group 3 chromosomes were deleted). The known biochemical pathways in wheat and ditelosomic lines were identified and genes controlling metabolite accumulation were analyzed using available draft wheat genome. The analyses revealed significant differences in the grain metabolome from Chinese Spring and ditelosomic line, while a total of 412 analytes were resolved between the metabolite profiles of mature wheat seed and ditelosomic lines from homoeologous group 3 chromosomes. The study further demonstrated the feasibility of wheat aneuploid lines to be a suitable experimental genetic system for validating metabolomics–genomics networks. Similarly, in another recent study conducted by Dhokane et al. [78], an integrated metabolic–-transcriptomic analysis identified candidate genes resistant to fusarium head blight of wheat, localized within the QTL-Fhb2 region [78]. In this study, recombinant inbred lines (RILs) carrying resistant and susceptible alleles of QTL-Fhb2 were subjected to metabolome and transcriptome profiling for identifying candidate genes within QTL-Fhb2 locus. The analysis identified 4-coumarate: CoA ligase, basic Helix Loop Helix 041 transcription factor, glutathione S-transferase, callose synthase, ABC transporter-4, and cinnamyl alcohol dehydrogenase as putative resistance genes localized within the QTL-Fhb2 region.

4 Online Resources for Wheat Research

Owing to the recent advances in wheat genomics research, the bioinformatics network for wheat resources has been well established and provides support and links to many of related web pages (Table 2). The International Wheat Genome Sequencing Consortium (IWGSC) (www.wheatgenome.org) established in 2005, is the major focus of the wheat society which connects a number of services that includes a Seq Repository to access chromosome survey sequences, reference sequences, physical map data, and transcriptome data. Recently, an integrated database, WheatGenome.info has been developed to support wheat research and crop

Table 2
Online resources for wheat research

Database	Description	URLs
WheatGenome.info	GBrowse2-based wheat genome viewer, BLAST search portal, wheat genetic maps using CMap and CMap3D, wheat genome Wiki	http://www.wheatgenome.info/
IWGSC	Genome sequence, transcript resources, physical maps	http://www.wheatgenome.org/
WheatNet	First genome-scale functional network for *T. aestivum*	www.inetbio.org/wheatnet
URGI	Physical and genetic maps, markers, QTLs, SNPs, gene annotation	https://wheat-urgi.versailles.inra.fr/
transPLANT	Triticeae genome resources, comparative genome analysis	http://www.transplantdb.eu
TriFLDB	Gene annotation	http://trifldb.psc.riken.jp/index.pl
Wheat genome database-PGSB	Genome sequence, Gene annotation	http://pgsb.helmholtz-muenchen.de/plant/wheat/uk454survey/index.jsp
PlaNet	Gene expression profiles based on the GeneChip	http://aranet.mpimp-golm.mpg.de/
CIMMYT	Ordering different accessions of wheat germplasms and stocks	http://www.cimmyt.org/
TriticeaeCAP	Genotyping and phenotyping different accessions of wheat germplasms and stocks	http://www.triticeaecao.org/
PlantCare	Plant *cis*-acting regulatory elements database	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
wDBTF	Wheat transcription factor database	http://wwwappli.nantes.inra.fr:8180/wDBFT/
GrainGenes	Gene annotation, EST blast, transcript resources, QTLs	http://wheat.pw.usda.gov/GG3/

improvement (http://www.wheatgenome.info/) [6]. Databases for the prediction of wheat transcription factors and *cis*-acting regulatory elements can be accessed on wDBTF and PlantCare web pages, respectively. Several small project-based wheat online resources such as Wheat Genomics, PlaNet, GrainGenes, and transPLANT are summarized in Table 2.

5 Combining Forward and Reverse Genetics

Over the years, both forward and reverse genetics approaches have been refined and complemented translational research in plants. The different strategies for gene knockout/down by combining mutagenesis and TILLING (Targeting Induced Local Lesions in Genomes) [79, 80], RNA interference (RNAi) [81–83], VIGS (virus induced gene silencing) [84], and gene editing by TALEN and CRISPR-Cas9 [23, 85] are accessible for wheat.

The combination of ethyl methanesulfonate (EMS)-mediated mutagenesis and TILLING technology has been a powerful reverse genetics approach in wheat to identify novel genetic variation without the need for direct selection of phenotypes [79, 86]. TILLING is a reverse genetic based approach that utilizes chemical mutagenesis for inducing variability and precise molecular analyses to identify point mutations responsible for phenotype alteration [87]. The polyploidy nature of wheat is well-suited for TILLING because of tolerance to the high mutation densities [86, 88]. TILLING is considered a non-GM (genetically modified) technology and its application in wheat provides access to new alleles in the A, B, and D genomes of the same cultivar for the development of new traits [86]. Recently, using TILLING approaches, nontransgenic durum, and bread wheat varieties with high amylose and resistant starch content have been successfully developed [89, 90]. Similarly other traits such as carotenoid content [91], grain hardness [92], grain length and width [93], seed dormancy [94], flowering [95], or vernalization [96], have also been targetted by TILLING in wheat. TILLING has also been reported to generate hexaploid bread wheat lines with enhanced resistance to the common powdery mildew disease [80]. In the study of Garcia et al. [80], TILLING was used to identify mutant in the *TaMlo* genes from EMS-mutagenized spring wheat. Both triple and double mutant lines, containing missense mutations in the *TaMlo-A1*, *TaMlo-B1*, and *TaMlo-D1* homoeologs conferred enhanced powdery mildew resistance were obtained. The nontransgenic wheat lines, generated through TILLING, thus represent an important step toward improving nutrition, quality and yield, and agronomically important traits of wheat. This approach, therefore, provides a rich and convenient resource for both basic functional genomics research and commercial crop improvement. A summary of events reported, so far, using TILLING in wheat is given in Table 3.

Table 3
A summary of studies involving reverse genetics approaches in wheat

Trait exploited	Technology employed	Results	Reference
Amylose and resistant starch	EMS-induced mutation	A set of 101 EMS-induced mutant lines (M4 generation) showing variation in amylose and resistant starch content in seed was develped in a bread wheat variety "C 306." Gene expression profiles of 20 starch metabolic genes in the two diverse mutant lines (low and high amylose mutants) indicated that in addition to key genes, several other genes (such as phosphorylases, isoamylases, and pullulanases) may also be involved in contributing to amylose/amylopectin biosynthesis	[97]
Grain weight and width	A tetraploid wheat TILLING population was screened to identify mutants in *TaGW2-A1* allele. The mutant allele was backcrossed into tetraploid and hexaploid wheat lines	Results indicated that the mutant allele consistently increased grain weight in all environments and across all grains of the spike in comparison to the wild type full length *TaGW2-A1* allele. The effect was detected as early as 5 days before anthesis in developing carpels	[93]
Resistance to powdery mildew disease caused by *Blumeria graminis* f.sp. *tritici* (*Bgt*)	TILLING	16 missense mutations were identified in the three wheat *TaMlo* homoeologs (*TaMlo-A1*, *TaMlo-B1*, and *TaMlo-D1*). Different missense mutants were functionnaly analyzed using transient gene expression assays in barley single cells. Triple and some double mutant lines showed enhanced powdery mildew resistance compared to wild-type (WT) plants	[80]
Kernel hardness and starch biosynthesis	TILLING	A set of EMS-induced mutant lines (M2 generation) was develped in a bread wheat variety "Longfumai 17." One novel allelic variation in the kernel hardness gene *Pinb* was obtained, and the frame shift and missense mutations of starch biosynthetic genes *waxy* and *SSIIa-A* had deleterious effects on their functions	[98]

(continued)

Table 3 (continued)

Trait exploited	Technology employed	Results	Reference
Dough handling characteristics	RNAi; A plasmid designed to downregulate secalin was introduced by biolistics into "Bobwhite," which carried a 1BL.1RS translocation	RNAi was used to decrease levels of omega secalins 3.7–5.5-fold in a 1BL.1RS wheat. Flour from the transgenic lines exhibited longer development times and greater tolerance to mixing	[99]
Resistance to *Fusarium* head blight (FHB) fungus *F. culmorum*	VIGS and HIGS	Wheat plants preinfected with *Barley stripe mosaic virus* (BSMV) strains containing antisense sequences against target genes of *F. culmorum* showed reduced disease symptoms. Stable RNAi transgenic wheat plants exhibited enhanced FHB resistance in leaves and spikes	[100]

Concurrently, RNA interference (RNAi)-induced gene silencing has been a significant approach for functional studies in hexaploid wheat. In polyploids, in particular, the technology provides an additional advantage of silencing all genes of a multigene family including homoeoloci for individual genes, which are often simultaneously expressed, leading to a high degree of functional gene redundancy. Practical use of RNAi-induced gene silencing for Petunia flower phenotypes was demonstrated by Jorgensen et al. [101]. Different studies have been reported for successful and stable RNAi transformation in wheat. For example, RNAi has been applied to modulate starch content in wheat. To increase amylose starch content in wheat, starch branching enzymes of class II (*SBEIIa*) genes were silenced using the RNAi technique in two cultivars of durum wheat [102]. The resulted transgenic wheat lines exhibited a remarkable increase in the amylose content. Similarly, RNAi-mediated downregulation of *Isoamylase1* (*Isa1*) has altered the starch composition in the durum wheat grain [103]. In the study of Sestilia et al. [103], RNAi *Isa1* transgenic wheat lines exhibited a reduction in the starch content and a moderate enhancement of both phytoglycogen and β-glucan in the wheat grain. Further, RNAi has been reported to target dough characteristics, grain width and grain weight in wheat. RNAi-mediated downregulation of Secalin proteins resulted in alteration of flour protein composition in wheat, leading to improved dough handling characteristics [99, 104]. Likewise, *TaGW2*-RNAi transgenic wheat lines exhibited a significant increase in the grain weight and size (width), suggesting that

TaGW2 negatively regulate the grain weight and width in bread wheat [105]. Subsequently, RNAi strategy has been successfully employed for improving defense mechanism against biotic stresses in wheat plants via virus-induced gene silencing (VIGS) (Table 3). This RNA-based plant defense response is triggered when dsRNA accumulates within cells. This response is transient since it depends on the virus replicating within the plant. This strategy has been demonstrated to be effective in wheat using either wheat streak mosaic virus (WSMV) or barley strip mosaic virus (BSMV) as a viral vector for posttranscriptional gene silencing [106, 107]. In the last few years, RNAi via host-induced gene silencing (HIGS) has gained importance in conferring disease/pest resistance in wheat and other crops [108, 109]. This approach is based on sequence information from potentially relevant genes of the pathogen and not on endogenous resistance genes in the host plant. Using this approach, small RNAs made in the plant silence the genes of pests or pathogens that attack the plant. For example, HIGS of a chitin synthase gene (*Chs3b*) conferred durable resistance to fusarium head blight and seedling blight diseases in wheat [110]. *Chs3b* gene has been identified as an essential survival gene in the fungal pathogen *Fusarium graminearum*, which causes Fusarium head blight and seedling blight diseases in wheat [110]. The results indicated that transgenic wheat plants expressing RNAi constructs in T_3–T_5 generations conferred highly durable and consistent resistance to both Fusarium head blight and Fusarium seedling blight. Thus, essential genes in the pathogen have been targeted by RNAi constructs expressed in wheat, that resulted in down-regulation and resistance against the specific pathogen species [100, 109, 110]. Reports involving VIGS and HIGS in wheat are summarized in Table 3.

Correspondingly, the recent advent of genome editing technologies has revolutionized functional studies in wheat. Genome editing begins with the introduction of a targeted DNA double-stranded break at a predetermined locus using a sequence-specific nuclease. Three types of sequence-specific nucleases are in general use in wheat: ZFNs, TALENs, and CRISPR/Cas [14, 15]. The targeting of specific sequences using ZFNs and TALENs is mediated by protein–DNA interactions, whereas CRISPR/Cas recruits a guide RNA via base-pairing to direct an endonuclease to a target DNA sequence. In the past few years, CRISPR/Cas-mediated genome editing has been applied for wheat genome engineering [20–22] thereby leading to crop improvement. For instance, TALENs and CRISPR/Cas-mediated targeted mutations in the *MILDEW-RESISTANCE LOCUS (MLO)* in hexaploid wheat conferred resistance to powdery mildew in transgenic wheat lines [23]. In this study, TALEN-induced mutation of all three *MLO* homoeologs (*TaMLO-A1*, *TaMLO-B1,* and *TaMLO-D1*) in the same plant has shown to confer heritable broad-

spectrum resistance to powdery mildew. Further, in the same study, CRISPR–Cas9 technology has been used to mutate selectively only one of the three homoeoalleles of *MLO-A1* in hexaploid wheat with a mutation frequency of 5.6%. Currently, substantial efforts have been undertaken in optimizing the CRISPR–Cas9 method for wheat genome engineering either through transient expression-based genome editing [24] or using CRISPR/Cas9 ribonucleoproteins (RNPs) [111].

Thus, the integrated use of newer genome editing technologies and the conventional functional genomics strategies will play an increasingly important role in characterizing the agronomically important genes in wheat functional genomics especially through leveraging the advances in genome sequencing [5].

6 Strategies for Generating Marker-Free Transgenic Wheat Plants

Selectable markers such as antibiotic or herbicide-resistant genes (e.g., *bar*, *kan*, and *hyg*) have repeatedly been used for *Agrobacterium tumefaciens*-mediated genetic transformation of transgenic crops. These selectable marker genes (SMGs) are needed for selecting transformed cells in the transformation process but are unnecessary after transformation, and are not useful to continuous transformation.

Due to the concerns about perceived risks of SMGs for human and environmental health, a series of marker-free strategies have been developed, such as biolistic [112] and *A. tumefaciens*-mediated cotransformation [113], site-specific recombination [114–116], multiautotransformation vector system [117, 118], transposon-mediated method [119], intrachromosomal recombination [120], and using a marker-free binary vector [121]. Different strategies for generating marker-free transgenic plants have been reviewed previously in detail [122–125].

Marker-free wheat plants have been successfully obtained employing Cre/lox site-specific recombination [126]. This was the first report of the simultaneous application of the minimal gene cassette and cold-inducible Cre/lox recombination system in wheat. This study has reported the elimination of bar selection and *cre*-recombinase genes from T_0 and T_1 transgenic lines with 44 and 51% efficiency. Another efficient wheat transformation system established by the Japan Tobacco Company has provided a remarkable opportunity for routinely obtaining marker-free transgenic hexaploid wheat plants using an *Agrobacterium*-mediated cotransformation system [127]. In cotransformation method using either *Agrobacterium*- or biolistics-mediated transformation, SMG and gene of interest are supplied either on two different constructs (for biolistic based transformation) or two binary vectors within the same *Agrobacterium* or two T-DNAs within the same binary

vector or with two different *Agrobacterium* strains. SMGs can be removed from the plant genome during segregation in next generation by selecting the transgene of interest only in the progeny. Thus, in a similar way, marker-free transgenic Fielder and Gladius wheat plants were generated with an efficiency of 3% [128]. Additionally, in a recent study, 15 commercial Chinese hexaploid wheat varieties were successfully transformed via an *Agrobacterium*-mediated cotransformation method, with efficiency of up to 37.7%, as confirmed by the use of quickstix strips, histochemical staining, PCR test, and DNA gel blotting [129]. In this study, a plasmid pWMB123 harboring two independent T-DNA regions was used for transformation. One T-DNA region harbors the *bar* selectable marker under the control of the *CaMV35S* promoter and the *NOS* terminator sequence, and the other T-DNA harbors the maize ubiquitin (*ubi*) promoter, *gus* (β-glucuronidase) reporter gene, and the *NOS* terminator sequence [129].

Similarly, marker-free transgenic wheat plants have been generated using the pCLEAN vectors [130]. In this study, *gusA* as a reporter gene and *bar* as a SMG has been used for the construction of modified vectors based on the pCLEAN vector system. Successful application of the pCLEAN vector series in wheat has established a useful platform that can facilitate the further development of precise wheat transformation technologies.

7 Conclusions

Together with the development of new technologies in wheat genomics and genetics, advances in "omics" technologies particularly transcriptomics and metabolomics has opened up new opportunities to decode, characterize, and study the genetic compositions, organizations, structures, functions, and interaction networks on a wide scale. The acquisition of genome sequence drafts for bread wheat and its progenitors represents a milestone for wheat improvement. The rapidly increasing sequence data sets and associated resources for wheat will potentially accelerate the gene discovery process and improve our understanding of their biological functions. The integrated use of transcriptomics, proteomics, and metabolomics approaches has revealed a variety of stress-responsive genes that can be used for functional studies in wheat, ultimately leading to improvement of this important crop.

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Chapter 2

What Will Be the Benefits of Biotech Wheat for European Agriculture?

Agnès E. Ricroch

Abstract

In European countries, wheat occupies the largest crop area with high yielding production. France, a major producer and exporter in Europe, ranks the fifth producer worldwide. Biotic stresses are European farmers' major challenges (fungal and viral diseases, and insect pests) followed by abiotic ones such as drought and grain protein composition. During the last 40 years, 1136 scientific articles on biotech wheat were published by USA followed by China, Australia, Canada, and European Union with the UK. European research focuses on pests and diseases resistances using widely marker-assisted selection (MAS). Transgenesis is used in basic research to develop resistance against some fungi (Fusarium head blight) while RNA interference (RNAi) silencing is used against some fungi and virus. Transgenic plants were also transformed with genes from various species for drought tolerance. The UK (mostly with transgenesis and site-specific nucleases) and France (with no transgenic tools but with MAS and site-specific nucleases) are the main countries carrying out research programs for both biotic stress and drought tolerance. Thus, few European countries used transgenesis for gluten protein composition and RNAi-mediated silencing in celiac disease. Because of vandalism field trials of transgenics dropped since 2000. No transgenic wheat is cultivated in Europe for political reasons.

Key words European Union, Biotic stress, Drought tolerance, Gluten protein composition, Celiac disease, MAS, Transgenesis, RNAi, CRISPR-cas9, Regulation

1 Introduction

Global food production needs to increase by an estimated 60% by 2050 [1]. Cereals, which include wheat, rice, barley, maize, rye, oats, sorghum, and millet, make up the majority of the production of the crop production. These are the most important food source for human consumption. Here, wheat includes both bread wheat (*Triticum aestivum*) and pasta (*Triticum turgidum* var. *durum*) wheat. Wheat is the most widely cultivated crop in the world (736 million tones, Mt., and approx. 200 million hectares, Mha) according to FAO in 2015 [2]. In 2015 China is the foremost producing country worldwide (127.5 Mt) followed by India (89 Mt), Russia (59 Mt), the USA (58.5 Mt), and France (41 Mt) [3]. The wheat

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global average yield of 2.5 t/ha in 2000 [4] shifted 2015 to roughly 3.07 t/ha in 2015 [5]. Increasing yield is a worldwide challenge since it needs reach a billion tones to feed the world's population by 2050.

In European countries wheat, maize and barley occupy the largest crop areas [2]. With its high yielding wheat production (7.9 t/ha in 2015), France is a major producer and exporter in Europe. Thus, France heads the Wheat Initiative [6] created in 2011 following endorsement from the G20 Agriculture Ministries. This Initiative provides a framework to establish strategic research and organization priorities for wheat international research in both developed and developing countries (16 countries, 2 international research centers, and private companies).

Regarding farmer challenges identified in our European survey [7], biotic stresses are their major challenges, followed by abiotic ones such as drought, frost, and nutritional enhancement. No transgenic wheat is cultivated in Europe. Maize resistant to European corn borer (*Ostrinia nubilalis*) and Western corn rootworm (*Diabrotica virgifera virgifera*) (the so-called Bt MON810 maize) is the only one transgenic crop allowed for cultivation in Europe.

In our literature research, over the 40-year period between January 1975 and December 2015, various countries published 1136 articles on biotech wheat. USA ranks the first (214 papers) following by China (97), Australia (76), Canada (59), and the UK. In the European Union (EU), the ranking is as follows: the UK (58), Germany (30), Spain (27), France (24), Switzerland (21), Italy (17), Poland and Belgium (15), Hungary (13), Denmark and The NL (11), Austria (9), Czech Rep. and Norway (5), Greece, Sweden and Portugal (4), Romania (2), and Bulgaria (1). We aim at examining research using biotech tools carried out in Europe and possible benefits of biotech wheat for both farmers and consumers.

2 Biotic Stress

Wheat is an important food crop in Europe and harvested in almost all European countries with various wheat areas. However, fungal diseases and pest insects are widespread which together with other factors contribute to general decrease of yields in many parts of Europe. Regarding biotic stress observed by farmers in European major countries 19 pests and diseases were identified by our survey [7]. Plant pests and diseases cause serious yield losses in fields depending on the country. For example, Septoria leaf blotch causes damage to half of the wheat area in the Czech Republic, France and Sweden.

Nine fungal diseases are observed by farmers: Leaf (brown) rust (*Puccinia triticina* (*Puccinia recondita* f. sp. *tritici*)), Stem (black) rust (*Puccina graminis*); Stripe (yellow) rust (*Puccinia striiformis*);

Bunts and smuts (*Ustilago tritici, Tilletia* spp., *Urocystis agropyri*); Fusarioses (incl. Fusarium head blight, *Fusarium graminearum* (Fusarium head blight)), Crown rot (*Fusarium* spp., *Microdochium* spp.); Powdery mildew (*Blumeria (Erysiphe) graminis*); Septoria leaf blotch (*Mycosphaerella graminicola* (anamorph: *Septoria tritici*)); Take-all (*Gaeumannomyces graminis* var. *tritici* (*Ophiobolus graminis*)); Eyespot (*Pseudocercosporella herpotrichoides*); Tan spot (yellow leaf spot) (*Drechslera* (*Pyrenophora*) *tritici-repentis*). In our literature search, a predominance of articles dealt with fungal diseases, notably Fusarium head blight (FHB), while pest insects are strikingly the least dealt with. FHB is a highly destructive disease of wheat, which causes serious mycotoxin contaminations of grain.

Seven insect pests are observed by farmers: Hessian fly (*Mayetiola destructor*); Sunn pest (*Eurygaster integriceps*); Corn ground beetle (*Zabrus tenebrioides*); Grain aphid (*Sitobion avenae*) and Rose-grain aphid (*Metopolophium dirhodum*); Frit fly (*Oscinella frit*); Chloropid gout fly (*Chlorops pumilionis*); Wheat blossom midge, orange wheat (*Sitodiplosis mosellana*); blossom midge, yellow wheat blossom midge (*Contarinia tritici*); Cereal leaf beetle (*Oulema melanopus* (*Lema melanopa*)). Two insect pests (Frit fly (*Oscinella frit*) and Chloropid gout fly (*Chlorops pumilionis*)) are "neglected" biotic stress, as they are not identified in the current European public research programs or in our literature search. Thus, impacts due to frit fly are seen in Czech Republic, Germany, Hungary, Romania, and Sweden. Impacts due to Chloropid gout fly are seen in Czech Republic, Hungary, and Romania. No papers were published on ground beetle (*Zabrus tenebrioides*) while it causes major damages in Romania. Only few scientific papers were published on Sunn pest (*Eurygaster integriceps*) which causes major damages in Romania and also in Bulgaria, on grain aphid (*Sitobion avenae*) which is present in major countries in Europe, and on leaf beetle (*Oulema melanopus* (*Lema melanopa*)) which is present in two countries only until now (Czech Republic and Italy).

Farmers observe two viral diseases: wheat dwarf virus (WDV) (leafhopper as vector) and barley yellow dwarf virus (BYDV) (aphid as vector). RNA silencing plays important roles in antiviral defense.

Current chemical used to manage plant diseases are vulnerable to pathogen evolution and therefore are not sustainable. Pathogen evolution is facilitated by the genetic uniformity underlying modern agroecosystems. Biocontrol with natural enemies can contribute to pest management, but biocontrol agents are often difficult to maintain at optimal levels over long periods of time. Thus, there is a need to develop new and sustainable ways to control plant diseases. In scientific papers, researchers used all biotech tools to develop genetic resistance against insect pests and fungal or virus diseases [7]. Research demonstrates a high genetic complexity of resistance in some cases. However, marker-assisted selection (MAS) is the most used technique for almost all pest and diseases. Transgenesis

is used with transgenes from wheat, rice, maize, barley, bean, radish, or potato to develop resistance against leaf rust, stripe rust, powdery mildew, Fusarium head blight, sharp eyespot, common root rot, and take-all. Biotech tools include RNA interference (RNAi) silencing to develop resistance against leaf rust, stripe rust, stem rust, powdery mildew, Hessian fly, aphids, grain aphid, and barley yellow dwarf virus. Barley stripe mosaic virus (BSMV) is used as a transient expression system *in planta* (BSMV-based virus induced gene silencing of pathogen gene). Leaf rust and Fusarium head blight resistance genes were cloned. The EMS is used to provide mutants that resistance to leaf rust, stem rust, stripe rust, and powdery mildew.

Plant breeding programs in Europe using biotechnologies intend to make a contribution toward resistance to pests and diseases. In Belgium (203,200 ha of wheat area), genetically modified (GM) fungal resistant varieties are in development (Syngenta) with field trials performed in different countries (not Belgium due to vandalism). In Czech Republic, for grass powdery mildew (*Blumeria graminis*) laboratory experiments are carried out for characterization of interaction between pathogen and host and positional cloning of gene for resistance; for powdery mildew (*Mycosphaerella graminicola*, sexual stage *Septoria tritici*) identification of wheat gene of resistance and study of plant defense are carried out (laboratory experiments have shown that most of the tested varieties, both commercial and with specific resistance genes had not susceptible response to mildew's isolate); for wheat dwarf virus (WDV) current research (started in 2012 and ended by 2015) aims to assessing the level of resistance in wheat and to identifying sources of resistance, with new techniques (RNAi).

In France (5,491,197 ha), the 9-year "Breedwheat" research program aims at strengthening the competitiveness of the French wheat breeding sector. "Breedwheat" with 26 public and private partners combine genetics, genomics, and ecophysiology analyses with high throughput phenotyping and genotyping to perform association studies and identify markers and candidate genes of factors impacting important agronomic traits such as yield, tolerance to biotic (Fusarium and Septoria) and abiotic stresses (nitrogen use efficiency and drought tolerance) and grain protein composition in the context of sustainable systems and climate change. In Germany (3,248,200 ha), basic public research concerns fungal resistance (with field trials from 2009–2011). Preliminary results (2012) were obtained for Mlo-resistance via gene transfer. In Italy (1,726,030 ha) laboratory research was carried out on the expression of polygalacturonase-inhibiting for resistance to Fusarium (no field trials). In the UK (1,969,000 ha) numerous research programs were carried out: Introduction of genes involved in avenacin biosynthesis from oats into wheat; Resistance gene isolated from peppermint plants and cloned into wheat (field trials planted

in 2012); Cloning resistance genes against wheat rusts in wild relatives of wheat with a view to mobilizing these in bread wheat as a transgene cassette containing multiple resistance genes (experiments in progress in the greenhouse/growth chamber facility).

In the UK, stably transformed wheat lines release (E)-beta-farnesene (Ebetaf), the alarm pheromone for many pest aphids, using a synthetic gene based on a sequence from peppermint with a plastid targeting amino acid sequence, with or without a gene for biosynthesis of the precursor farnesyl diphosphate. In laboratory behavioral assays, three species of cereal aphids were repelled while field trials showed no reduction in aphids or increase in parasitism [8].

Despite the fact that MAS is widely utilized in public research even in Europe, transferring desirable genetic traits can take a long time. Moreover the political context is not optimal for genetic engineering research in certain European countries (*see* Subheading 5). Unfortunately, to date, only products developed through the application of MAS have been placed on the market.

3 Abiotic Stress

Water stress continues to be a major limiting factor hindering world wheat productivity under dry and hot weather conditions or facing diminished groundwater resources [9]. Drought and high temperature commonly occur in combination. Increasing yield under these abiotic conditions and improvement in crop water-use efficiency (WUE) are major goals of wheat breeding including genetic engineering. The International Maize and Wheat Improvement Center (CIMMYT) aims to genetically enhancing drought tolerance in wheat. Breeding for drought tolerance is further complicated since this trait is quantitative with a complex phenotype. Although marker-assisted selection (MAS) is now widely deployed in wheat, it has not contributed significantly to improvement for adaptation to low-yielding difficult environments and breeding has relied largely on direct phenotypic selection. The limited success of the physiological and molecular breeding approaches suggested that a careful rethink is needed in order to understand better and breed for drought tolerance [10]. Research programs for drought tolerance tackled the problem in a multidisciplinary approach, integrating functional genomics; physiological dissection of drought-tolerance traits; and genetic tools, such as quantitative trait loci (QTL), positional cloning, microarrays, and genetic engineering. Germplasm and genetic resources remain crucial.

Some promising results are now available. Protein kinases play major roles in response to abiotic stress signals. TMKP1 has been found as a novel drought stress responsive mitogen-activated protein kinase phosphatase in wheat [11]. The accumulation of

osmolytes in leaf tissues and the abscisic acid-induced stomatal closure are well-recognized mechanisms associated with drought tolerance. The leaf proline content is seen to be a suitable indicator for selecting drought-tolerant wheat genotypes [12].

In addition to physiology and phenology research, researchers on wheat genetics have discovered many QTLs affecting yield under drought or the expression of drought tolerance-related traits. Researchers have provided genes useful either as candidate sequences to dissect QTLs or to develop transgenic wheat varieties. Some main results can be mentioned. Wheat plants transformed with a gene from *Vigna aconitifolia* show accumulation of proline, which appears to be a promising approach to maintain the productivity under water stress condition [13]. Anther culture-derived haploid embryos were used as explants for *Agrobacterium*-mediated genetic transformation of bread wheat using barley HVA1 gene for drought tolerance (doubled haploid technology for development of stable drought tolerant transgenics) [14]. Drought- and salt-tolerant wheat plants were created by transferring genes from barley into a local wheat variety [15]. A cotton (*Gossypium hirsutum*) DRE-binding transcription factor gene confers enhanced tolerance to drought in transgenic wheat [16]. The expression of AlSAP (stress-associated protein) gene from the halophyte grass *Aeluropus littoralis* improves tolerance to drought in transgenic durum wheat [17].

No scientific papers on drought tolerance were published by European research institutes yet. In Hungary the Biological Research Centre (Hungarian Academy of Sciences, Szeged) had isolated an alfalfa aldose reductase gene, which confers tolerance to abiotic stress. This gene has been incorporated in spring wheat for testing for drought tolerance. This current research used both transgenesis and new techniques of plant breeding. GM plants are currently evaluated in vitro and in glasshouses. A French Florimond Desprez company and Bioceres (a venture named Trigall Genetics [18]) develop and market GM wheat varieties with next generation biotechnologies (HB4® technology), for the Latin-American region. Argentine researchers have isolated the drought tolerance gene (HB4) from sunflower, which has been inserted in wheat, maize, and soybeans varieties with promising results. This HB4® technology will help increase average crop yields between 10 and 15% in areas where crops are unreliable due to soil salinity or frequent water deficits. Trigall Genetics is targeting a 2016 launch for these drought-tolerant wheat varieties in Argentina. Perhaps this technology will be used in Europe if the political context allows it but nothing could be less certain (*see* Subheading 5). In the UK, an ongoing research program aims to genetically engineering the wheat nuclear and chloroplast genomes in order to improve total crop biomass and grain yield through increased photosynthetic efficiency, altered canopy and root architecture, modified seed

development and enhanced nutrient utilization efficiency. An English and Chinese team increased grain yields and grain protein concentrations in transgenic wheat plants overexpressing NAC1-type transcription factor (TaNAC-S) resulting in delayed leaf senescence [19].

4 Nutritional Properties

Another major priority for wheat breeding is to improve nutritional quality, including vitamin and mineral contents for the developing world and starch digestibility and dietary fibre content and composition for developed countries. Glutens, the storage proteins in grains, are a major source of protein in human nutrition. The protein composition of wheat has therefore been an important focus of research using genetic engineering and omics [20] used to understand the impact of specific gluten proteins on wheat quality. The use of transgenesis and RNAi silencing aims at improving the quality of gluten.

In the UK, the use of genetic engineering aims at studying qualitative and quantitative changes in glutenin subunit composition and to manipulating the patterns of gene expression and pathways of protein trafficking and deposition to develop novel wheat varieties with improved processing properties. Basic research carries out the use of transgenic lines with epitope tagged forms of gluten proteins and tagged compartments of the secretory pathway, to identify gene function and monitor the impact of environmental stresses, such as high temperature and drought, on the morphology and physiology of these organelles, and the way this affect gluten protein deposition and glutenin polymers assembly. Genetic engineering via upregulation and RNAi-mediated silencing will be one strategy used to enhance the contents and bioavailability of minerals (experiments in progress in the greenhouse/growth chamber facility).

Genetic transformation provides a precise tool to alter the composition of wheat grain by expressing new genes or by down-regulating groups of proteins encoded by gliadins (multigene families). They contain clusters of epitopes that are active groups in triggering celiac disease. Indeed, the majority of the epitopes responsible reside in the gliadin fraction of gluten. Celiac disease is an enteropathy triggered by the ingestion of these gluten proteins. The inflammatory reaction is controlled by T cells that recognize gluten peptides in the context of human leukocyte antigen (HLA) DQ2 or HLA-DQ8 molecules. The only treatment for the celiac disease is a lifelong gluten-exclusion diet. For a decade, biotechnology has been used to produce novel gluten phenotypes. In Spain, a new approach to silence multiple gliadin genes was used to produce GM lines of bread wheat containing combinations of endosperm-

specific promoters and different inverted repeat sequences to silence fractions of gliadins by RNA interference [21]. A French and American team used two-dimensional immunoblot analysis for assessing the allergenic potential of transgenic wheat lines in which, omega 5-gliadin genes were silenced by RNAi [22]. The omega 5-gliadins are known to be the major sensitizing allergens in wheat-dependent exercise-induced anaphylaxis (WDEIA). In the UK, GM bread and pasta wheats with entire gliadin pathway downregulated are developed for consumption of celiac and gluten-intolerants. This technology is developed at public research institutes and now licensed by PBL to a multinational agribusiness company in the UK. The downregulation of gliadins by RNAi can be used to obtain wheat lines with very low levels of toxicity for patients. A Spanish team targeted prolamins by RNAi for silencing fragment combinations and obtained lines devoid of celiac disease epitopes from highly immunogenic gliadins [23].

5 Conclusion

There are 58 GM foods and feed authorized in the EU for food and feed, and only one GM crop (Bt maize, a GM maize with MON 810 event) for cultivation [24]. In the EU, authorizations of GM crops are valid for a maximum of 10 years (renewable). In March 2015 the European Council and the Parliament agreed on an amendment of the GMO legal framework to allow Member States to restrict or prohibit the cultivation of authorized GMOs (Directive 2015/412/EU) [25]. Member States would have to justify that their opt-out measures are compatible with the internal market. Opt-outs shall be based on legitimate reasons other than those assessed at EU level by EFSA, i.e., risk to human or animal health or the environment. In April 2015, the European Parliament adopted the possibility for individual Member States to ban the cultivation of GM crops approved at EU level, not based on scientific criteria and evidence [26]. This new Directive (EU) 2015/412 called "opt-out" is be based on grounds other than scientific safety assessment of health and the environment, criteria that are assessed by the European Food Safety Agency (EFSA). But, in September 2015, the agriculture committee of the European Parliament rejected the European Commission proposal on the possibility for the Member States to restrict or prohibit the use of EU-approved GM food or feed on their territory [27]. Such a ban would have consequences for the competitiveness and freedom of choice for farmers, for the scientific potential of the EU, and for the EU contribution to European and global food security.

To date, 19 European countries restrict the cultivation of GM crops under the Directive (EU) 2015/412 or Regulation (EC) No 1829/2003: Austria, Bulgaria, France, Germany, Greece,

Hungary, Italy, Luxemburg, and Poland, along with Croatia, Cyprus, Denmark, Latvia, Lithuania, Malta, the Netherlands, Slovenia, and four regions in two countries (Wallonia in Belgium; Northern Ireland, Scotland, and Wales in the UK). None of the five European countries that currently grow Bt maize decided to "opt out" of GM crop cultivation for all or part of their territories: Spain (representing more than 90% of the total area of Bt maize in the EU), the Czech Republic, Portugal, Romania, and Slovakia. In the other countries (Ireland, Sweden, Finland, and Estonia) and regions (Flanders in Belgium, England in the UK), cultivation is still allowed while no Bt maize is grown.

It appears in this literature search that in Europe wheat research is underrepresented. Although conventional breeding (MAS) will continue to play a major role in increasing wheat yield and quality, genetic transformation to introduce novel genes into wheat and genome editing will be essential in complementing existing breeding tools (MAS) [28]. In Europe, only few research programs at a very early stage focus on developing and improving methods for targeted insertion of genes and the targeted modification of plant genes using site-specific nucleases (including CRISPR-cas9). In France, zinc finger nucleases along with CRISPR-cas9 are being used on wheat in "Genius" project (2012–2019) for proof of concept of various stresses including biotic stress and nitrogen use efficiency. In the UK, research programs have started testing CRISPR-cas9 in barley for targeted mutagenesis and hopefully later in wheat. In Belgium and Hungary, oligonucleotide-directed mutagenesis (ODM) is developed but not on wheat until now. A Hungarian team reports simultaneous application of the minimal gene cassette and cold-inducible Cre/lox recombination system in wheat, which produces selection gene-free transgenic wheat lines [29].

In Europe, few public research programs are dealing with biotic and abiotic stresses, agronomic properties, and nutritional quality. Climate instability will not only make some parts of the world hotter and drier, but it will change the whole pattern of pests and diseases. However, the current political situation in certain EU countries is an impediment to genetic engineering research (e.g., transgenesis and new breeding techniques which include nucleases and ODM) in order to address these agricultural challenges in the future [7]. The decline of experimental field trials number in the EU from 2000 [30] reflects a consequence of vandalism [31] and the political restrictions on genetic engineering research efficiency. In a multidisciplinary approach, promising new genetic tools for breeding would improve wheat resistance to pests and diseases, tolerance to drought, and grain quality in European countries.

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Chapter 3

Overview of the Wheat Genetic Transformation and Breeding Status in China

Jiapeng Han, Xiaofen Yu, Junli Chang, Guangxiao Yang, and Guangyuan He

Abstract

In the past two decades, Chinese scientists have achieved significant progress on three aspects of wheat genetic transformation. First, the wheat transformation platform has been established and optimized to improve the transformation efficiency, shorten the time required from starting of transformation procedure to the fertile transgenic wheat plants obtained as well as to overcome the problem of genotype-dependent for wheat genetic transformation in wide range of wheat elite varieties. Second, with the help of many emerging techniques such as CRISPR/cas9 function of over 100 wheat genes has been investigated. Finally, modern technology has been combined with the traditional breeding technique such as crossing to accelerate the application of wheat transformation. Overall, the wheat end-use quality and the characteristics of wheat stress tolerance have been improved by wheat genetic engineering technique. So far, wheat transgenic lines integrated with quality-improved genes and stress tolerant genes have been on the way of Production Test stage in the field. The debates and the future studies on wheat transformation have been discussed, and the brief summary of Chinese wheat breeding research history has also been provided in this review.

Key words Wheat, Genetic transformation, Breeding, End-use quality, Stress tolerance

1 Introduction

Wheat, as one of the three major cereal crops, as well as one of the earliest cultivated crops in the world, is an essential source of calories in human diets and animal feed [1]. About 40% human population lives on wheat and wheat foods all over the world. Asia has the most distribution of wheat production compared to other areas, accounting for 45% and total wheat cultivation and production of other areas is about 55%. Several studies claimed that the annual growth rate of global wheat production has to be maintained at about 2% to meet the growing demand. However, the present situation is that the arable land for wheat cultivation is quite limited, resulting in the more challenges to satisfy the demand of wheat production worldwide.

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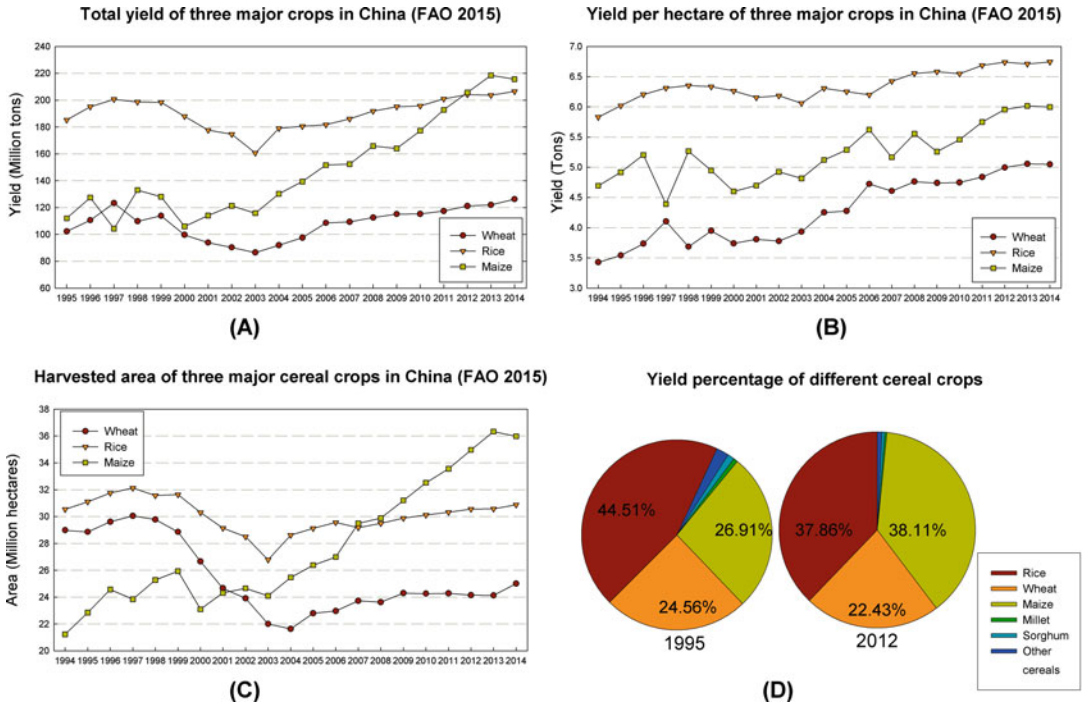

Fig. 1 Production summary of main cereal crops in China. (**A**–**C**) The total yield, yield of per hectare, and harvested area of three major crops (maize, rice, and wheat) from 1995 to 2014 in China (Data from FAO 2015), respectively. (**D**) The yield percentage changes of different cereal crops including maize, rice, wheat, millet, sorghum, and other cereals from 1995 to 2012 (data from National Bureau of Statistics of China in 2015)

China is a largest wheat production country. Data published by FAO (Food and Agriculture Organization of the United Nations, 2015) and National Bureau of Statistics of China in 2015 showed that wheat production in 2014 has increased by 3% comparing to that of 2013, reaching 126.2 million tons, which accounts for 22.6% of the cereals yield. The harvested area of wheat in China has reduced by 13.7% from 1994 to 2014, whereas the yield of each hectare has been up to approximately 1.47 times (Fig. 1). In 2012, the harvested area of wheat in China was 24.1 million hectares and the productivity was about 121 million tons, which was the first time that the yield of wheat had exceeded 120 million tons since 1997.

However, various challenges, such as pathogens, pests, and changeable climate as well as the conflict between agricultural land demand and the land for urbanization and industrialization are still threatening wheat production in China. Moreover, emerging challenges due to improving living standards have more requirements on wheat nutrition. Therefore, both of wheat productivity and end-use quality need to be increased and improved through various breeding methods. Varieties with increased grain

yield, improved edible quality, better end-use property, enhanced tolerance to abiotic stresses and resistance to diseases and pests are still urgently needed. Wheat breeding methods, including traditional cross breeding and molecular breeding, have contributed to improve the Chinese wheat production capacity over the past several years [2].

In this review, we outline the development history of wheat genetic breeding in China, discuss the progress of different wheat transformation methods as well as their application in wheat genetic breeding, including developing new wheat varieties with improved quality, enhanced stress tolerance and increased grain yield. The prospect of wheat transformation in China has also been proposed.

2 Brief Summary of the Development History of Wheat Genetic Breeding in China

It has been more than 5000 years since ancient Chinese agronomists began to select crops with excellent traits on breeding purpose. It was not until the end of the nineteenth century when new techniques spread into China and Chinese scientists started to learn and use modern scientific methods for wheat breeding. Then crop breeding reached a new stage, in which the heterosis had been made the most of. Cross breeding refers to the hybridization between individuals with different populations and different wheat genotypes, and selecting homozygous varieties from offspring generations, where the breeding of backbone parents plays a key role in the yield potential increase. Yield potentials can be improved through increasing numbers of grain per panicle, improving plant shapes, enhancing lodging resistance, and raising harvest indexes, though the improvement of potentials of yield components can be different in different areas. In 1926, the first improved wheat variety, Jinda 26, was successfully cultivated through cross breeding technology in China. Then, more and more wheat varieties were developed with the help of new breeding methods and the application of statistics theory. In 1950s and 1960s, great efforts were made by Chinese government aimed at improving wheat breeding, including encouraging breeders to try new strategies on breeding at Chinese National Work Conferences, and great progress has been made by exploitation of cross-fertilized, radiation induced mutation breeding and pollen culture techniques, respectively. The potential of wheat genetic diversity was sufficiently explored and about 570 cultivars such as Bima 1 and Nada 2419 with high yield and good resistance to pathogens were developed during 1949–1979 in China. Currently, crossbreeding is still a major method for wheat breeding in China. During the past two decades, the yield potentials of wheat cultivars in China, particularly in Henan and Shandong provinces, have been continuously

increasing. Numbers of grain per panicle and harvest indexes in both places have been significantly increased [3].

The continuous development of modern molecular biology brings new opportunities to wheat breeding. Genetic modification (GM) technology is an emerging and powerful tool for crop improvement, as well as for functional study of genes, which has made it possible to develop the varieties of crops within a relatively short time. The first transgenic wheat was obtained in 1992 by biolistic approach [4]. In 1993, Chinese scientists obtained the transgenic wheat with laser micro-beam method [5], while the first transgenic wheat by biolistic approach was obtained in China in 1994 [6]. Since then, great progress has been made in wheat genetic transformation technology. Chinese government has initiated the National Genetically Modified New Varieties of Major Projects of China since 2008, aiming at promoting new varieties of GM staple crop breeding [7]. With the Chinese government's support, great progress has been made in the GM wheat research in China. However, due to the difficulties associated with gene delivery and its low efficiency, wheat is still a recalcitrant species for genetic transformation [7, 8]. Although wheat genetic transformation method has been rarely applied to agricultural industry in China because of the transgenic food safety issues, wheat transgenic research has made great progress in recent years, and numerous transgenic wheat lines with better properties than common wheat varieties have been obtained. For example, *TaNAC-5A*-overexpressing transgenic wheat lines have been proved to accumulate higher nitrogen and have higher grain production in field experiments, which made the fertilization more efficient [9]. The resistance to *Bipolaris sorokiniana* and drought stress condition was enhanced significantly in wheat lines overexpressed *TaPIMP1* [10]. The sodium dodecyl sulfate sedimentation volume has been increased and the flour mixing characteristic of dough has also been improved in transgenic wheat lines overexpressed *avenin-like b* gene [11]. A summary of different wheat transformation methods used in China is listed in Table 1.

3 Current Status of Different Wheat Genetic Transformation Methods

Over the past two decades, plenty of wheat transformation methods, such as biolistic bombardment, *Agrobacterium*-mediated transformation, pollen-tube pathway, ion implantation, laser micro-beam puncture, treatment with polyethylene glycol (PEG) and ultrasonic wave, and electroporation have been reported resulting in significant increase in wheat transformation efficiency [5, 18]. Through biolistic bombardment, Zhao et al. [13] transformed pAHC25-*DREB1A* into young spikes of wheat *cv.* H311 and H6756 with the transformation efficiencies of 2.53% and

Table 1
Summary of different wheat transformation methods used in China

Transformation methods	Wheat genotypes	Explants type[a]	Plasmid or gene	Transformation efficiency (%)	Reference
Biolistic bombardment	Zhong-60,634	Precultured IE	pAHC25	0.88	[12]
	H311	YS	pAHC25-DREB1A	2.53	[13]
	H6756	YS	pAHC25-DREB1A	5.36	[13]
	Emai 12	IE	Cassettes, pAHC25, pCal-neo	1.1, 0.4, 0.5	[14, 15]
	Emai 12	ME	pCal-neo	0.3	[15]
	Mianyang 19	Callus from IE	pHMW1Bx14	0.28	[16]
	Baofeng 104	IE	Gcn5RNAi	1.80	[17]
	Liaochun10	IE	GUS	1.35	[17]
	Kenong 199	IE	pAHC25	2.45	[18]
Agrobacterium-mediated transformation	Yangmai 10	Callus from IE	pPTN254 (C58c1)	0.8	[19]
	Yan 103, Hesen no. 3	Callus from IE	*AtNHX1* (GV3101)	1.3, 2.9	[20]
	Bainong 160	Callus from ME	(LBA4404)	2.36	[21]
	Jinan 17	SAM	pUN-1301-ubi-TaCHP	6.7	[22]
	Jimai 22	SAM	pUN-1301-ubi-TaCHP	5.8	[22]
Pollen-tube pathway transformation	Shi 4185		*DNAj* from *Foxtail millet*	0.142	[23]
Electroporation	Shi 5093, Jinghong 5		pAHC25	6.0, 7.5	[24]
	Jimai 19		pWMB002	1.79	[25]
Protoplast transformation	Hartog		pEmuGN, pEmuPAT	73	[26]

[a] *IE* immature embryo, *ME* mature embryo, *YS* young spikes, *SAM* shoot apical meristem

5.36%, respectively. Wu et al. [27] transformed the durum wheat variety Ofanto with *Agrobacterium* strain *AGL1* and the transformation efficiencies were between 0.6 and 9.7%. Liu et al. [22] introduced salt tolerance gene *TaCHP* into shoot apical meristem of wheat seedlings of Jinan 17 and Jimai 22 by *Agrobacterium* infection with the average transformation efficiency of 6.7% and 5.8%, respectively. The problem of genotype dependent for wheat genetic transformation has already been overcome in wide range of wheat varieties.

3.1 Biolistic-Mediated Transformation

Biolistic-mediated transformation method is regarded not to be restricted by the receptor plant resources, making this method be widely applied in wheat genetic transformation study. In 1992, the wheat immature embryo calli were successfully used to achieve the first wheat transformation by particle bombardment [4]. Since then, the particle bombardment has been developed as a robust, versatile, and relatively efficient method for wheat transformation.

Many factors, such as bombardment parameters, explant types chosen for wheat transformation, precultured conditions, and screening process or regeneration rate of resistance plant may have an impact on the efficiency of particle bombardment [8]. To increase transformation efficiency, the particle bombardment transformation system has been gradually optimized. Yao et al. [14] transformed the immature embryos of wheat variety Emai 12 with different bombardment parameters and found that the acceleration pressure, target distance, gold particle size could obviously affect the transient expression of *gus* gene. When the immature embryos were transformed at pressure of 650 psi, target distance of 6 cm and gold particle diameter of 0.6 μm, the stable transformation efficiency increased to 1.1% for linear gene cassettes and 0.4% for pAHC25 plasmid [14]. Min et al. [18] determined the parameters of 0.6 μm gold particle diameter, 5.5 cm target distance, and 650 psi acceleration pressure as the optimum to transform the immature embryo of wheat variety Kenong 199 with an average efficiency of 2.45%.

The explants for wheat transformation are generally immature embryos or embryogenic calli derived from immature embryos. Immature embryos can only be collected within a very short period during wheat life cycle [28], largely restricting the application of transgenic wheat greatly. Few reports about using mature embryos or embryogenic calli derived from mature embryos as explants have been published [29]. Zhao et al. [13] compared the quality of calli from young spikes with that from immature embryos of 15 different wheat genotypes, showing that young spikes were better explants in wheat tissue culture with higher plantlet regeneration rate. Ding et al. [15] optimized the mature embryo transformation system by using a bombardment pressure of 900 psi at a distance of 6 cm and 20–40 mg/L G418 for selection with transformation efficiency up to 0.3–0.9%. In addition, Dong et al. [30] preliminary established the biolistic-mediated wheat transformation of shoot apical point with the pressure of 1100 psi and target distance of 9 cm. Liu et al. [16] transformed a linear minimal expression cassette with the HMW-GS *1Bx14* gene into the immature embryos of wheat cultivar Mianyang 19 by microprojectile bombardment with an efficiency of 0.28%.

Precultured conditions before the bombardment are also critical to the biolistic-mediated wheat transformation efficiency. Liang et al. [12] found that the transformation efficiency of the fresh

immature embryos scutella precultured at 25 °C in darkness for 4 days was 0.88%, and 2 mg/L dicamba in the medium contributed to a higher differentiation rate. Ren et al. [31] found that when precultured for 10–15 days and treated with 0.4 mol/L mannitol, the immature embryos exhibited higher plant regeneration frequency. Li et al. [17] modified the preparation of embryo isolation and screened five genotypes with relative higher regeneration frequency of the mature embryos, which varied from 57.60 to 73.11%. The results showed that embryos after 4 °C overnight soaking with sterile water could improve the induction and regeneration frequency of calli in wheat mature embryo culture system. Then they transformed *gus* and *Gcn5RNAi* genes into the precultured mature embryos of Liaochun 10 and Baofeng 104 by bombardment with the efficiencies of 1.35% and 1.80%, respectively [17]. Cai et al. [32] investigated the effects of different medium on biolistic transformation efficiency, found that the calli could have the best percentage (65%) of transgenic plantlets with the *KN2* gene when induced on medium containing Cu^{2+} and precultured for 4 days, then cultured on R medium. New optimized methods for wheat immature embryos transformation have been extensively applied in wheat genetic engineering research and a wide range of transgenic wheat lines has been successfully generated by biolistic-mediated transformation [33].

3.2 Agrobacterium-Mediated Transformation

The *Agrobacterium*-mediated transformation system relies on the unique insertion mode of *A. tumefaciens* and *A. rhizogenes* to transfer and integrate T-DNA from Ti or Ri plasmids into the genomes of recipient plants [34]. Unlike biolistic transformation method, *Agrobacterium*-mediated transformation allows insertion of a well-defined DNA segment into the host genome [35] and presents stable heredity and expression of exogenous genes in transgenic plantlets. Efforts have been made to establish a simple and efficient *Agrobacterium*-mediated transformation system in monocotyledon plants since it was successfully established in dicotyledonous plants. First efficient transformation system mediated by *A. tumefaciens* in rice [36] and maize [37] were established, followed by the *Agrobacterium*-mediated transformation of wheat that was reported in 1997 [38]. However, the efficiency of *Agrobacterium*-mediated transformation for wheat stays at a relatively low level until now, to some extent, influencing the research and application of wheat and wheat products [39].

In recent years, several cases of *Agrobacterium*-mediated wheat transformation have been reported in China. Current recipient materials under investigation include young spikes, anthers and immature embryos. Various exogenous genes involved herbicide resistance, disease and insect resistance, drought and salt tolerance, as well as factors affecting the efficiency of *Agrobacterium*-mediated transformation have also been discussed [39–43]. It is generally

recognized that wheat genotype also plays a vital role in transformation efficiency. Ye et al. [19] transformed target genes contained in *Agrobacterium* strain *C58c1* into 4 days precultured embryos of wheat varieties Bobwhite and Yangmai 10 with an average efficiency of 0.82%, showing that the growing status and genotypes of explants had remarkably different influences on wheat transformation. Xue et al. [20] used *A. tumefaciens* carrying *NPTII* gene and *AtNHX1* gene to invade calli derived from three wheat varieties, and the efficiencies turned out to be 0, 1.3%, and 2.9%, respectively, confirming that genotypes could indeed influence wheat transformation efficiency. Wang et al. [40] used *Agrobacterium* stain *C58c1* to infect explants with different genotypes from different cultivation areas, finding that wheat transformation could be affected by not only genotypes, but the coculture approaches during the infection process as well.

Using *Agrobacterium* to infect embryonic calli of immature embryos, Bi et al. [44] constructed transgenic wheat lines with the overexpression of *cowpeatrysin inhibitor* (*CpTI*) gene and found that the resistance of transgenic lines to the wheat storage pest was improved. Ding et al. [45] first investigated the influence factors of *Agrobacterium*-mediated wheat transformation via mature embryos from Emai 12. Then through culturing wheat mature embryos on the precultured medium for 14 days, treating in infiltration suspension for 3 h following coculturing the explants wrapped up in wet sterilized filter paper for 3 days, they showed that the percentage of stable GUS expression on calli and G418 resistant plantlets was 15.83 and 0.28% [45]. Yang et al. [41] analyzed the transient expression of *uidA* gene in wheat seedlings of different ages by infecting the wounded apical meristems with *A. tumefaciens* and found that the inoculation time showed more significant potential in improving the transformation efficiency [41].

He et al. [21] studied the transformation efficiency of the mature embryo calli of wheat variety Bainong 160 with *Agrobacterium* strain *LBA4404* under different conditions, including *Agrobacterium* concentrations, infection periods, and concentrations of acetosyringone and cephalosporin. The highest efficiency turned out to be 2.36% [21]. Song et al. [43] used mature embryo calli of six different varieties of wheat infected by *A. tumefaciens* strains (*EHA105* and *LBA4404*) and the results showed that differentiation ratios, induction time of calli differed in different wheat varieties, and also the regeneration frequencies could be influenced by infection time and concentrations of *Agrobacterium*, while little difference was exhibited between strains *EHA105* and *LBA4404* [43].

3.3 Pollen-Tube Pathway Transformation

The pollen-tube pathway (PTP) transformation, which can transfer foreign DNA into ovule by flowing down the pollen tube after pollination, was applied in rice transformation for the first time in 1988 [46], and it was also successfully put into use in wheat in 1994

[47]. Qiu et al. [48] transformed an exogenous DNA labeled with FITC (fluorescein isothiocyanate) into wheat via the PTP, and the results showed that the transformation buffer containing 0.05% Silwet L-77 and 5% sucrose promoted the exogenous DNA into embryo sacs; nonetheless, there was no improvement in the transformation efficiency. Wang et al. [23] introduced a drought and heat tolerance related gene *DNAj* from *Foxtail millet* into four wheat cultivars via the PTP with the transgenic efficiency of 0.142%. Ou et al. [49] introduced the genome DNA of sorghum into the soft-grain wheat varieties which were highly infectable to stripe rust via the PTP and obtained two stable lines with stripe-rust resistance and quality improvement. Zhang et al. [50] transformed *alfAFP* (alfalfa antifungal peptide) gene, *LTP* (lipid transfer protein) gene and antibacterial peptide *spCEMA* gene into wheat cultivars Xiaoyan 22, Xiaoyan 6, and Xinong 1376 by PTP, respectively, and obtained a transgenic efficiency of 0.135% on average.

3.4 Electroporation Transformation

Electroporation of cells is another way to introduce foreign DNA into bacterial, fungal, animal, and plant cells for transformation. Liang et al. [24] successfully delivered plasmid DNA with *bar* and *gus* genes into the wheat immature embryos via electroporation at 770 V/cm field strength and 800 μF capacitor. The transformation efficiency was 7.5% verified by PCR and Southern blot confirmations. Zhang et al. [25] optimized the pollen electroporation transformation and obtained transgenic plants in wheat variety Jimai 19. Now pollen electroporation transformation system for common wheat has been successfully established.

3.5 Protoplast Transformation

Plant protoplast, whose cell wall is removed partially or completely by mechanical or enzymatic means, possesses all the characteristics of living cells. The main source of protoplasts includes leaves, root tips, pollen, and callus cells. The plant regeneration of protoplast culture generally goes through four stages: cell wall regeneration, cell division, callus (or embryonic form), and plant regeneration. An efficient approach for genetic transformation is proposed to transfer exogenous plasmids with the target gene into protoplasts by electroporation or treating with PEG. With respect to wheat transformation, pollens are frequently chosen to prepare the protoplasts. He et al. [26] obtained the positive regenerated wheat plants through isolating protoplasts from wheat suspension cultures, then electroporating plasmids with *gus* and *bar* genes into the protoplasts, establishing a way for wheat transformation by directly transferring gene into protoplasts. In addition, pollen can also be used to isolate protoplasts. Haliloglu et al. [51] explored the delivery of target plasmid DNA into embryos derived from wheat anther culture using electroporation experiment and obtained four regenerated plants on selection medium containing different concentrations of PPT.

With the development of protoplast transgenic technology, it has been applied in the transient expression of exogenous genes on a large scale. Xu et al. [52] transferred the target genes into the protoplasts isolated from mesophyll tissue of 1-week-old wheat seedlings. The detective GFP fluorescence indicated that the target proteins were located in the nuclei [52]. Huang et al. [53] also used the wheat protoplast transient expression to analyze subcellular localization of TaNAC29 in wheat cells.

4 Advanced Progress in Wheat Transgenic Breeding

In China, the problem of genotype dependent of wheat genetic transformation has been eased and more than 50 elite wheat varieties have been used for genetic transformation studies, including Bainong 160, Baofeng 104, Emai 12, Jimai 22, Jinan 17, Kenong 199, Liaochun 10, Xinong 1376, Xiaoyan 6, and Xiaoyan 22 [14, 17, 18, 21, 22, 50]. Since the first transgenic wheat was generated by particle bombardment in 1992, the herbicide resistance related genes, which could be selective marker genes, such as *bar* [4], *NPT-II* [54], *GOX*, *CP4* [55], *bxn* [56], and *EPSPs* [57] have been extensively used in wheat transformation. Recently, in the study of wheat transformation, functional genes introduced into wheat are mainly relevant with quality improvement, resistance to pathogens and insects, abiotic stress tolerance, male sterility, yield improvement, etc. A summary of different genotypes and genes for wheat transformation in China is listed in Table 2.

4.1 Progress in Wheat Quality Improvement

Currently, there is an increasing requirement for high quality food, especially for wheat-based food products. Genetic engineering makes it possible to explore the molecular and biochemical basis of the wheat bread-making quality, to develop wheat varieties with different protein subunit combinations and to generate lines with improved or novel properties. Based on the capacity of the elasticity and viscosity of dough, also described as strength, bread wheat can be made into various foods, such as noodles, steamed bread, pasta, and biscuit [84, 85]. In China, however, wheat cultivars with high quality dough are scarce and most elite wheat varieties are not bread-making wheat. Nevertheless, early studies have demonstrated that wheat storage proteins, like gluten proteins, are the most important components, which govern dough characteristics and influence end-use quality and utilization of wheat. The fraction of glutenin is also critical to wheat dough elasticity [86–90]. It has been confirmed that the bread-making quality is influenced by the high molecular weight glutenin subunits (HMW-GS) combinations such as 1Dx5 + 1Dyl0, 1Ax1, 1Ax2* and 1Bxl7 + 1Byl8 [87, 90–93]. Therefore, a set of transgenic wheat lines expressing one or two extra HMW-GS were generated to improve the bread-

Table 2
Summary of different genotypes and genes for wheat transformation in China

Wheat genotypes	Transformed genes	Gene function	Reference
Chuan 89-107, Emai 18	*1Dx5*	Improving bread-making performance	[58]
Chuan 89-107, Chuanmai 107, Emai 12	*1Ax1*	Improving bread-making performance	[58–60]
Emai 12	*Avenin-like b*	Improving flour mixing properties	[11, 61]
Luna, Venusia	*PinA*	Controlling grain hardness	[62, 63]
G8901	*NP-1*	Biotic stress	[64]
Yangmai 11	*Antifungal peptides (AFPs)*	Biotic stress	[65]
Yangmai 12	*TiMYB2R-1*, *TiERF1*	Biotic stress	[66, 67]
Yangmai 12	*TaPIMP1*	Biotic stress and drought	[10]
Yangmai 18	*TaLTP5*, *Snakin-1*	Biotic stress	[68, 69]
Alondra, Yangmai12	*AtDREB2A*, *OsSNAC1*	Improving the salt and drought tolerance	[70, 71]
Bobwhite, Xin Chun 9	*ZmED-Tu*	Ameliorating negative effects of heat stress	[72]
Kenong 199	*TaSUT1A*	Drought resistance	[73]
Xiaoyan 54	*TaSCL14*	Involved in plant growth, photosynthesis, tolerance to photo-oxidative stress, and senescence	[74]
Yangmai 10, Lumai 23	*GhDREB*	Improving the tolerance of drought, high salt, and freezing stresses	[75]
Yangmai 12	*TaERF3*	Responding to salt and drought stresses	[76]
Yunmai 34	*TaBTF3*	Impairing tolerance to freezing and drought stresses	[77]
Bobwhite	*crtB*, *crtI*, *TaHYD*	Increasing carotenoid contents	[78]
Emai 12	*Zmy1*, *crtI*	Increasing carotenoid contents	[79]
Shi 4185	*TaGW2*	Negatively regulating the grain width and weight	[80]
Shaan 512	*TaCYP78A5*	Affecting seed size	[81]
Yumai 34	*TaLSUI*	Enhancing the starch biosynthesis	[82]
Zhoumai 19	*ZmPEPC*	Increasing grain yield	[83]

making quality. By the combination of transgenic with conventional crossing and backcrossing techniques, *1Ax1* was introgressed into commercial elite wheat cultivars from model wheat lines with the results showing that gluten elasticity and viscosity during early mixing were significantly increased and the overall dough mixing performance was improved as well [58–60, 94].

Low molecular weight glutenins make up 14–27% of total wheat seed proteins, which can form highly polymorphic protein complex. Additionally, low molecular weight glutenins have abundant allelic variations, making them good candidates for the major functional markers of gluten quality [95]. With the development of electrophoresis technology, more and more studies have proved that low molecular weight glutenin subunits (LMW-GS) play an essential role in determining wheat functional properties, such as the viscoelasticity of durum wheat gluten [96]. Zhang et al. [97] investigated 16 Aroona (*T. aestivum* L.) near-isogenic lines (NILs) by determining the dough properties and bread-making quality of individual NILs. They identified 18 LMW-GS genes in individual NILs and found that LMW-GS alleles affected both dough extensibility and bread quality [97]. Si et al. [98] isolated eight LMW-GS from landrace (*T. aestivum* L.) and analyzed the protein sequence and structure indicating that the Glu-B3-1a could form more disulfide bonds which had the potential as a candidate gene for improving bread-making quality.

Moreover, another storage protein named avenin-like has been characterized, which possibly contributes to the functional properties of wheat flour [99, 100]. Overexpression of *avenin-like b* gene in Emai 12 by particle bombardment-mediated wheat immature embryo transformation confirmed that avenin-like b proteins could form interchain disulfide bonds to crosslink with glutenin polymers, thus improving flour mixing properties [11, 61].

Grain hardness affects milling, baking, and other end-use qualities of bread wheat. It has been demonstrated that friabilin consisting of puroindoline-a (Pin-a) and puroindoline-b (Pin-b) proteins is the main determinant of grain hardness [101]. Puroindolines have strong impacts on flour yield, starch damage and the water absorption of dough. Xia et al. [102] observed that hardness values of wheat transgenic lines with the knockout of *Pin-a* were in the range of hard wheat, while overexpression of *Pin-a* made kernel texture softer than control. Li et al. [62] found that coexpression of *1Ax1* and *Pin-a* in durum wheat showed better dough mixing properties comparing with transgenic lines expressing either *1Ax1* or *Pin-a*.

Cong et al. [79] introduced *Zm-y1* gene encoding phytoene synthase and carotene desaturase gene *crtI* from *Erwinia uredovora* into the wheat variety Emai 12 and obtained the transgenic wheat with light yellow color endosperms. Wang et al. [78] cotransformed the bacterial phytoene synthase gene (*crtB*) and *crtI* into

Bobwhite and obtained transgenic wheat lines coexpressing both genes with an increase of approximately eightfold of total carotenoid, 65-fold of beta-carotene, and 76-fold of provitamin A content comparing with wild-type wheat. Zeng et al. [103, 104] cloned a genuine lycopene cyclase gene *TaLCYB* from Chinese Spring. The *TaLCYB* was downregulated expression through RNA interference (RNAi), resulting in a decrease in the content of beta-carotene and lutein as well as the accumulation of lycopene. They also found that endosperm-specific silencing of the carotenoid hydroxylase gene (*TaHYD*) and overexpression of *crtB* in transgenic wheat both increased beta-carotene. In addition, combination of overexpressing *crtB* and silencing *TaHYD* obtained significant accumulation of beta-carotene, which was increased up to 31-fold to 5.06 μg/g [103, 104].

4.2 Resistance to Biotic Stress in Transgenic Wheat Lines

A variety of diseases especially caused by fungus, such as rust diseases, powdery mildew, and root rot, could have devastating consequences on wheat yields and quality. Conventional plant protection and breeding strategies, like fungicides are easy and effective, but not environmentally friendly and affordable. Identification and selection of resistant genes for wheat breeding to improve the resistance to pathogen have been urgent. Although wheat transgenic lines have been on the way of Production Test stage, there is still a long way before the application of transgenic wheat in commercial market. Rabbit defensin *NP-1* gene, which has a broad resistance spectrum to pathogens, was introduced into the wheat cultivar G8901 via the PTP. Four transgenic plants overexpressing *NP-1* gene exhibited higher resistance to powdery mildew, leaf rust and stripe rust [64]. Liu et al. [65] cotransformed different antifungal peptides (AFPs) into an elite wheat cultivar Yangmai 11 by biolistic bombardment and obtained transgenic wheat with an improved overall resistance to Fusarium pathogens. Zhu et al. [68] introduced *TaLTP5* (lipid transfer protein) gene into wheat *cv.* Yangmai 18 by biolistic bombardment and it turned out that the resistance to *Cochliobolus sativus* and *Fusarium graminearum* was significantly enhanced in all six transgenic lines. By bombardment-mediated transformation, the gene *Snakin-1* (*SN1*) amplified from potato was transformed into wheat *cv.* Yangmai18. Five transgenic wheat lines obtained exhibited significantly enhanced resistance to take-all, which is a devastating root disease of wheat and caused by *Gaeumannomyces graminis var. tritici* [69].

On the other hand, overexpression of transcription factors, such as ERF (ethylene response factor) and MYB, in commercial wheat remarkably enhanced the resistance of transgenic wheat to various diseases. For example, overexpression of ethylene response factor gene *TiERF1* and a MYB gene *TiMYB2R-1* in wheat cultivar Yangmai 12 observed significantly enhanced resistance to sharp eyespot and take-all [66, 105]. Overexpression of another MYB

gene *TaPIMP1* enhanced the resistance of transgenic wheat lines to *Bipolaris sorokiniana* and drought stresses, whereas *TaPIMP1*-underexpressing wheat lines were more susceptive to the stresses, indicating that TaPIMP1 positively regulates *B. sorokiniana* and drought stress responses in wheat [10].

4.3 Improving Wheat Tolerance to Abiotic Stresses

Intricate environmental stresses, such as extreme temperatures, drought, and salinity, severely threaten plant growth and development as well as the final production of cereal crops including wheat [106, 107]. Therefore, it is necessary to find out the molecular mechanisms of abiotic stress responses in wheat. Recently, many candidate genes involved in abiotic stress response have been identified in wheat, including genes from different gene families such as *bZIP*, *CBL*, *CDPK*, *CIPK*, *NAC*, *SnRK2*, and *WRKY*[108–113]. It has been reported that more than 30 genes among them have been transformed into model plants like Arabidopsis and tobacco in China. In *Arabidopsis thaliana*, overexpression of *TaSnRK2.8*, *TaMYB33*, *TaPI4KIIgamma* (Phosphatidylinositol 4-kinase), *TaGBF1* (G-box binding factors), and *TaNAC29*, respectively, revealed that all of these could enhance salt and drought stress tolerance [53, 67, 114–117]. The ectopic expression of wheat genes, which included *TaASR1*, *TaCIPK14*, *TaCIPK29*, and *TaNHX3*, conferred various stresses tolerance to transgenic tobacco plants [118–121], which laid a foundation for creating new abiotic stress tolerance varieties in wheat.

Furthermore, transgenic wheat lines are also on the way of Production Test stage in the field, although no permissions of commercial transgenic wheat is issued until now. Fu et al. [72] discovered that overexpression of *ED-Tu* improved heat resistant capability of the transgenic wheat. Overexpression of *TaSUT1A* can significantly improve tolerance to drought stress in transgenic wheat plants [73]. Barley stripe mosaic virus (BSMV)-based virus-induced gene silencing (VIGS) of *TaBTF3* in wheat plants could reduce tolerance to freezing and drought [77]. In *TaERF3*-overexpressing transgenic wheat lines, the tolerance of seedlings to salt and drought stresses was significantly enhanced, while the *TaERF3*-silencing plants were more sensitive to salt and drought stress conditions, indicating that TaERF3 positively regulated wheat adaptative responses to salt and drought stresses [76]. Silencing of *TaSCL14* in wheat variety Xiaoyan 54 exhibited negative influence to plant growth, photosynthetic capacity and tolerance to photo-oxidative stress [74].

Genes from other plants can also be transformed to improve the tolerance of GM wheat. It has been reported that ectopic expression of *GhDREB* from cotton *cv.* Simian 3 successfully improved the tolerance to drought, high salt, and freezing stresses of the transgenic wheat lines [75]. Zhou et al. [70] generated transgenic wheat of *AtDREB2A* gene from *A. thaliana* through

particle bombardment, which were promising for developing salt and drought tolerant wheat germplasm. The field trial in a high salt field showed that transgenic wheat lines, generated by introducing a *betA* gene from *Escherichia coli* into wheat via *Agrobacterium*-mediated transformation, had higher germination rates, more tillers and higher grain yields than control [122]. Saad et al. [71] introduced the rice *SNAC1* gene with the maize *ubiquitin* promoter into Yangmai 12 and found that the overexpression of *SNAC1* significantly enhanced tolerance of transgenic lines to drought and salinity.

4.4 Transgenic Breeding for Yield Improvement

Improving yield is one of the main targets in wheat breeding, which could come true by enlarging the seed size and weight and increasing starch content in wheat endosperm. Seed size is an important agronomic trait as well as a dominant component of grain weight in cereals. The identification of candidate genes associated with grain size and weight is extremely important for wheat high-yield breeding. Hong et al. [80] transformed *TaGW2*-RNAi cassette into wheat variety Shi 4185, demonstrating that TaGW2 negatively controlled grain width and weight. The *TaCYP78A5* silence reduced the cell numbers in seed coat, resulting in reduction of seed size in wheat *cv.* Shaan 512, while *TaCYP78A5* overexpression significantly reduced seed set and resulted in seed enlargement in Arabidopsis [81]. Overexpression of maize phosphoenolpyruvate carboxylase (*PEPC*) gene could increase grain yield in transgenic wheat plants under drought-stress conditions [83]. The overexpression of the *TaLSUI* gene in wheat cultivar, Yumai 34 with an endosperm-specific promoter remarkably enhanced the starch biosynthesis [82].

5 Conclusions and Future Prospects

Genetic transformation is a powerful tool to introduce exogenous genes of superior traits into wheat for creating new varieties with desirable characters. Over the past few years, the Chinese government has set up the "The Breeding Research for Transgenic New Varieties of Plant and Animal, the National S&T Major Project of the People's Republic of China" program to promote transgenic studies. Remarkable progress in wheat transformation has been made during the last two decades. Several patents related to wheat transgenic breeding have been approved [7]. Although wheat transformation efficiency has been greatly improved and the problem of genotype dependent for transformation have been eased, wheat transgenic breeding is still not routine and almost all transgenic wheat lines have been merely generated for theoretical study.

There still exists urgent need for the stable and highly efficient wheat transformation system. So far, the particle bombardment and the *Agrobacterium*-mediated transformation have been established as the main methods for wheat transformation. The transformation efficiency of particle bombardment is diverse among different varieties and culture conditions of recipient materials, which is 0–5% in China (Table 1). Although it has been reported that the transformation efficiency of particle bombardment could be above 60% in Bobwhite [123], this result could not be repeated in other commercial wheat varieties. The efficiency of *Agrobacterium*-mediated transformation is higher than that of particle bombardment transformation, but that still could not meet the demand of production. Researchers have tried majority of approaches such as combining multiple transgenic techniques to improve the transformation efficiency and shorten transformation procedure. Xiao et al. [124] put forward a hypothesis that the linear T-DNA containing LB and RB could be transformed into wheat by particle bombardment, followed by *Agrobacterium* infection to assist the transportation and integration of T-DNA in wheat cells, which could protect the integrity of exogenous DNA and improve the transformation efficiency. Furthermore, conventional breeding strategy is still an effective solution to cultivate new wheat varieties. Genes with excellent agronomic performance can be introgressed into commercial varieties from model wheat by the combination of conventional cross/backcrossing breeding and wheat genetic transformation [60, 125]. Min et al. [126] used transgenic wheat *cv.* Xinchun 9, which was transformed with *TaEBP* genes, to hybrid with seven widely used wheat cultivars, suggesting that drought resistance of three transgenic introgression lines from Xingmai 6 BC_3F_2 generations of backcross introgression line seedlings was increased obviously under PEG-6000 stress.

The type and amount of functional genes that control important agronomic traits for wheat genetic breeding are limited. In wheat transformation study, searching for exogenous genes with breeding potential is still one of the major works. Now, genes from Arabidopsis, rice, barley, soybean, and millet have been transformed into wheat successfully. Genes for transgenic wheat construction in China include herbicide resistance genes for transformation selection and genes related to quality improvement, resistance to pathogens and insects, abiotic stress tolerance, yield improvement, high photosynthetic efficiency, and male sterility as well. However, the bread wheat has the largest genome among the three major cereal crops, composing of three genomes, A, B, and D, which is almost five times of human genome and 40 times of rice genome. The high similarity between these three genomes and high complexity with amounts of repeat sequences of wheat genome make it difficult to completely detect all the chromosome sequences, thereby restricting wheat genes identification and functional studies. So far, it has

been reported that the whole genome sequencing of more than 100 plant species or varieties have been finished, making it possible to identify and clone important genes related to quality improvement, stress resistance and yield improvement for wheat transformation. Recently, great progress has also been made by researchers to figure out the structure and sequences of whole wheat genome since the first genome draft of bread wheat was published in 2012 [127–129]. Due to the continuous efforts of the International Wheat Genome Sequencing Consortium (IWGSC), accompanied by the improvement of sequencing techniques and innovation of sequencing strategies, the physical maps of most chromosomes of bread wheat have been generated with 124,201 gene loci annotated and the establishment of physical maps of rest chromosomes is on the way [130–136]. The progress of wheat genome sequencing promotes the speed of screening target genes with breeding potential. In addition, next generation sequencing (NGS) technique can be used to analyze expression of a large number of genes on transcriptome level and identify target genes involved in important agronomic traits in a short time [137, 138]. Dong et al. [139] used the single-molecule sequencing technology PacBio RSII to investigate transcripts in common wheat and two full-length transcripts of gluten genes which control the end-use quality of common wheat have been identified.

Biosafety concern is another problem that influences wheat transformation study and the development of transgenic technology. The public concerns that the escape of herbicide resistance genes such as *bar*, *NPT-II*, which are used as selective marker genes in wheat transformation, may threaten the ecological security and have side effects to consumers. The development of modern molecular biotechnology has provided a new horizon to improve wheat transformation. Genome editing based on engineered nucleases has been a powerful tool to target and edit gene sequences precisely in the genome of a variety of plants, including wheat. Three main types of engineered nucleases for genome editing, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) 9, can induce a double-stranded DNA break (DSB) at the target DNA site, which can be repaired by homologous recombination (HR) or nonhomologous end joining (NHEJ) [140]. Shan et al. [141, 142] accomplished genome editing through the CRISPR/Cas9 system in rice and common wheat for the first time in the world. Li et al. [143] tested seven target sequences of five genes in *Arabidopsis* and *N. benthamiana*, resulting in targeted mutagenesis by using sgRNA:Cas9 technology. Wang et al. [144] used TALEN and CRISPR-Cas9 technologies in bread wheat to modify the MILDEW-RESISTANCE LOCUS (MLO) alleles and generate transgenic plants conferred heritable resistance to powdery mildew.

The emerging plant genome editing tools, especially CRISPR/Cas9, is promising to accelerate plant breeding by introducing precise and predictable modifications directly in an elite variety. Since no marker gene is required, they can also help resolve the contradiction between reality requirement and concerns about biosafety risk.

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Chapter 4

Wheat Improvement in India: Present and Future

Sonia Goel, Kalpana Singh, and N.K. Singh

Abstract

Wheat (*Triticum aestivum* L.) contributes substantially to global food and nutritional security. With the growing demands under the constraints of depleting natural resources, environmental fluctuation, and increased risk of epidemic outbreaks, the task of increasing wheat production has become daunting. The factors responsible for first green revolution seem to be exhausting rapidly, and there is an immediate need to develop the technologies which can not only increase the wheat production but also sustain the same at a higher level without adversely affecting the natural resources. Understanding abiotic stress factors such as temperature, drought tolerance, and biotic stress tolerance traits such as insect pest and pathogen resistance in combination with high yield in plants is of paramount importance to counter climate change related adverse effects on the productivity of wheat crops. Thus, an important goal of wheat breeding is to develop high-yielding varieties with better nutritional quality and resistance to major diseases. Therefore, in this chapter, we present a judicious mixture of basic as well as applied research outlooks. We trust that the information covered in this chapter would bridge the much-researched area of stress in plants with the information to breed climate-ready crop cultivars to ensure food security in the future.

Key words Wheat, Abiotic stress, Molecular breeding, Germplasm conservation, Wheat QLTs, Markers, MAS

1 Introduction

Grain production needs to be doubled to feed an increasing world population which is estimated to reach approximately nine billion by 2050. The existing trends in wheat yield increase are inadequate to meet this projected demand. Commercially cultivated wheat is basically of two types, i.e., durum wheat (*Triticum turgidum* L.) and bread wheat (*Triticum aestivum* L.) that differ in their genetic complexity, adaptation as well as use. Bread wheat is one of the most important crops providing one-fifth of the total calories for the world's population. The current major challenges facing future wheat production in India are increasing heat stress; declining water supplies for irrigation; growing risk of new virulence of disease such as wheat rusts (yellow, brown, and black) and leaf blight; continuous adoption of rice–wheat system on around 11 million hectares;

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changes in urbanization pattern; and demand for better quality wheat [1]. Breeding gains rely on access to useful genetic variations from crops' gene pools. Gene banks are the repositories of the beneficial gene(s)/alleles from crop's primary, secondary or tertiary gene pools which should be harnessed for present and future wheat genetic improvement programs. Underutilized but useful gene bank variation, when channeled into elite breeding materials using effective pre-breeding strategies, can provide diverse benefits including increased stress tolerance, yield potential and improving nutritional and processing quality.

During the Green Revolution era, global increases in wheat yield potential were achieved by deploying plant height genes (*Rht1* and *Rht2*) as well as numerous genes for disease resistances. The semidwarf, fertilizer-responsive, lodging-resistant, and high-yielding green revolution varieties replaced landraces and traditional varieties grown by the farmers. As a consequence, the genetic diversity in most of the world's wheat producing regions became limited. Even today this remains as one of the major challenges for wheat improvement as modern high-yielding wheat cultivars possess genes or gene combinations pyramided by breeders using well-adapted cultivars. There is need to introgress new variations and gene combinations from landraces and wild species.

2 History of Wheat in India: Green Revolution

India achieved remarkable progress in wheat production during the last four decades and is the second largest wheat producer in the world. In India, scientific plant breeding probably began in the first decade of the twentieth century [2]. A lot of exotic material was used in crossing, and several high-yielding varieties of the Pb and NP series such as NP4, NP6, NP12, Pb8, Pb8A, Pb9D, Pb11, K13, K46, AO13, AO85, AO90, Bansi, Motia, and Gulab series were developed. Among which NP4 won international award for its grain quality and also became popular in countries like Australia, South Africa, and Hungary. Thereafter, recombination breeding between pure lines led to the development of varieties like PbC518, PbC591, NP52, NP80-5, NP120, NP125, NP165, Niphad4, AO68, AO113, and AO115 however, with little emphasis on disease resistance. The higher incidence of rusts, particularly black and brown rusts in cultivated wheat, forced breeders to incorporate disease resistance in the breeding material and notably improved material such as NP809 (resistant to all three rusts) has been developed. Some other popular old varieties were PbC 228, C273, C281, C519, Hyb11, Hyb23, Hyb38, RS31-1, Kenphad28 etc. Despite all this progress, average wheat productivity has been quite low. In 1962, Dr. B.P. Pal and Dr. M.S. Swaminathan

observed photothermal insensitive, dwarf, input-responsive wheat lines in the material initially developed by Dr. N.E. Borlaug and supplied by the United States Department of Agriculture (USDA) which laid the foundation for the so-called "green revolution." The selection of amber-grained, input-responsive, high-yielding varieties such as PV18, Kalyan Sona, Sonalika, Chhoti Lerma, and Safed Lerma, and their faster adoption by farmers, led to unparalleled growth in wheat production in India and by 1970, wheat production in India reached 20 Mt. The path of progress was further cemented by the release and cultivation of varieties like HD2009, WL711, WH147, UP262, Lok1, HD2285, and HD2329. This was again followed by a period of despair and frustration when researchers failed to breed over and above HD2329 for a very long period. During the last 40 years, ~312 wheat varieties have been released in India's six wheat zones. The continuous efforts of breeders and other wheat workers led to the more sharp gain in total factor productivity in the post Green Revolution period than in Green Revolution era.

The momentum of growth in wheat production could not keep pace with demand in the last 5 years or so. Total factor productivity (TFP) and yield growth have slowed in the most productive wheat belt of India [3]. This along with declining land quality, declining investment, and increased intellectual property protection has made the task of increasing the production more complicated. To ensure gradual gains in wheat production with a shrinking land base, breeding wheat cultivars with increased grain yield potential, enhanced water use efficiency, heat tolerance, end-use quality, and durable resistance to important diseases and pests can contribute to meeting at least half of the desired production increases. The remaining half must come through better agronomic and soil management practices and incentive policies. To meet the daunting task of increasing yield potential in a sustainable way, the breeding efficiency has to be improved significantly [4]. In 1990, Swaminathan used the term "Evergreen Revolution" to emphasize the need for enhancing productivity without harming the environment. According to him, in densely populated countries like India, China, and Bangladesh, production increase has to come from diminishing arable land, irrigation water and increasing biotic and abiotic stresses [5].

3 Drought Tolerance: Physiological and Molecular Analysis

Wheat production is affected by several abiotic factors like drought that affects 50% of the area under production in the developing countries such as India. Drought tolerance (DT) is a complex trait having low heritability, higher genotype–environment interaction which hampers the progress towards DT breeding. Variation and

unpredictability of environmental condition have hindered breeders' efforts to select for DT. Changing weather patterns indicate that even irrigated wheat will have to be produced with less applied water. Irrigated wheat in most parts of India is already cultivated under partial irrigation, receiving 1–2 irrigations over a cropping season [6]. Water will become a limiting factor for sustained production of wheat and other crops, and this includes the water-rich Indo-Gangetic Plains [7]. Therefore, breeding for enhanced DT is essential for achieving improved crop productivity and greater food security [8]. The current wheat production in South Asia is around 95 million tones and demand for 2020 is estimated to be around 137 million tones. The demand for wheat is increasing, but there is no further increase in an area mainly due to growing urbanization and diversification [9].

Several traits have been identified which has been responsible for DT are heritable, additive in nature, and display continuous variation; this is an indication that there is considerable room for improvement in DT [10]. However, progress in breeding drought-tolerant wheat varieties has been slow. For instance, C306, a variety released in 1969, is still considered the most dependable wheat variety under drought and is used as the standard check in trials throughout India [6]. Some of drought tolerant wheat has been released by government of India in 2015 such as PBW 527, HI 1531, HI 8627, HD 2888, HPW 349, PBW 644, WH 1080, HD 3043, PBW 396, K 9465, K 8962, MP 3288, HD 4672, NIAW 1415, and HD 2987. Derivatives of PBW343 and Inquilab are in advanced stage of the trial in South Asia, and some of them have been found superior to local checks [11]. In this direction, unexploited germplasm such as synthetic wheat is also being used [12]. Also, the winter–spring wheat hybridization, interspecific/wide hybridization for introgression of useful genes from related wheat species may also be highly effective [13]. Some of these unexploited germplasms have shown promise in Indian wheat improvement program, and substantial variability has been observed for yield components in advanced lines of buitre, synthetic hexaploids, or Chinese cum compacted ear types. DT is controlled by several small effect genes or QTLs and is often confounded by differences in plants phenology [14]. To address the complexity of plant responses to drought, it is crucial to understand the physiological and genetic basis of this response.

Development of high-density molecular linkage map provides a tool for dissecting the genetic basis of such complex traits into component QTLs through genomic associations. Although a substantial amount of information has been generated on the genetics of aboveground traits under water stress, limited attention has been paid to below the ground root traits in wheat. The creation of suitable mapping populations and the development of molecular markers have enabled linkage studies in wheat, and many QTLs

have been identified for yield and related traits under drought environments [15]. Linkage studies have shown that QTLs for grain yield reside in several chromosomal regions and measurements of yield components allow us to dissect complex traits to smaller genetic components more amenable for building our knowledge of trait architecture which will inform our future strategies for exploitation. Detection of QTLs containing the genes conferring quantitative traits including DT has revolutionized the selection process towards marker-assisted and genomic selection [16].

To date, several putative QTLs for DT related traits have been mapped in wheat, particularly on the A and B genomes where most of relevant QTLs seem to be localized on chromosomes 2B, 3A, 4A, 4B, 7A, and 7B. Several molecular markers have been used, of which, sequence-based DNA markers, notably single nucleotide polymorphisms (SNPs), are gaining popularity and are expected to advance the dissection of complex traits on complex genomes due to their high linkage with heritable variation [17, 18]. Kadam et al. [19] studied differentially expressed genes under drought stress in WL711/C306 wheat RIL population between parents and the drought tolerant, and drought susceptible RIL bulks through transcriptome profiling. On Chromosome 3BS, a differentially expressed gene encoding *glutathione S-transferase* was identified which was downregulated 2.36-fold in the DT parent C306 and the tolerant RIL bulk. Glutathione is an important antioxidant, redox buffer, and detoxifier [20]. Four differentially expressed genes identified in this region of chromosome 3BL included *b-glucosidase* while the remaining genes coded for a hypothetical protein with unknown function. Gene *b-glucosidase* has upregulated 2.5-fold in the DT parent C306 and the tolerant RIL bulk as compared to the DS parent WL711 and the sensitive RIL bulk. Gene *b-glucosidases* have been implicated in the release of sugars for remobilization and completion of energy-dependent senescence program under drought-induced senescence in Arabidopsis [21].

Shukla et al. [22] identified several major and novel genomic regions on chromosomes 3BS, 4AL, 4BS, 6AS, 7BL for yield and yield related under water deficit stress treatment in WL711/C306 wheat RIL population. The yield enhancing allele under drought stress at *qGYWD.3B.1* locus was contributed by DT parent C306. This genomic region explained 18.7% of phenotypic variation for grain yield under drought and co-located with genomic regions for grain yield components. Fine mapping of the major QTLs on chromosome 3BS will enable identification of robust markers and candidate genes for marker-assisted breeding for DT in wheat. Furthermore, several phenotypic DT traits in wheat have been correlated with molecular markers allowing precise mapping of their respective QTLs on chromosomes [23]. However, QTL identification for tracing DT remains a challenge due to the large

number of genes influencing the trait, instability of some QTLs, the large size of the wheat genome, and epistatic QTL interactions, among other constraints [24]. Knowledge of the candidate genes responsible for DT is useful for understanding the functional basis of DT and assists in their subsequent use after validation in molecular breeding through MAS [25].

4 Heat Stress Tolerance: Physiological and Molecular Analysis

Abiotic stress, especially heat stress, induces complex morphophysiological phenomenon in plants which decrease the yield and quality of wheat grain [26]. An unfavorable effect from a climate change scenario to wheat production is South Asia, which has alarmed CIMMYT and the Indian Government, resulting in a new agreement to establish the Borlaug Institute for South Asia (BISA) with three strategic field sites. BISA has a primary objective of developing more productive, climate-resilient wheat varieties with National and International Partners. The three BISA locations are Ludhiana in Northwest India, Jabalpur in Central India, and Pusa in Northeast India. These locations are strategically placed throughout the Indo-Gangetic Plains and target main growing regions/environments for wheat in South Asia. Jabalpur and Pusa sites are highly heat-stressed with night and day temperatures significantly warmer than more optimal production area in the Punjab region.

Furthermore, current estimates indicate that in India alone, more than 13.5 million ha of wheat growing area is heat stressed [6]. Both the proximity to the equator and the popular rice-wheat cropping system, which involve late sowing of wheat, are the major causes of exposure of wheat in India and other neighboring countries to high temperatures during grain filling [27]. The current trends in India indicate that the 'cool period' for the wheat crop is shrinking, while the threat of terminal heat stress is increasing [7]. High temperature may cause changes in phenological, physiological, biochemical, and morphoanatomical behavior in wheat thereby affecting its growth and development [28]. Heat stress was reported to reduce the grain filling duration and change the starch composition and activity profiles of starch biosynthesis enzymes. In India, wheat is cultivated during the winter season from October to April. The Indian wheat germplasm is not only adapted to cooler areas of the north but also to the warmer area of central and peninsular parts. The productivity of wheat in the country is higher in the north than down south, due to temperature difference as well as longer duration and availability of water for irrigation in the northern region. The early heat stress at tillering and terminal heat stress at grain filling are a major concern for wheat productivity in the country. Early heat stress is mainly

experienced is central and peninsular India, but terminal heat stress is noticed to affect crop area by 10% for every 1 °C rise in temperature [29].

High temperatures affect crop growth at many stages of development and through several different mechanisms. High temperatures can also increase the rate of grain filling, but only slightly at temperatures above 20 °C, which fails to compensate for the shortened duration and leads to an overall reduction in grain size. Above 30 °C, warming can slow grain-filling rates, in part because the leaf photosynthetic apparatus can be damaged at extreme canopy temperatures, resulting in an acceleration of senescence [30]. Various physiological traits have been studied to ascertain their input to heat tolerance (HT), i.e., higher photosynthetic rates, stay-green, chlorophyll content (Chl), chlorophyll fluorescence (CFL), etc. These traits are heritable, additive in nature and display continuous variation and indicate that there is considerable scope for improvement in HT [10]. Breeding programs may include such traits to assist in the selection of heat-tolerant parents, segregating generations or advanced lines. Therefore, breeding for HT in wheat is a major objective in India and around the world. Hence, it is important to incorporate late HT into wheat germplasm. Breeding for HT is still in its infancy stage and warrants more attention in future [31]. However, significant variation for HT exists among wheat performed using indirect selection for GFD, 1000 grain weight (TGW), and canopy temperature depression (CTD) [32]. HT is now a major concern for crop production and approaches for sustaining high yields of crop plants under HT stress are important agricultural goals.

The major cooperating centers established in the country for screening materials for HT are Karnal and Hisar for North Western Porphyry Zone (NWPZ); Varanasi and Sabour in North Eastern Porphyry Zone (NEPZ); Indore, Jabalpur, Bilaspur, Sagar, Vijapur, and Junagarh in central zone; and Dharwad, Akola, and Pune in the peninsular zone. The efforts made under coordinated system resulted in the identification of sources of HT such as WH730, CBW12, NIAW34, NIAW845, RAJ4037, and HD2808 which are being used breeding program. Also, genotypes HW4250, RAJ3765, RAJ 4101, WH1021, LOK54, HW2004, RAJ4250, GW432, and HD3095 were found to have least heat sensitivity index under multilocation HT trials.

Plants possess some adaptive, avoidance, or acclimation mechanisms to cope with HT situations. Also, major tolerance mechanisms that employ ion transporters, proteins, osmoprotectants, antioxidants, and other factors involved in signaling cascades and transcriptional control are activated to offset stress-induced biochemical and physiological alterations. Plant survival under HT stress depends on the ability to perceive the HT stimulus, generate and transmit the signal, and initiate appropriate

physiological and biochemical changes. HT-induced gene expression and metabolite synthesis also substantially improve tolerance. The physiological and biochemical responses to heat stress are active research areas, and the molecular approaches are being adopted for developing HT in plants [33]. The activity of the enzymes glutathione S-transferase, ascorbate peroxidase, and catalase were more enhanced in the cultivar showed better tolerance to heat stress and projection against ROS production [34].

HT is quantitative in nature and is controlled by some genes/QTL which may sometimes be involved in interactions with each other (QTL × QTL interaction) and/or with the environment (E) (QTL × E and QTL × QTL × E interactions) [35]. QTL for HT in wheat were reported using different traits like GFD, CTD and yield and senescence-related traits [36]. Efforts are now being made to identify QTL using other important traits, such as yield, TGW and CTD. There are very few reports involving QTL analysis for the heat susceptibility index (HSI) of yield, TGW and GFD as well as CTD (under heat stressed conditions) using a mapping population specially developed for HT and evaluated under natural field conditions of heat stress. However, HSI has been used effectively for measuring flooding tolerance in soybean [37] and drought and HT in wheat [38]. Mason et al. [39] reported five stable QTL for HSI of single grain weight (1A and 2A), grain weight (3B), and grain number (2B and 3B) contributing 11.1–22.6% of phenotypic variation. However, their phenotypic assessment was performed on 65 lines of F5 generation material in controlled conditions for a short period (3 days) of heat stress.

Upregulation of many genes has been reported to help the plant to withstand the stress conditions which leads to plant adaptation [40]. Some broad group of those are the Ca-dependent protein kinases (CDPKs), mitogen-activated protein kinase (MAPK/MPKs), NO, sugar (as signaling molecule), phytohormones [41]. These molecules together with transcriptional factors activate stress responsive genes. Under heat stress condition, plants perceive the external and internal signals through different independent or interlinked pathways which are used to regulate various responses for its tolerance development [42]. Transcriptome analysis of developing grain and flag leaves is of much relevance in identifying genes and pathways for HT in wheat as heat stress causes grain shriveling and significant yield reduction [43]. Chauhan et al. [44] identified differentially expressed sequence tags (ESTs) of wheat under heat stress from the three forward and one reverse subtractive cDNA libraries constructed from seedling and flowering stages. Some of the ESTs were found specific to heat stress. However, expressions of many were also detected in response to other abiotic stresses which indicates their putative role while interacting with other abiotic stresses. Majoul et al. [45] analyzed the effect of heat stress on hexaploid wheat grain proteome using two-

dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). A total of 43 proteins differentially expressed under heat stress, consisted of enzymes of carbohydrate metabolism, the ATP synthase β-chain that was related to four heat-decreased proteins, heat shock proteins, and some other defense proteins. The expression of HSPs was not surprising as they perform some well-established functions like preventing denaturation of existing protein or misfolding of newly synthesized proteins. However, the exact role of many HSPs in wheat under heat stress is not clearly understood.

In conclusion, during recent years, new parameters for heat stress tolerance, e.g., Heat Sensitivity Index (HSI) for TKW, GFD have been developed. These parameters allow selection of best heat tolerant plant/lines in combination with physiological and biochemical approaches. Various mapping approaches and genetic studies have contributed greatly to a better understanding of the genetic bases of plant stress-tolerance, which led to the development of plants with enhanced tolerance to abiotic stress. In recent past, several molecular markers associated with heat tolerance have been developed. Screening of wheat germplasm for heat stress tolerance using marker assisted selection (MAS) approach for the known tolerance genes might be the right approach to assist a conventional breeder for developing heat stress tolerance which would play an important role to minimize the yield losses. Also, the applications of genomics, proteomics, and transcriptomics approach for developing a better understanding of the mechanism of heat stress tolerance is also required. Molecular knowledge of response and tolerance mechanisms will pave the way for engineering plants that can tolerate heat stress and could be the basis for production of crops which can produce economic yield under heat-stress conditions. The induction of signaling cascades leading to profound changes in specific gene expression is considered an important heat stress adaptation. These changes emphasize the importance of physiological and molecular studies to reveal the mechanisms underlying stress responses. In addition, understanding the nature of the signaling cascades as well as the specific genes expressed in response to HT will be valuable for developing stress tolerant plants. Molecular approaches that uncover the response and tolerance mechanisms will pave the way to engineering plants capable of tolerating HT and could be the basis for development of crop varieties capable of producing economic yields under HT [46]. At the field level, managing or manipulating cultural practices, such as the timing and methods for sowing, irrigation management, and selection of cultivars and species, can also considerably decrease the adverse effects of HT stress. In recent decades, exogenous applications of protectants such as osmoprotectants, phytohormones, signaling molecules, trace elements, etc. have shown

beneficial effects on plants growing under HT, due to the growth promoting and antioxidant activities of these compounds. Engineering plants to synthesize these compounds may be an alternative way of developing thermotolerance in important crop plants and represents a potentially important area of research on thermotolerance. However, most of the experiments on HT effects currently carried out in different parts of the world are still limited to laboratory conditions and short-term studies only. Field experiments that explore different biochemical and molecular approaches and agronomic management practices are needed to investigate the actual HT responses and their effects on final crop yield.

5 End Product Quality and Nutritional Improvement

Wheat with its high protein content is an important source of plant protein in the human diet. Among the cereals, the flour of bread wheat has a superior capability of forming leavened bread. Majorly wheat is consumed in India in the form of unleavened bread called chapati [6]. However, with growing industrialization, the demand for bread, biscuit, and related products is growing throughout the world including India [47]. In today's scenario, works on both quantity and quality of the wheat are required. The quality requirements of wheat for this product differ, i.e., hard wheat with strong gluten (>60 ml sedimentation value), >12.0% protein, 5 + 10 high molecular weight glutenin subunit with a 9 or 10 Glu-1 score is required for making good bread. This is due to the structure and composition of its seed storage proteins, which upon hydration can interact to form gluten, an insoluble, but highly hydrated, viscoelastic aggregate that endows the wheat dough with its unique properties. So the HMW glutenins are necessary to create the strong dough, which is essential for making high-quality bread. HMW-glutenin proteins are majorly responsible for high dough strength for bread-making traits. Different alleles of HMW-glutenin proteins, LMW-glutenin proteins, albumins, and gliadins have been exploited for their impact on bread and chapati-making quality in near isogenic lines of wheat variety HD2329 [48, 49].

Many Indian varieties have been characterized for various end products such as C306, WH147, UP262, Sujata, and PBW226 for chapati; HI977, K9107, HD2285, GW120, GW190, DWR195, and NI5439 for bread; Sonalika for biscuit, and durum varieties PDW233, WH896, Raj1555, HI8498, GW1139, HI8381, and MACS2846 for pasta products. Physicochemical and rheological properties of Indian wheat cultivars varying in HMW-GS were evaluated and observed that the cultivars having subunits of: 20 (Glu-B1) and 2 + 12 (Glu-D1) exhibited very weak dough stability; 2 + 12 (Glu-D1) and 7 + 9 (Glu-B1) as well as 5 + 10 (Glu-D1) with 7 or 7 + 9 (Glu-B1) were weak and 17 + 18 (Glu-B1)

with 2 + 12 or 5 + 10 (Glu-D1) and 7 + 8 (Glu-B1) with 5 + 10 (Glu-D1) were strong dough stability. Allelic diversity of protein subunits was studied in 35 bread wheat genotypes, and their effect was observed on bread-making quality. Clustering of the genotypes revealed the institutional, zonal, and temporal prevalence of particular subunit combinations in the varieties [50].

A novel HMW glutenin subunit designated as "17*" reported earlier was confirmed in the land race Pissi-local. Allelic variation at the Glu-D1 locus was found to be an important determinant of bread-making quality and allele of Glu-D1 encoding the subunits "5 + 10" was superior to its allelic counterpart, encoding "2 + 12". Novel variants were observed in HMW-GS in Indian wheat landraces one coded by the Glu-Blx locus and two novel subunit pairs at the Glu-D1 locus. New high yielding varieties were assessed for the allelic difference at high molecular weight glutenin-Glu D1 (Singh et al., 2007). Also, 5 + 10 HMW-GS at Glu-D1 was already quoted for good bread-making quality, but it was observed that the role of 2 + 12 glutenin subunits is equally relevant in imparting good bread quality [51]. Durum wheat which is known for Semolina formation was studied under different conditioning treatments on milling quality and found that variety DWL-5023 have the maximum amount of semolina with highest protein, carotenoids [52]. Scientists are incorporating multigrain to improve the nutritional quality of bread. The whole wheat high nutritional bread using varying flours comprising wheat, oats, quality protein maize, and soybean were assessed for the flour quality traits, and highly acceptable composite bread was obtained by combining the optimized level of multi ingredients. The resultant bread obtained was endowed with high protein content and crude fiber and was overall more desirable [53].

Exploiting exotic germplasm for the diverse gene pool for improving end product quality is an important aspect of current research scenario in India. *Aegilops geniculata* (M^gU^g), a wild wheat relative with several useful traits was exploited for wheat improvement [54]. Protein characteristics and peroxidase and pentosans activities on chapati-making quality have also been observed to improve the quality end product. The effect of added arabinoxylans on rheological properties of dough and chapati-making quality was observed [55]. Pasting is one of the most important properties of wheat starch determining the flour quality and functionality. New Plant Type (NPT) wheat derivatives along with three checks (PBW343, HD2329, and Raj3765) were studied for their starch pasting properties. Although all flour pasting characteristics varied, breakdown viscosity (BV) and setback viscosity (SV) exhibited greater variability across environments. Hardness is also the main quality trait for bread-making, as hard wheat has good milling characters. The hardness of *T. aestivum* genetic stocks and a collection of 35 durum germplasm lines and varieties were observed by

SKCS (single kernel characterization system) by Sharma et al. [56]. The relationship between protein molecular weight distribution, quality characteristics, and muffin-making properties among Indian wheat varieties were evaluated, and it was found that flours from varieties with higher grain weight showed a lower proportion of fine particles. Another study on noodle-making characteristics from durum wheat showed varieties having higher grain weight had lower hardness and higher yellow pigment content. Gluten performance index showed a positive correlation with α-helix and negative with intermolecular + antiparallel-β-sheets in gluten. Dough strength was found to be related to the proportion of extracted polymeric proteins [53].

Wheat quality components are quantitative in nature which is influenced by various environmental factors and showed high genotype–environment interaction. In a particular genetic background, QTL analysis allows the presence of QTLs to be identified, thereby providing breeders with an opportunity to use MAS to increase breeding efficiency. QTL analysis for some quantitative traits related to grain protein content in bread wheat was done by Pushpendra et al. [57]. They observed QTL related to grain protein on chromosome arms 1AS, 2BS, and 7AS. Four QTLs were observed for growth-related traits. QTL analysis for grain protein content using SSR markers and validation studies was also done by using NILs in bread wheat [58]. Further QTL mapping of 1000-kernel weight, kernel length, and kernel width in bread wheat was done in 185 recombinant inbred lines from the cross of Rye Selection 111 × Chinese Spring. For 1000-kernel weight, ten QTLs were identified on wheat chromosomes 1A, 1D, 2B, 2D, 4B, 5B, and 6B, whereas six QTLs for kernel length were detected on 1A, 2B, 2D, 5A, 5B, and 5D. Chromosomes 1D, 2B, 2D, 4B, 5B, and 5D had nine QTLs for kernel width [59].

Malnutrition is one of the raising issues in the developing country like India. Wheat is one of the major sources of food and nutrition for the people of India. Here diet is majorly based on dal (pulses) and roti (chapati). Wheat is rich in protein but limited in essential amino acids like lysine, tryptophan, and threonine. Hence, there is a need for nutritional quality improvement of wheat using modern functional genomics tools. One of the target proteins for this is triticin, which is a minor storage protein of wheat endosperm with good essential amino-acid balance, but it contributes only less than 5% of the total seed proteins. It has a lysine-rich decapeptide repeat motif in its hypervariable region (HVR). A detailed study on this was done for its use in the improvement of wheat bread at genetic and biochemical levels [60]. The genetic manipulation of *triticin* gene in increasing lysine content can be made possible either by enhancing the expression using more efficient seed storage protein promoters or by manipulating the hypervariable region. Triticin alleles have been assessed for the quality traits in

segregating population, and a positive effect was observed on bread and chapati-making quality which was comparable to well-known HMW allele Glu-D1d. Triticin was observed as a product of D genome from Chinese Spring wheat variety by comparing sequence data from different wild wheat accessions [49]. Cloning and characterization of full-length triticin cDNA have been carried out in wheat varieties K-68 and Chinese Spring. Comparative analysis of the nucleotide sequences of the triticin genes with the cDNA clone λTri-25 revealed the presence of a stretch of 31 nucleotides in the 5′ -UTR of λTri-25 having exact complementarity with a stretch of nucleotides of the same length in the 3′ -UTR of the full-length triticin genes cloned from the wheat varieties K-68 (Tri-gK68) and Chinese Spring (Tri-gCS). Different wild wheat progenitors were also exploited for the presence of variation in the allelic pattern of triticin protein [61].

A RIL population comprising of 306 lines derived from parents PBW343 and popular wheat variety Hitherto were evaluated for grain iron (Fe) and zinc (Zn) concentration and considerable amount of genetic variation was observed explaining 37.42% and 57.78% of the total sum of squares respectively. Genotype–environment interaction (G × E) for Fe and Zn accounted for 29.46% and 23.24% of the total sum of squares, respectively. The magnitude of G × E interaction was relatively high. High heritability was observed for iron (0.81) and zinc (0.71) concentrations reflecting the non-crossover type of interaction. Additive main effects and multiplicative interaction biplot and environmental indices indicated the most favorable environment for Fe to be at Delhi, which was the second most favorable environment after Samastipur in Bihar for Zn. A poor environment for grain Fe and Zn accumulation was at Pantnagar. Four stable RILs each for grain Fe and grain Zn concentration were identified by Gopalareddy et al. [62].

Improvement of processing qualities for various products and reduction of the adverse health effect of storage proteins are becoming more important. We need to develop more sophisticated tools and to upgrade the resolution of current technologies to move forward. Although progress has been made in this context, HMW-GS at Glu-1 and LMW-GS at Glu-3 explain only about 60% of the variability in dough properties and end-use quality. Therefore, the identification of other major QTLs is needed for a better understanding of wheat processing qualities. The international wheat genome Sequencing Consortium (IWGSC) efforts and genome-wide association studies (GWAS) with modern marker technologies such as genotyping by sequencing and SNPs will be crucially important. Completion of the wheat DNA sequencing project will greatly enhance our capacities in gene discovery, gene cloning, and marker development.

6 Biotic Stress: Disease and Pest Resistance

The impact of climate change on wheat diseases is most devastating in a developing country like India because it can influence the growth of the crop, host–pathogen interactions and alter the susceptibility window [63]. Temperature rises above normal and increased humidity and provides a potent way for pathogen action on the crop. Wheat crop is one of the victims, in this case, one such threat observed in Punjab state change in temperature and humidity will reduce the importance of yellow rust (*P. striiformis*) and Karnal bunt (*T. indica*); the importance of leaf rust, foliar blights, Fusarium head blight, and stem rust may increase in the future, particularly in the absence of resistance in wheat cultivars [64]. The majority of currently grown wheat varieties are susceptible to these diseases, presumably because of high pathogenic variability occurring in these fungi and narrow genetic base for resistance in currently available wheat varieties. So as a tool of control measure, the demand of resistant cultivars is growing day by day. Initially, wheat breeders must have new genes for overcoming this situation [65].

Wheat cultivars that have remained resistant for a long time, which carry durable or race-nonspecific resistance, are known to occur. Resistance from such genotypes can be transferred in a planned manner to the susceptible cultivars, either through the selected bulk approach [65] or single backcross breeding approach: these are the two efficient selection methodologies currently used by CIMMYT that allow the simultaneous accumulation of the desired number of slow rusting genes concurrently with increased grain yield potential and other traits [66]. An aggressive strategy to promote these resistant cultivars in farmers' fields through large-scale quality seed production is the only viable option as the resource for poor farmers in most of South Asia cannot afford to use chemical control.

Molecular tools have opened up new dimensions in the area of disease resistant varieties by knowing the availability of linked molecular markers; an effective selection strategy can be made for the development of multiple disease resistance varieties. Once, markers are established for different resistant genes conferring resistance against multiple diseases; these markers can be used in selecting multiple disease resistant varieties through MAS. Also, stay green trait was also showed positive linkage with spot blotch resistance [7]. These morphological markers are successfully being used in markers assisted selection of spot blotch resistance germplasm lines. *Lr19* is a resistance gene against leaf rust (*Puccinia triticina*) originally transferred from *Agropyron elongatum* to wheat (*Triticum aestivum* L.), was found to be effective worldwide against this disease but Bhardwaj et al. [67] reported a new pathotype of *P. triticina* virulent on *Lr19* from India. Validation of molecular

markers for Lr19 and Lr24 was done by Singh et al. [66]. STS markers detected the presence of the leaf rust resistance gene Lr19 in a Thatcher NIL (Tc*Lr19) and Inia66//CMH81A575 and of the gene Lr24 in the genotypes Arkan, Blue Boy II, Agent, and CI17907. Wheat genotypes carry specific rust resistance genes have been identified using molecular markers for Lr19 and Lr24 [66].

A highly virulent race of stem rust of wheat caused by *Puccinia graminis* was detected in 1999 in Uganda and may pose a major threat to wheat crop in many countries including India in coming years. This race is commonly known as *Ug99* and designated as TTKSK based on North American system of nomenclature. The race *Ug99* carries virulence to several genes commonly present in global wheat germplasm including *Sr31*, known to be present in several leading spring and winter wheat cultivars and germplasm. Indian wheat program in collaboration with CIMMYT and Kenya has identified some wheat cultivars and genetic stocks that are resistant to *Ug99*. Although several alien genes can provide resistance, the long-term strategy should focus on achieving durable resistance by rebuilding the *Sr2*complex [68]. Some of the Indian wheat lines were observed for the resistance to Ug99 like UP 2338, HUW 510, GW 273, GW 322, HD 2781, HD 4672, HI 1500, HI 8498, MACS 2846, and MP 4010. Although many reports of tagging and mapping of several disease resistance genes and QTLs are available in wheat [69], not many reports are available for spot blotch. For this disease, the association of resistance with microsatellite markers in bulks of susceptible and resistant progeny lines was reported [70]. The QTLs for spot blotch resistance in the Chinese wheat variety, "Yangmai 6" were mapped on chromosome 2A, 2B, 5B, and 6D [71]. However, more information with respect to the identification of QTLs in different genetic background was generated when QTLs were mapped in two other resistance sources, i.e., "Ning 8201" and "Chirya 3"and to compare the chromosomal locations of QTLs with "Yangmai 6" to identify diagnostic markers that can be used for marker-assisted selection and to make an effective breeding program [72].

Potential QTL associated with spot blotch resistance and to determine the potential association of Lr34 and Lr46 with resistance to this disease was done. *Lr34* was found to constitute the main locus for spot blotch resistance and explained as much as 55% of the phenotypic variation in the mean disease data across the six environments. Based on the large effect, the spot blotch resistance at this locus has been given the gene designation Sb1. Two minor QTLs were detected on 7DS, and on 1BL. *Lr34* and *Lr46A*, which are widely used in wheat breeding to improve resistance to rust diseases and powdery mildew, were found to have a beneficial effect on spot blotch [73]. Relationship of plant height and days to maturity with resistance to spot blotch in wheat was also observed [74].

Powdery mildew is another important disease of wheat in the North Western plains zone of India, especially in the context of the present day varieties that are being cultivated in this zone. The various planting options were studied to see the effect on disease and various aspects of microclimate and epidemiology were discussed under influence of tillage practices [75]. Landraces and wild relatives of wheat have played an important role as genetic resources for the improvement of disease resistance. The use of molecular approaches, particularly molecular markers has allowed better characterization of the genetic diversity in wheat germplasm. Allele mining and eco-tilling are some of the important tools for the characterization of what disease resistance. Kaur et al. [76] employed different approaches, resources, and potential tools to characterize and utilize the naturally occurring resistance diversity in wheat. Wheat Pm3 powdery mildew resistance locus was identified in a set of 1320 landraces assembled by ecogeographical criteria. They identified a set of resistant lines where new potentially functional alleles would be present. Newly identified resistance alleles will enrich the genetic basis of resistance in breeding programs and contribute to wheat breeding.

7 Conclusions and Future Prospects

In recent years, the necessity for a change in focus in plant biotic and abiotic stress research has become apparent. As plants have finite resources which must be balanced between growth and defense against stresses, often both natural and induced stress tolerance comes with a growth or yield penalty, making it agriculturally disadvantageous. Rather than developing crops that can survive extreme stress events, it may therefore be more beneficial to focus on producing crops which are stress tolerant but which maintain high photosynthesis, growth rates, and yield. The transgenic approach has been used for successfully introducing and overexpressing the barley *HVA1* gene encoding for a late embryogenesis abundant (LEA) protein into wheat by particle bombardment [77]. Most of the transgenic lines tested displayed improvement in important agronomic traits, including total dry mass and water use efficiency, shoot dry weight, root fresh and dry weights, when plants are grown under soil water deficit conditions. In general, this investigation showed that the transgenic lines expressing the *HVA1* gene had improved growth characteristics including an enhanced biomass yield under water deficit conditions. The discovery of novel genes, determination of their expression pattern in response to abiotic stress and an improved understanding of their roles in stress adaptation (obtained by the use of functional genomics) will provide the basis of effective

engineering strategies leading to greater stress tolerance. The technological advance has not only improved the understanding of genetic control and regulation of traits but has also widened the scope of its application. The improvement of transformation protocols, development of high-density molecular marker maps [78], the cloning of several wheat genes [79, 80], statistical tools for characterization of genotype–environment interactions [81] and virus-induced gene silencing systems for candidate gene evaluation have the potential to revolutionize the wheat breeding [82].

Production of transgenic wheat incorporating disease resistance genes is a potent option for preventing yield losses. Transgenic wheat plants with specific differences in their grain quality traits will highly enhance the end-use properties of wheat [83]. This needs the application of reliable and robust transformation methods for introduction of additional genes. However, non-acceptance of transgenic wheat by the public is the biggest impediment in the knowledge application. Herbicide-resistant transgenic wheat has been developed in India, but controversies remain regarding the spread of transgene to wild relatives. Inheritance and stable expression of the transgene over a generation is important in creating drought-tolerant crops for agricultural purpose. Wheat transformed using the biolistic method with the pathogenesis-related chitinase and β-1,3-glucanase genes under the control of the maize ubiquitin promoter showed gene silencing in T3 generation plants [84].

Besides ethical issues for non-adoption of genetically modified material, higher costs are also an impediment. However, to overcome the increased level of a barrier for genetic improvement, the success with the traditional breeding method is more likely to be economically unviable and unsustainable. The newer tools and technologies like molecular marker technologies provided a range of opportunities to plant breeders to improve their selection efficiency. MAS is a new technique making use of knowledge generated at genomic level to assist plant breeders in the selection of plants with desirable traits. Much of plants hidden potential were unknown in the past as most of the selection were done by phenotype, but MAS hold the potential to unlock the hidden variability for important traits like yield and resistance to biotic and abiotic stresses.

Similarly, most of the phenotype we observe arises from the interaction between genes, RNA, proteins and environmental signals and therefore breeding techniques have been more successful where single gene harbors the desired trait. MAS, on the other hand, hold the potential to deal with sets of interacting genes for complex traits. The information generated by the location and numbers of genes controlling the traits of interest through comparative mapping and QTL studies can speed up the end product-specific variety. The good news is Triticeae genome is rapidly

evolving and quite heterogeneous [85], thus offering opportunities to plant breeders to continuously search for novel genetic variations [86]. Marker-assisted backcrossing can routinely be integrated into the breeding programs to introgress the different disease and pest resistant genes as well as genes for quality improvement. MAS can also be very helpful in selection of parental lines for crossing in making complex crosses, particularly where the traits to be transferred recessively inherited and thus removes the need for progeny testing. With boom and bust cycle associated with all the three rusts, it becomes imperative to pyramid the number of multiple effective genes in the single genetic background, which is impossible without using MAS. Another area where MAS can be useful in improving the breeding efficiency is the early generation selection. With increasing throughput analysis, the large material can be screened by marker assisted selection in the early generation for multiple traits. Lesser and lesser materials, therefore, will be advanced for replicated, multilocation testing, which is more expensive and time-consuming.

The initial use of MAS in tracking the gene from donor parent is still relevant, but the selection of background in the absence of sufficiently large number of reliable markers and whole genome analysis discourage the use of this technique. However, for most of the complex traits like yield, accurate detection and estimation of QTL effect, undesirable linkage of already identified QTL and the differential value of multiple QTL alleles are the issues which are identified a decade ago but are still relevant today. Despite its all advantages, the decision to invest in cutting edge technologies is governed by the availability of resources and economic return offered by the application of these technologies. Moreover, linkage and exchange of ideas and material between the traditional plant breeders and molecular breeders in India is needed, and therefore a strong joint effort will prove more rewarding.

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Chapter 5

Overview of Methods for Assessing Salinity and Drought Tolerance of Transgenic Wheat Lines

Rohit Joshi, Khalid Anwar, Priyanka Das, Sneh L. Singla-Pareek, and Ashwani Pareek

Abstract

Salinity and drought are interconnected, causing phenotypic, physiological, biochemical, and molecular changes in a cell. These stresses are the major factors adversely affecting growth and productivity in cereals. Genetic engineering methods have advanced to enable development of genotypes with improved salinity and drought tolerance. The resulting transgenic plant produces a group of progenies which includes moderate to high-stress tolerant transgenic lines. Development of reproducible screening methods to identify high-stress tolerant germplasm under laboratory, greenhouse, or field conditions is must. Further, field level demonstration of improved phenotypes and yield under salinity and drought stress conditions is both challenging and expensive. Fast and efficient screening techniques that could be used to screen transgenic lines under greenhouse conditions, for salt and drought stress tolerance, may contribute toward the identification of promising lines for field conditions. This chapter provides information on various approaches which can be developed during different stages of plant development for selecting salinity and drought tolerant plants in cereals, especially wheat.

Key words Salinity, Drought, Wheat, Rice, Transgenic plants, Screening methods

1 Introduction

Soil salinization and drought are considered to be the major threats to food security and to fulfill the objective to mitigate the increasing demand for food [1, 2]. However, cereal grains such as wheat and rice are considerably sensitive to salinity and drought during different stages of development [3]. After rice, wheat is the second most important crop in developing countries and is grown over a large latitudinal range throughout the world [4]. The effects of drought and salinity with the future climate change on growth and development of plant and agricultural productivity have become a major environmental issue in developed as well as developing countries. A high level of similarity has been observed in salinity and drought stress responses with regard to their physiological, molecular, and

Prem L. Bhalla and Mohan B. Singh (eds.), *Wheat Biotechnology: Methods and Protocols*, Methods in Molecular Biology, vol. 1679, DOI 10.1007/978-1-4939-7337-8_5,

genetic effects [5]. Drought and salinity have common mechanism in plant cells to alter redox homeostasis by disrupting essential metabolic processes.

Among various abiotic factors challenging global crop productivity, salinity is one of the most significant constraints on crop yield in agriculture, especially in those areas where salinization has developed due to deforestation, inefficient irrigation, or poor drainage [6]. It is estimated that 230 Mha (19.5%) of irrigated land and almost 45 Mha (2.1%) of dry land agriculture is affected by salt globally [7]. In Australia, two-third of the agricultural area has a potential for transient salinity. Salt-affected soils excessively accumulate a complex combination of soluble salts. Plants possess a very fine signaling system to rapidly sense, respond and adapt to salt stress [8]. Elevated levels of salt in the soil limit the water uptake because of low water potential, thereby initiating drought stress [9].

Similarly, drought is another environmental factor affecting the plant development, as periodic drought affects more than 50% of the wheat cultivated area [10, 11]. With increasing global environmental vagaries, the incidence of drought is more likely to occur at escalated frequency (up to 30% by 2070) particularly in southwest Australia and Central and South America [12]. Drought stress leads to nearly 40% annual loss in productivity of South and Southeast Asia, resulting in 58% income loss [13, 14]. Plants manifest multiple impairments upon exposure to drought, including reactive oxygen species (ROS)-mediated cell damage and higher intercellular temperature, which increases the viscosity of cytosol, modulates protein–protein interactions, protein aggregation, and denaturation [15]. A distinct alteration in cellular volume followed by cytosolic contraction is an instant symptom of dehydration. Besides, enhanced solute accumulation results in toxicity and impairment of enzymatic functions, leading to decrease in photosynthetic rate and water use efficiency (WUE). However, under prolonged dehydration, leaf rolling occurs, followed by bleaching and wilting that causes plant death [16]. Reproductive stages, i.e., flowering and seed development are more sensitive toward drought stress [13, 17]. It has already been reported earlier that higher pollen sterility, accumulation of abscisic acid in spikes and reduced dry matter partitioning and grain filling occur in drought stress susceptible genotypes leading to severe grain loss [11]. Recently, leaf characteristics such as area, weight, and growth rate were also found to correlate well with the breeding genotypes under drought-afflicted Mediterranean regions [18]. Extensive development has already been made to boost crop productivity through conventional breeding techniques. However, development of cultivars for drought tolerance using breeding approach is limited due to low heritability of drought tolerance, constricted genetic

variation in the genetic pool, complex and polygenic nature of this trait, a large size of wheat genome (17 Gbp) and the great extent of environmental interactions [19].

It is clear that development of cultivars with improved tolerance is need of the hour. Unfortunately, the mechanisms contributing toward tolerance to these abiotic stresses in crop plants are not entirely understood. At the same time, protocols to screen wheat genotypes for salt, as well as drought stress tolerance under field conditions, are also needed. However, this is often difficult due to the highly variable environmental conditions. Screening protocols well suited for the controlled environment have therefore often been used which are readily acceptable for an effecient screening of a large number of genotypes. The protocols presented in the current chapter gives an easy-to-follow procedure to select salt and drought-tolerant wheat transgenics for subsequent field testing. We describe here screening methods for transgenic vis-à-vis wild-type (WT) plants at different stages of plant development. The goal is to investigate the possibility of how cereals can be screened for salinity and drought tolerance with less time and money, to support the breeding programs.

2 Screening Methods for Salinity and Drought Tolerance at Seed Germination, Vegetative Stage, and Reproductive Stage

Drought and salt tolerance is a complex stage-specific trait, and it depends not only on the intensity of stress but also on the particular stage in which it occurs [20]. The methods of screening presented below are focused basically on screening at three major stages of crop development, i.e., germination, vegetative, and reproductive stages (Fig. 1). Work from our laboratory has shown the usefulness of a few of these methods to screen a large population of rice for tolerance toward salinity [30].

2.1 High-Throughput and Automated Phenotyping

Development of crops for stress tolerance and yield stability is efficiently supported by identifying quantitative trait loci (QTLs) correlated with phenotypes of transgenic plants [21]. The development of phenomics as an independent discipline opens new horizons for plant sciences by utilizing sophisticated, nondestructive, high-throughput automated phenotyping systems for capturing, storing and analyzing a vast amount of data. This allows for nonintrusive high spatial resolution spectral imagery for large-scale monitoring and quantification of physiological parameters, i.e., plant height, leaf and head size, canopy temperature, hydration status, pigment composition, and agronomic traits such as biomass and yield [22, 23]. Near or far-infrared reflectance based remote sensing techniques through digital sensors, thermometers, thermal imaging cameras were found to accurately measure various

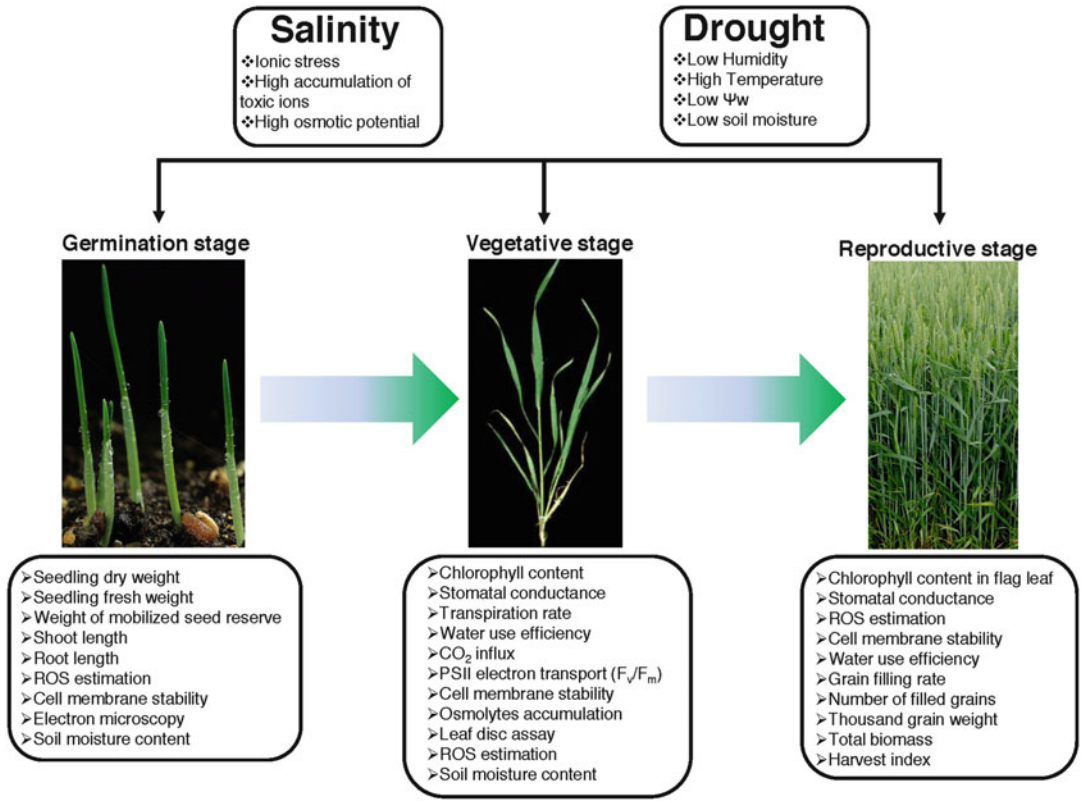

Fig. 1 A schematic depiction of rapid screening methods during different development stages of wheat under abiotic stresses

phenotypic traits like complex root architecture [24]. These advanced phenotyping technologies can be used to create and manage large databases [19].

2.2 Screening at Juvenile Stage

Salinity and drought are the major abiotic stresses affecting the productivity of major cereals and predicted environmental changes might cause serious salinization of more than 50% of arable lands by 2050 [25]. A reduction in seedling growth, fraction of seed reserve utilization, increase in mobilization of stored reserve due to higher β-amylase activity and protein modulation in germinating embryos was observed with increasing salinity and drought [26]. The decline in seedling dry weight in response to salinity and drought is a consequence of a reduction in weight of mobilized seed reserve, not seed reserve utilization efficiency. Further, seed germination has been shown to be extremely sensitive in durum wheat cultivars for abiotic stresses [27]. Previous studies thus, established that salinity stress significantly reduces seed germination in wheat either by limiting water absorption by creating higher osmotic potential or by specific ion toxicity [28]. The dry weight of germinating seedling is always less than the mobilized substrates, because of

enhanced respiration rate [29]. Seedling growth under salt stress is inhibited through reductions in seed reserve utilization and/or efficiency. However, under drought stress, a drastic reduction in fresh weight occurs. Earlier reports have established that wheat is moderately tolerant to salt stress during seedling stage, as an injury at this stage can be recovered during later stages of development [30, 31]. Similarly, seed development is also sensitive to drought stress [32, 33]. Upon exposure to drought, plants accumulate reactive oxygen species (ROS), generated mostly in chloroplast and to some extent in mitochondria, causing oxidative stress [4, 13]. To test the germination percentage of wheat under salinity and drought stressed conditions, we use sodium chloride [34] and a high molecular weight osmotic substance, i.e., polyethylene glycol (PEG) respectively [35].

2.3 Leaf Disc Senescence Assay

Various physiological parameters, such as plant height, fresh weight, chlorophyll content, photosynthetic rate, antioxidant activity, and total yield, are directly correlated with the stress tolerance ability of any given genotype. Stress tolerance potential of a plant can also be evaluated rapidly through a quick leaf disc assay which is a widely acceptable technique with least requirement of instruments [5, 36]. Leaf disks from leaves of a similar age are floated in control (Yoshida medium) or saline (200 mM NaCl solution) or drought (5% PEG-6000) medium, and the extent of chlorosis indicates the decline in photosynthesis caused by stress [2, 37, 51]. When compared to different transgenic with their corresponding WT plants, this technique provides a quick assessment of the ability of the tissue to tolerate Na^+ accumulation. It has been demonstrated earlier in wheat that salinization leads to increased apoplastic osmotic potential, which would reduce wall extensibility and turgor inside the cell [38]. Similarly, drought stress during reproductive stage accelerates leaf senescence rate of flag leaves which contributes about 30–50% assimilates in wheat grains [39].

2.4 Estimation of Photosynthetic Efficiency

Chlorophyll (Chl) content is the primary photosynthetic pigment and an important agronomic trait directly correlated with photosynthetic rate [40]. Earlier, researchers have already reported that high-yielding genotypes possess more leaf Chl and have a higher photosynthetic rate which aid in the increase in plant biomass and yield [32]. Chlorophyll a is essential in photochemistry, while chlorophyll b is a critical component of light-harvesting chlorophyll binding proteins [41]. Drought stress decreases chlorophyll content, ATP synthase expression, cytchrome b6f expression and increases xanthophyll pool [42]. Spectrophotometric technique requires destructive analysis, as they consume leaf tissues and involve lengthy procedures for chlorophyll measurement. Hand-held SPAD chlorophyll meter (Konica-Minolta, Japan) gives an

indirect measurement of leaf chlorophyll contents in a rapid, non-destructive, and convenient manner. The SPAD 502 Plus chlorophyll meter is a widely used tool for fast determination of chlorophyll concentration as well as photosynthetic rate. The SPAD 502 Plus is based on two light-emitting diodes and a silicon photodiode receptor, that determines the relative amount of chlorophyll in two wavelength regions, i.e., transmittance in the red (650 nm; the measuring wavelength) and infrared (940 nm; a reference wavelength used as standard for nonspecific differences between samples) regions. These transmittance values are sensed by the device to derive a relative SPAD value (ranging from 0.0 to 50.0) that directly reflect the chlorophyll content of the sample [43].

Stomatal conductance influences both transpiration and photosynthesis, thereby correlating both carbon and water cycles affecting soil–plant–atmosphere continuum [44]. However, during water deficit, stomatal opening rate act as a limiting factor to this process [45, 46]. Stomatal conductance varies widely across species, and the biophysical factors regulating the rate of response are still in vague [46]. Photosynthetic and water-use efficiency (WUE) are also related to the dynamic change in stomatal conductance. Under control conditions with less evapotranspiration rate and high radiance, the upper limit of the CO_2 assimilation rate is determined by the maximum operating stomatal conductance. In contrast, under severe water deficits and/or dry soil, resulting in high evapotranspiration, where plants depend on complete stomatal closure and a highly water-impermeable leaf cuticle for a reduction in water loss [47]. It directly affects metabolic activities by decreasing CO_2 influx into mesophyll cells, reduced ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, impaired ATP synthesis at initial stages [48]. Drought stress also decreases PSII electron transport, but light absorbance by PSI increases and generates a proton-motive force to activate nonphotochemical quenching of energy [42]. However, in wheat, drought stress at later stages results in tissue dehydration, decreased Rubisco activity in flag leaves leading to reductions in net photosynthetic rate [49]. But this does not affect water status of spikelets, suggesting that desiccation of reproductive organs is not related to decreased grain filling rate [50]. Thus, the stay-green character of the flag leaf in wheat is positively correlated with higher photosynthetic activity, water use efficiency and harvest index [51]. The stomatal conductance can be measured using Leaf Porometer (SC-1; Decagon, USA), while other photosynthetic parameters, i.e., intercellular CO_2 concentration (Ci), stomatal conductance (gs), net photosynthetic rate (PN), chlorophyll fluorescence (Fv/Fm), electron transport rate (ETR), and transpiration rate can be measured from the youngest fully expanded leaves (in triplicate) with a portable photosynthesis system (LI-6400XT, LICOR, USA) [6, 52].

2.5 Reactive Oxygen Species and Antioxidant Enzyme Activity

Oxidative stress symptoms such as accumulation of reactive oxygen species (ROS) are often exhibited by plants suffering from salinity or drought stress [53, 54]. These superoxide ions and other ROS are generated in photosystem I (PSI) either due to stomatal closure leading to the reduction in CO_2 influx or because of excessive light followed by electron movement to molecular oxygen [49]. Excessive light energy decreases the thylakoid lumen pH and activates qE by protonating the protein PsbS [42]. These ROS further damage the photosynthetic apparatus thus leading to a considerable decrease in carbon fixation. A straightforward and rapid histochemical staining method using nitro-blue-tetrazolium (NBT) for determining the intercellular accumulation of superoxide anions and 3,3′-diaminobenzidine (DAB) for detection of H_2O_2 in the leaves of stress-treated plants can be used as directed earlier [55]. In cereals, antioxidant defense activities are modulated by enzymatic (glutathione reductase, catalase, ascorbate peroxidase, and superoxide dismutase) and nonenzymatic (β-carotene, reduced glutathione, ascorbic acid, and α-tocopherol) antioxidants [49]. To determine the antioxidant enzyme activity, protein contents can be measured, and enzyme assays can be performed in the salinity and drought-treated WT as well as transgenic lines [55].

2.6 Membrane Stability and Ionic Content Determination

Electrolyte leakage defines the status of the stability of plasma membrane under stress response at the cellular level. The electrolyte leakage is variable among different species, tissues, and cell types, and can be elicited by all the main stresses [56]. It is the primary response of K^+ efflux and influx of the so-called counter ions (Cl^-, HPO_4^{2-}, NO_3^-, citrate^{3-}, and malate^{2-}) which maintain cellular homeostasis. This is a peculiar response among roots, where irreversible loss of K^+ due to leakage is during a stress response. The control of Na^+ and Cl^- transport is a crucial phenomenon in plants for salinity tolerance. The accumulation of these ions in a certain amount is essential for ion homeostasis in plants. However, during salinity stress, these ions can accumulate to toxic levels in the cells. Thus, salinity tolerance mechanism in plants is defined by the Na^+ and Cl^- partitioning into the vacuoles and the accumulation of K^+ or organic solutes in the cytoplasm [57]. These ions are primarily localized in soluble form inside cells and are recovered using dilute acid and are detected either by a flame photometer or through much sensitive atomic absorption spectrophotometer (AAS). Flame photometry is a device utilized for the detection of inorganic ions like Na^+ and K^+. Readily available hydrocarbon gas such as propane is used to generate a controlled flame to vaporize elements. Comparison of emission radiation from samples with that of a calibration curve of known concentration solution gave the quantitative analysis of the element of interest [57]. Similarly, atomic absorption spectrometry is a highly sensitive analytical method to determine specific ion concentration. A

hollow cathode lamp act as light source and an air–acetylene mixture flame are used as an atomizer to produce 2300 °C temperature into which samples are sprayed. The electrons of the atoms move to higher energy levels for few nanoseconds. The radiation flux with and without the sample is measured by the detector and the light absorbed by that element is converted to analyze concentration according to Beer–Lambert Law [57, 58].

2.7 Estimation of Osmolytes

Sugars (sucrose), sugar alcohols (trehalose, mannitol), amino acid (proline), and quaternary ammonium compounds (glycinebetaine, polyamines) are the main osmotic substances in plants [4]. Proline accumulation is a common physiological response in plants toward various environmental stresses. Progressive drought stress induces a considerably higher proline accumulation in stressed plants. Proline can act as an osmolyte, and a signaling molecule that influences mitochondrial functions, modulates cell proliferation as well as cell death, and induces specific gene expression that is essential for plant recovery from stress [35]. Proline primarily accumulates in the cytosol and substantially contributes to the osmotic adjustment by modulating protein degradation by preserving the quarternary structure of complex proteins, maintaining membrane integrity and reducing membranous lipid oxidation or photoinhibition under dehydration stress. In addition, it also contributes in stabilizing subcellular organelles, ROS scavenging, and buffering cellular redox potential during stress [2]. The most commonly used method for proline estimation is based on spectrophotometer [2, 55, 59, 60].

2.8 Relative Water Content and Water Use Efficiency

It is already known that the differences in drought tolerance among different genotypes chiefly reflect variations in their plant water status and turgor under stress conditions. Plant water status, measured as leaf relative water content (RWC), can vary significantly among genotypes exposed for similar periods of water elimination [61]. In cereals, these differences are coupled with the variations in stomatal conductance during transpiration, water evaporation, and differences in canopy size under stress conditions. Thus, factors affecting the ability to maintain inner water potential primarily influence plant productivity under stress conditions as a rapid decrease in plant water status do not permit time for osmotic adjustments [48]. Furthermore, RWC is closely related to leaf water potential and leaf rolling under drought stress [61].

Water use efficiency (WUE) is the ratio of the biomass produced per unit water transpired over a specified period. It deals primarily with soil water efficiently used for biomass production and thus considered as “drought avoidance” trait. Improving this trait has been shown to be quite relevant in crop improvement. Maximum utilization of available soil water increased biomass productivity per unit water use and maximum biomass conversion into

economic yield under limited water conditions are the main targets of drought-related studies [62]. Physiologically, water use efficiency (WUE) is defined at either single leaf level or whole plant level and or at canopy level.

$$\text{Water use efficiency for crop grain} = (\text{grain yield/water applied at different level of requirement}) \text{ expressed in } \text{kg/m}^3 \text{ or kg/ha/mm}$$

$$\text{Water use efficiency for crop biomass} = (\text{Total Biomass/water applied at different level of requirement}).$$

WUE is mainly determined either by a gravimetric method in which whole plant WUE is determined as follows:

$$\text{WUE } \left(\text{g/L}^2\right) = (\text{dry weight of final biomass} - \text{dry weight of initial biomass/total water consumed}).$$

Carbon isotope composition method using IRMS (isotope ratio mass spectrometer): Isotope ratio mass spectrometer can be used for the quantification of stable isotope ratios of carbon ($^{13}C/^{12}C$), oxygen ($^{18}O/^{16}O$), nitrogen ($^{15}N/^{14}N$), and deuterium ($^{2}H/^{1}H$) in plant samples and organic materials. The stable isotope ratio analysis involves sample preparation, isotope ratio quantification, and data interpretation [63].

2.9 Soil Water (Moisture) Content

Soil moisture availability directly affects plant growth rate, root development, soil strength, and crop yield [64]. However, reliable determination of this variable at regional scales through standard point measurements is complicated. In the past decades, several methods were developed for soil moisture measurement such as gravimetric method [65], X-ray micro-computed tomography (μCT) [66], satellite-based techniques [67] and used soil moisture sensor. Soil volumetric water content probes (sometimes referred to as soil moisture sensors) measure the water content of soil. These probes can also be used for estimation of the stored water in the particular soil sample so that the irrigation requirement is calculated. These probes can be used both for rapid analysis or installed for continuous measurements for a long time. Satellite-based techniques are based on the interpretations using optical–thermal sensors and microwave sensors. Through this technique, large-area soil moisture can be estimated and interpreted for the interactions between land surface and atmospheric process, canopy water stress, land surface temperature, and vegetation indices [68–70]. X-ray μCT is a nondestructive method for high throughput root phenotyping including soil matrix, air-filled pores, water-filled pores, plant material, and organic matter [71, 72].

3 Conclusion

Crop phenotype under affected field conditions is the ultimate decisive factor enabling the user to determine whether a genetically altered plant is suitable to grow or not. The screening methodologies for abiotic stress tolerance bridge the gap between molecular mechanisms and abiotic stress response of a stable transformant. In this chapter, we discuss rapid screening techniques and to present their correlation with various stress responsive traits associated with either increased (ectopic expression and overexpression) or decreased (mutants, knockouts, and RNAi knockdown) gene products. At present, several high-throughput molecular techniques are available for rapid and precise detection of the zygosity status of a transgene locus, but the phenotypic response of the transformants give the most reliable and consistent tool to test the homozygosity of transgenic events. Thus, these rapid techniques available for screening various traits have been used widely in recent past years thus helping in the identification and better understanding of abiotic stress tolerance phenotypes. These techniques provide researchers the valuable tool for basic and applied research to address critical global food security.

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Chapter 6

Allergenicity Assessment of Transgenic Wheat Lines In Silico

Ankita Mishra and Naveen Arora

Abstract

Agriculture biotechnology is a promising tool for developing varieties with enhanced quality and quantity. Transgenic proteins expressed by genetically modified (GM) food crops improve crop characteristics like nutritional value, taste, and texture, and endow plants with resistance against fungus, pests, and insects. Despite such potential benefits, there are concerns regarding possible adverse effects of GM crops on human health, animals and the environment. Among the proposed guidelines for GM food safety testing—the weight-of-evidence approach proposed by the Codex Alimentarius Commission (ALINORM 03/34A) is the most recent. Till date, several transgenic wheat lines have been developed and research is underway for further improvement. However, GM wheat is not being grown or consumed in any part of the world. In the present study, in silico tools were employed for safety testing of eight transgenes used for the development of transgenic wheat lines. Among the genes studied, none of them shared sequence homology with the reported allergens and may be safe for use in genetic engineering. In conclusion, gene selection for developing transgenic wheat lines should be done with utmost care to ensure its safety for feed and fodder.

Key words Wheat allergy, Celiac disease, Transgenes, Allergenicity, Safety testing

1 Introduction

Conventional breeding involves random mixing of many genes present in a plant with various other genes from different crops. However, genetic engineering implies transfer of a single gene, or a couple of genes in a precise and controllable pattern to obtain desired results [1]. These genetically modified crops are superior to their natural counterparts and endow the host plant with several advantages like improved taste, texture, yield, and nutritional value [2–4]. A wide variety of crops have been engineered for enhanced resistance against herbicides, insecticides, viral diseases and also abiotic factors like salt, drought, and extreme temperatures [5]. However, GM foods obtained from transgenic crops require safety testing for evaluating their tendency to provoke an allergenic

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reaction, stability in the environment and unintended effects which could result from the insertion of the new genes.

2 Food Allergy

Food allergy is an adverse immune response to a food component (usually a protein) mediated by IgE antibodies and is characteristic of type 1-hypersensitivity reactions. Reaction mechanism involves preferential production of allergen specific IgE antibodies, which possess high affinity for its receptors on the surface of mast cells and basophils. Subsequent exposure to the same allergen results in cross-linking of the cell bound IgE(s) and release of mediators like histamine, cytokines, and serotonins. Food allergies are estimated to affect 1–2% of the adult population and 6–8% of children with growing evidence of an increase in prevalence [6]. Previous studies suggest that milk, egg, peanut, tree nuts, fish, shellfish, soy, and wheat account for 90% of all food-allergic reactions worldwide [7]. Wheat, milk, egg, and peanut account for majority of IgE mediated reactions in young children, whereas peanuts, tree nuts, and seafood (fish and shellfish) account for the IgE-mediated reactions in adults. Common allergic manifestations include eczema, hives, allergic rhinitis, asthma, gastrointestinal tract allergies, and reactions to the venom of stinging insects such as wasps and bees. In some people, severe allergies to environmental or dietary allergens or to medication may result in life-threatening anaphylactic reactions and death.

Whereas, non IgE-mediated (cell mediated) food allergy is less common and involves generation of T cells against the protein, leading to the release of mediators, involved in a variety of subacute and chronic diseases. These reactions generally affect the gastrointestinal (GI) tract and skin. Non IgE mediated food allergy reactions manifests into some chronic diseases like eosinophilic GI disease (EGIDs), food protein-induced enterocolitis syndrome (FPIES), systemic (whole body) contact dermatitis, and celiac disease.

3 Wheat

Wheat is a cereal grain consumed as a staple food by millions across the globe [8]. It is generally rich in proteins, vitamins, dietary fibers, minerals, and carbohydrates and is considered to be an essential part of the balanced diet. Wheat grain is generally processed into flour before consumption and the common dietary intake is in the form of bread, pasta, noodles, biscuits and other bakery items, etc. [9]. Till date, more than 20,000 cultivars of wheat are known, and is the cumulative result of conventional

breeding [10]. Wheat is grown in almost all parts of the world including Europe, North and South America, North Africa and the Indian subcontinent. Nowadays, two of the most commonly grown species include—*Triticum aestivum* (bread wheat) and *Triticum durum* (durum or macaroni wheat) [11]. World leaders in cultivation of wheat are China followed by India and the USA. But, China and India both produce for their own consumption. On the contrary some countries like the USA, Canada, Australia, and Argentina export a significant portion of their produce to the world. As per the records, USA produces around 1.3–2 billion bushels of wheat per year and exports half of it, to other nations [12]. The ever increasing global population and constantly changing lifestyle has made it mandatory for the regulatory authorities to come up with a solution to meet the demands for major cereal crops like wheat, maize, and rice.

Genetic engineering is a promising tool for developing varieties resistant to biological infestations and tolerant to natural inconsistencies. Researchers are currently developing protocols for conferring resistance to fungal and viral diseases and are also testing their effectiveness on wheat. Field trials for GM wheat are underway in many countries, in 2013 around 34 such events had taken place in Europe and 419 in the USA. The GM wheat strains tested had genetic modifications for developing resistance to herbicides, insects, fungal pathogens (especially *Fusarium*), and viruses, and develop tolerance to drought, high soil salinity and heat. Some other desired traits for developing GM wheat include—high glutenin content, enhanced nutritional quality, and improved crops for use as—biofuels and feed stocks [9]. Till date, several research groups have developed many GM wheat lines to meet the consumer demands. However, none of them are being or have been cultivated in the past due to safety concerns. GM wheat is neither grown nor eaten anywhere in the world, and it has never been in market. Only four major GM crops have been grown for commercialization and these include—soy, corn, canola, and cotton. And account for almost 99% of the world's GM crop produce. Some of the genes used for development of transgenic wheat lines are mentioned in Table 1.

4 Celiac Disease

Some food allergic reactions are categorized as delayed type hypersensitivity responses like cell-mediated reactions involving sensitized lymphocytes in tissues rather than IgEs [13]. The exact mechanism for cell-mediated reactions is not known, however the prevalence is high among infants [14]. The onset of symptoms occurs more than 8 h after ingestion of the offending food. The most commonly encountered cell-mediated hypersensitivity

Table 1
Some of the commonly used genes for developing transgenic wheat

S. No.	Gene	Source	Function
1	*Ac*	Maize	Plants expressing the transposase
2	*Ama 1*	*Amaranthus hypochondriacus*	Improve nutritional quality
3	*Bar*	*Streptomyces hygroscopicus*	Resistance to herbicides
4	*BetA*	*Escherichia coli*	Drought tolerance
5	*BLF*	Bovines	Resistance to head blight
6	*Chi 2*	Barley	Resistance to *Erysiphe graminis*
7	*Coat protein (CP)*	WSM virus	Resistance to WSM virus
8	*DREB1A*	*Arabidopsis thaliana*	Drought tolerance
9	*FsTRI101*	*Fusarium sporotrichioides*	Resistance to *F. graminearum*
10	*GmDREB*	Soybean	Drought and salt
11	*GhDREB*	Cotton	Drought, salt, freeze stress tolerance
12	*HVA1*	Barley	Drought tolerance
13	*KP4*	*Ustilago maydis* infested with virus	Resistance against stinking smut
14	*NPR1*	*Arabidopsis thaliana*	Resistance to *Fusarium* head blight
15	*P5CS*	*Vigna aconitifolia*	Increased tolerance to salt
16	*PhyA*	*Aspergillus niger*	Accumulation of phytase in seeds
17	*PIN2*	Potato	Cereal cyst nematode infestation
18	*RIP*	Barley	Resistance to *Erysiphe graminis*
19	*Rnc 70*	*Barley stripe mosaic virus*	Resistance to *stripe mosaic virus*
20	*Stilbene synthase (Vst1)*	*Vitis vinifera*	Production of phytoalexin resveratrol

reaction affecting all age groups is celiac disease, also known as gluten-sensitive enteropathy. Along with wheat, delayed food-induced enteropathy has also been observed for some other food allergens like milk and soy.

Celiac disease affects 1 in every 300–3000 individuals in a population depending upon the geographical disparity. Gluten-sensitive enteropathy or celiac disease is a T cell-mediated immunological response elicited by gluten (gliadin) and affects atopic individuals. Gluten-sensitive enteropathy is said to occur on ingestion of gliadin, a prolamin (gluten protein) found in wheat, and also due to similar proteins found in other crops like barley and rye. Upon exposure to gliadin, the immune system initiates an inflammatory reaction leading to truncation of the "villi," present in the lining of

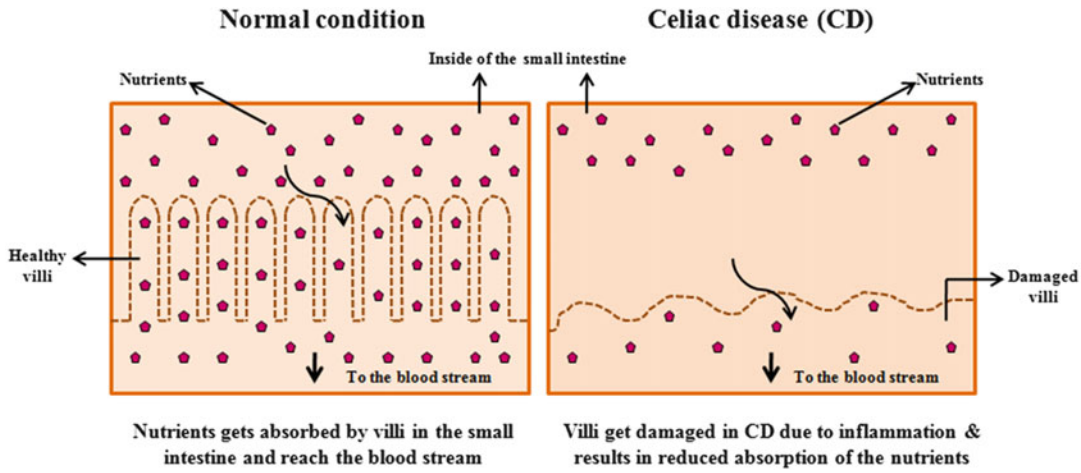

Fig. 1 Effect of celiac disease (CD) on the nutrient absorption in the intestines

the small intestines (Fig. 1). This condition is referred as "villous atrophy." The active phase of the disease consists of an inflammatory process where the small intestine is incapable of absorbing the nutrients present in the consumed food, leading to the development of anemia, diarrhea, vitamin deficiency, and fatigue [15, 16]. The only known effective treatment is lifelong avoidance of gluten from wheat, rye, barley, and related cereals. It is noteworthy that although the disease occurs due to ingestion of wheat proteins it is usually classified as different from the other forms of wheat allergy due to lack of IgE immunoglobulins.

5 Wheat: A Major Food Allergen

Recent population-based studies have shown that the prevalence of wheat allergy and its sensitization has increased since the last few decades. Allergic reactions can result from consuming wheat, and in some cases, only by inhaling wheat flour. The wheat grain comprises of four different types of protein (classified on the basis of solubility) and these are—(1) water-soluble albumins, (2) salt-soluble globulins, (3) ethanol-soluble gliadins, and (4) detergent, or KOH-soluble glutenins. Wheat allergy is an IgE mediated immune reaction and is different from "celiac disease" which is a gluten-sensitive enteropathy, caused by ingestion of the gliadin fractions. Wheat-dependent exercise-induced anaphylaxis is commonly found in adults [17]. The major allergens present in wheat are ω-5 gliadin (Tri a 19), α/β-gliadins (Tri a 21), and HMW glutenin (Tri a 26). Furthermore, wheat LTP (lipid transfer protein; Tri a 14) have also been identified to play an important role in the development of wheat-induced anaphylaxis [18]. As per the

Table 2
Major and minor wheat allergens (as per IUIS) isolated and characterized

Allergen	Molecular weight in kDa	Food allergen (Y)/(N)	Property	Isoforms
Tri a 12	14	Y	Profilin	4
Tri a 14	9	Y	Nonspecific lipid transfer protein	2
Tri a 15	–	N	Monomeric α-amylase inhibitor	–
Tri a 19	65	Y	Omega-5 gliadin	–
Tri a 20	35 to 38	–	Gamma gliadin	–
Tri a 21	–	N	Alpha-beta-gliadin	–
Tri a 25	–	Y	Thioredoxin protein	–
Tri a 26	High molecular weight glutenin	Y	Glutenin	2
Tri a 27	27	N	Thiol reductase homolog	–
Tri a 28	13	N	Alpha-amylase inhibitor	–
Tri a 29	13	N	Tetrameric α-amylase inhibitor	2
Tri a 30	16	N	Tetrameric α-amylase inhibitor	–
Tri a 31	–	N	Triosephosphate-isomerase	–
Tri a 32	–	N	1-cys-peroxiredoxin	–
Tri a 33	–	N	Serpin	–
Tri a 34	–	N	Glyceraldehyde-3-phosphate-dehydrogenase	–
Tri a 35	–	N	Dehydrin	–
Tri a 36	40	Y	Low mol. wt. glutenin GluB3-23	–
Tri a 37	12	Y	Alpha purothionin	–
Tri a 39	–	N	Serine protease inhibitor like protein	–

IUIS, the major and minor wheat allergens isolated and characterized (Table 2) till date, include:

Tri a 12: Tri a 12 is a profilin of molecular weight 14 kDa and exists in four isoforms namely: Tri a 12.0101; Tri a 12.0102; Tri a 12.0103, and Tri a 12.0104. Previous studies have shown that specific IgE against Tri a 12 has been found in patients suffering from bakers' asthma, wheat induced food allergy and also in some patients with grass pollen allergy [19]. Profilins are ubiquitous proteins with a highly conserved 3D structure responsible for wide cross-reactivity among pollen, latex, and plant foods [20].

Information on the 3D structure of Tri a 12 can be of help in identifying potential B and T cell epitopes and subsequently develop hypoallergenic variants. However, contrary to other plant profilins, the 3D structure of Tri a 12 is not known.

Tri a 14: Tri a 14 is a nonspecific lipid transfer protein 1 of molecular weight 9 kDa and exists in two isoforms namely: Tri a 14.0101 and Tri a 14.0201. It is one of the major allergens responsible for eliciting allergic sensitization in baker's asthma. LTP's are low molecular weight proteins ranging from 9 to 10 kDa and display thermal stability and pepsin resistance. In a study of 40 patients with occupational asthma due to wheat-flour inhalation, specific IgE to purified Tri a 14 was found in 24 patients, and the purified allergen elicited positive skin-prick test reactions in 14 of these 24 patients [21, 22]. None of the 40 patients with baker's asthma described symptoms after eating wheat-derived products further confirming the involvement of Tri a 14 in baker's asthma.

Tri a 15: Tri a 15 is a monomeric alpha-amylase inhibitor 0.28 and is not considered as a food allergen.

Tri a 19: Tri a 19 is an omega-5 gliadin (seed storage protein) of molecular weight 65 kDa. Tri a 19 is a major allergen among water/salt-insoluble proteins. Previous studies have identified that IgE antibody against fast omega-gliadin cross-react with gamma-gliadin and slow omega-gliadin [23]. Some studies also suggest that gamma-70 and gamma-35 secalins in rye and gamma-3 hordein in barley cross-react with omega-5 gliadin, and, therefore, rye and barley may elicit symptoms in patients sensitized to omega-5 gliadin. Among the wheat proteins, omega-5 gliadin has been reported to be a major allergen in wheat-dependent exercise-induced anaphylaxis (WDEIA), however, the exact mechanism for WDEIA is not known.

Tri a 20: Tri a 20 is a gamma gliadin of molecular weight in the range of 35–38 kDa.

Tri a 21: Tri a 21 is an alpha-beta-gliadin and is not considered as a food allergen.

Tri a 25: Tri a 25 is a thioredoxin protein and IgE reactivity has been found among 47% of the bakers with occupational asthma and 35% among patients with grass pollen allergy, without any clinical history of cereal allergy. In the same study, it was also observed that maize thioredoxin Zea m 25 shares about 74% sequence identity with wheat Tri a 25 and display significant IgE cross-reactivity [24]. Thioredoxins expressed in different grasses may account for cross-reactivity between wheat and grass pollen allergens, due to conserved regions.

Tri a 26: Tri a 26 is a high molecular weight glutenin and exists in two isoforms namely: Tri a 26.0101 and Tri a 26.0201. Tri a 26 and wheat omega-5 gliadin are major allergens responsible for eliciting allergic symptoms in wheat-dependent exercise-induced anaphylaxis. Simultaneous detection of specific IgEs to the epitopic

sequences of both the proteins is considered to be a reliable method for diagnosis of wheat-dependent exercise-induced anaphylaxis. However, the IgE-binding epitopes of HMW-glutenin remains unknown [25].

Tri a 27: Tri a 27 is a thiol reductase homolog of molecular weight 27 kDa and 6 of 38 subjects with baker's asthma had IgE binding (ELISA) with rTri a 27.0101 fusion protein. And, it is not a food allergen.

Tri a 28: Tri a 28 is a dimeric alpha-amylase inhibitor 0.19 of molecular weight 13 kDa and 10 of 40 subjects with baker's asthma had IgE binding to rTri a 28 fusion protein (maltose binding protein), based on ELISA. And, it is not a food allergen.

Tri a 29: Tri a 29 is a tetrameric alpha-amylase inhibitor CM1/CM2 of molecular weight 13 kDa and exists in two isoforms, namely Tri a 29.0101 and Tri a 29.0201. And it is not a food allergen.

Tri a 30: Tri a 30 is a tetrameric alpha-amylase inhibitor CM3 of molecular weight 16 kDa and is not a food allergen.

Tri a 31 (triose phosphate isomerase), Tri a 32 (1-cys-peroxiredoxin), Tri a 33 (Serpin), Tri a 34 (Glyceraldehyde-3-phosphate dehydrogenase), and Tri a 35 (Dehydrin) are wheat allergens for which the exact molecular weight is not known and they are not considered as food allergens.

Tri a 36: Tri a 36 is a low molecular weight glutenin GluB3-23 of 40 kDa molecular weight required for seed germination. Previous SPT studies with Tri a 36 have identified it to be a potent allergen because of its ability to induce immediate skin reactions in sensitized patients. Basophil histamine release assay studies with Tri a 36 have also confirmed the allergenic potency of the r-Tri a 36. Wheat prolamin Tri a 36 shares sequence identity with other plant prolamins like rye, 76%; barley, 64%; oat, 48%; rice, 40% and may also share cross-reactive epitopes. However, more information is required to investigate the clinically relevant cross-reactivity [26].

Tri a 37: Tri a 37 is a recently identified novel wheat allergen of 12 kDa molecular weight and is an alpha purothionin. IgE reactivity to Tri a 37 is generally associated with high risk of developing severe wheat induced anaphylaxis, as seen in 20% of wheat food allergic patients. Due to its involvement in development of severe wheat-induced anaphylaxis, it may act as a class I allergen capable of sensitization via the GI tract [27]. The class I food allergens are generally 10–70 kDa in size and are resistant to heat, acid, or protease treatments [28]. These are also referred as "complete food allergens" and common examples include milk (caseins), peanut (vicillins), egg (ovomucoid), and nonspecific lipid transfer proteins [29].

Tri a 39: Tri a 39 is a serine protease inhibitor-like protein of wheat and is not considered as a food allergen. Out of 101 European baker's 18, had IgE binding to recombinant Tri a 39 in Immunocap.

6 Allergenicity Testing of Transgenic Wheat Lines

It is difficult to assess the allergenicity associated with GM food crops. However, the preliminary testing procedures involve evaluating the source of the transgene followed by in vitro and in vivo assessment of the transgenic protein. Transgenes used for the development of GM crops should be from a nonallergenic host. There are chances that the gene transfer/vector insertion may result in creation of a new allergen de novo or may enhance the expression of a minor allergen in the GM crop. The transgenic protein may sometimes have an adjuvant effect on a food protein of previously low allergenic potential or vice versa. Therefore, national and international regulatory bodies have designed protocols for safety testing of GM food crops. Among the recommended protocols, two of the most widely used are FAO/WHO, 2001 [30] decision tree and Codex, 2003 "weight of evidence" approach [31]. As per these guidelines, initial evaluation involves in silico studies like database search for the source of the gene and sequence homology study, where the primary amino acid sequence of the query protein is compared with that of the reported food and other allergens. Depending on the results obtained for in silico studies, further in vitro and in vivo testing is performed. However, here emphasis will be given to the in silico tools used for the safety testing of GM wheat varieties.

6.1 In Silico Studies to Assess the Allergenic Potential of Transgenes Used for Developing Transgenic Wheat Lines

Bioinformatics provides us with a platform where novel proteins can be compared to already known allergens, to find out sequence similarity, conserved motifs, or 3D structural similarity. The primary structure of proteins can be compared to deduce information essential for identifying probable homologs [32]. FASTA [33] and BLAST [34] are two commonly used alignment tools for comparing protein sequences. The information obtained may be used for predicting functional similarity or clinically relevant cross-reactivity among proteins. Previous studies have shown that proteins sharing reasonable linear sequence identity, may share structural motifs [35] and in some cases cross-reactive epitopes. Sequence homology studies are the main basis of bioinformatic methods being performed for safety testing of GM crops. The criterion recommended by FAO/WHO (2001) and Codex (2003) states that protein(s) having greater than 35% identity over any segment of 80 amino acids, between the GM protein and any allergen, depicts that the transgenic protein sequence may be a potential allergen and should be subjected to further testing. In the present study, the allergenic potential of eight transgenes (used for the development of GM wheat) was evaluated by SDAP server using 80 amino acid sliding window search mode (as described previously) [36].

Table 3
List of transgenes studied by SDAP server

S. No.	Transgene	Species	Accession no.
1	*NPR1*	*Arabidopsis thaliana*	GenBank: ABY88391.1
2	*phyA*	*Aspergillus niger*	GenBank: CAA78904.1
3	*Bar*	*Streptomyces hygroscopicus*	PRF: 225701
4	*DREB 1A*	*Arabidopsis thaliana*	GenBank: BAA33434.1
5	*RIP*	*Hordeum vulgare*	PDB: 4FBH_A
6	*Stilbene synthase (Vst1)*	*Vitis vinifera*	NP_001267939.1
7	*HVA1*	*Hordeum vulgare*	GenBank: CAA55041.1
8	*P5CS*	*Vigna aconitifolia*	Swiss-Prot: P32296.1

6.1.1 Selection of Transgenes Used for the Study

Eight transgenes were randomly selected from the genes listed in Table 1. Moreover, these genes have been used by different research groups for the development of transgenic wheat lines. Protein sequences for the genes *NPR1* and *DREB1A* from *Arabidopsis thaliana*, *phy A* from *Aspergillus niger*, *Bar* from *Streptomyces hygroscopicus*, *RIP* and *HVA1* from *Hordeum vulgare*, stilbene synthase (*Vst1*) from *Vitis vinifera*, and *P5CS* from *Vigna aconitifolia* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) (Table 3). Other genes were not included in the study due to unavailability of the amino acid sequences in the NCBI server.

As per the proposed guidelines sequence identity of >35% between query protein and reported allergen is indicative of homology among the proteins. However, for clinically relevant cross-reactivity proteins should share high degree of identity. Among the genes studied, none of them displayed greater than 35% identity with any of the reported allergens (Table 4). However, *NPR1* gene from *Arabidopsis thaliana* shared 35% identity with Sol i 4 venom allergen from *Solenopsis invicta* (fire ant) and *phy A* gene from *Aspergillus niger* shared 37.50% identify with Asp n 25 allergen from *Aspergillus niger* (Table 4). These findings confirm that the genes used for the development of transgenic wheat lines are safe and may be used for other GM crops as well.

In conclusion, eight genes studied have shown negligible homology with known allergens and may not contribute for any clinically important food allergic reactions. These findings may also serve as a guide for selection of transgenes for developing GM wheat lines.

Table 4
Sequence homology study of the genes employed for GM wheat lines by SDAP sever

SDAP—80 aa sliding window	
Allergens	**Sequence identity %**
Gene 1. NPR1 gene from Arabidopsis thaliana	
Gal d 5	22.50
Sol i 4	35.00
Gene 2. phyA gene from Aspergillus niger	
Api d 1.0101	25.00
Asp n 25	37.50
Hev b 1	27.50
Gene 3. Bar gene from Streptomyces hygroscopicus	
Mala f 2	32.50
Bom p 4.0101	22.50
Gene 4. DREB1A gene from Arabidopsis thaliana	
Lyc e NP24	23.75
Jun v 3.010101	22.50
Bos d 8	22.50
Gene 5. RIP gene from Hordeum vulgare	
Ani s 7.0101	25.00
Asp f 9	22.50
Ara h 8.0201	25.00
Tri a 34.0101	25.00
Gene 6. Stilbene synthase (Vst1) from Vitis vinifera	
Asp f 10	28.75
Cas s 5	23.75
Bla g 6.0101	27.50
Ara h 11.0101	25.00
Pen c 24	32.50
Ory s 33kD	25.00
Gene 7. HVA1 gene from Hordeum vulgare	
Tri a 35.0101	28.75
Hom s 2	27.50
Act d a	31.25
Hev b 5	32.50
Alt a 4	33.75
Poa p 5	31.25
Gene 8. P5CS gene from Vigna aconitifolia	
Asp f 8	31.25
Cyp c 1.01	28.75
Lep s 1	26.25
Ory s 1	26.25
Ole e 2	25.00
Syr v 3	26.25

Note: Some of the (80 amino acid sliding window) hits observed for the eight genes have not been considered due to space limitation. And these will not affect the data interpretation

6.1.2 Some of the Algorithms Employed for Safety Testing of Transgenes

Full length FASTA alignment: This algorithm evaluates the extent of similarity between protein sequences on the basis of percent identity and *E*-values (expectation value). An *E*-value is a degree of similarity between pairs of sequences based on matches of identical or functionally similar amino acids. Very small *E*-values indicate probable evolutionary homology, and structural similarity.

80 amino acid sliding window: Transgenic protein sequences are evaluated for potential cross-reactivity with known allergens in the databases. FASTA alignment is performed to compare all possible contiguous amino acid segments of the transgenic proteins against the allergen sequences listed in the databases. Each contiguous 80 amino acid sequence of an individual protein is searched starting with 1–80 aa, then 2–81 aa, and so on until the last 80 amino acid segment of each protein is compared with the database. An alignment of >35% identity over segments as short as 80 amino acids with known allergen(s) depicts that the transgenic protein may be a potential allergen and should be subjected to further testing.

6.2 Prediction of Probable B and T Cell Epitopes

In silico tools have complemented the conventional techniques in understanding the antigen–antibody interactions—by simulating the 3D structures for known allergens, identifying the probable antigenic determinants for numerous allergens and by elucidating the cross-reactivity among proteins. The allergenic potential of a protein can be investigated through analysis of the primary amino acid sequence, structural motifs, and epitopes of an allergen. Homology modeling/comparative modeling is an efficient tool used for engineering 3D structures for allergens (with unknown structures) based on known protein structures having high sequence homology to the allergen [37]. Computational algorithms offer fairly accurate and rapid determination of epitopes on the allergen molecules. Information obtained from experimentally derived epitopes for several allergens, has been used for designing algorithms for predicting epitopes of uncharacterized proteins. And this has formed the basis of many in silico epitope prediction severs like Immune Epitope Database (IEDB) [38], ABCpred [39], and BCPREDS [40].

B cell epitope prediction: B cell epitopes are antigenic determinants that are recognized and bound by soluble and membrane-bound antibodies on the surface of B lymphocytes and are classified as continuous (linear) and discontinuous (conformational). Most of the existing B-cell epitope prediction methods are based on physicochemical properties of amino acids like hydrophilicity, flexibility/mobility, solvent accessibility, polarity, exposed surface, turns, and antigenicity. Linear epitopes are segments on the protein sequence recognized by IgE antibodies present in patient sera. On the contrary, a discontinuous or conformational epitope is composed of several fragments scattered along the protein sequence and

these are brought together in spatial proximity when the protein exists in its native structure. Structure-based methods like "Conformational Epitope Prediction (CEP)" [41] and "Discotope" [42] use three-dimensional structural data for predicting probable IgE binding regions and possess improved efficiency compared to methods based solely on sequence information.

T cell epitope prediction: A range of computational algorithms has been developed to predict T-cell epitopes for known allergen. The accurate identification of T-cell epitopes remains a critical step, since the most selective requirement for a peptide to be immunogenic is the ability of the peptide to bind to the MHC molecule. X-ray crystallographic studies have demonstrated that the MHC class II epitope binding site consists of a groove, which is open at both ends and can accommodate 9-mer core region of 13–25 residues long peptides. The prediction algorithms vary in complexity and accuracy and most are trained to recognize peptide motifs that are required for binding to a particular MHC molecule [43]. Some others use a weight-matrix method to identify amino acids that occur at a higher-than-expected frequency at specific epitope positions [44]. However, the most accurate methods available use logistic regression [45] and, more generally, artificial neural networks.

7 Summary

Safety testing of GM crops involves evaluating potential allergenicity and toxicity associated with transgenic proteins being expressed in GM crops. Among the proposed guidelines for GM food safety testing—the weight-of-evidence approach proposed by the Codex Alimentarius Commission (ALINORM 03/34A) is the most recent. This approach has been formulated because no single endpoint is sufficiently predictive of the allergenic potential of a novel protein. It evaluates the source of the gene, investigates the sequence similarity of the transgenic protein to known allergens, and lastly studies the stability of the protein in simulated digestive fluids. Based on the results of these studies, additional studies (like IgE sera screening studies with relevant allergic patient serum) may be necessary in determining if a protein has characteristics similar to known allergenic proteins.

In silico methods used for safety testing of transgenic proteins are less time consuming and easy to practice in comparison to rigorous in vitro experiments. The prediction results obtained from servers are quite reliable and generally form the basis of future in vitro and in vivo experiments. In the present study, different search modes used for in silico studies are discussed and one of the commonly used search parameter, i.e., 80 amino acid sliding window has been used for testing potential allergenicity of eight

transgenes used for the development of transgenic wheat lines. Among the genes studied none of the genes shared sequence homology with the reported allergens and may be safe for use in genetic engineering.

Till date, the available testing protocols used for the safety assessment of genetically modified foods are not completely predictive of the allergenicity of novel proteins. Future research activities need to focus on the improvement and refinement of various components of these protocols, including bioinformatics study designs.

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Chapter 7

Agribusiness Perspectives on Transgenic Wheat

Bill Malcolm

Abstract

Declining yields of the major human food crops, looming growth in global population and rise of populism, and ill-founded bans on agricultural and horticultural crops and foodstuffs which are genetically modified have potentially serious implications. It makes the chance less than otherwise would be the case that agribusiness value chains in the future will meet the growing demand around the world for more and different foods from more and wealthier people. In the agribusiness value chain, transgenic wheat, meeting a consumer "trigger need" also must meet the "experience" and "credence," risk-related criteria of well-informed consumers. Public policy that rejects science-based evidence about the reductions in costs of production and price of genetically modified agricultural products and the science about the safety of genetically modified foods, including transgenic wheat, has imposed significant costs on producers and consumers. If the science-based evidence is accepted, transgenic wheat has potential to improve significantly the well-being of grain growers and consumers all over the world.

Ke ywords Agribusiness, Transgenic wheat, Wheat, Value chain

1 Introduction

By 1994, progress made in research and development in agricultural biotechnology meant that genetically engineered crops had become commercial propositions. Since then, there has been widespread adoption around the world of genetically modified (GM) corn, soybeans, and canola for animal feed and oil, and cotton, with significant benefits to producers and consumers in the agribusiness sector of the economy and the natural environment. At the same time, the resistance of consumers based on poor understanding, distrust of the science on which agricultural biotechnology is based, or sometimes more faith in pseudoscience than genuine scientific endeavors, have resulted in populist policy bans on GM food. Thus, GM technology improving the quantity and quality of wheat, rice, fruit, and vegetables while lowering costs of production and environmental costs is not commercially used to produce food. At the same time, the growth in world population and in world income over the next 30 years means that the increased demand for food

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that will result will pose the greatest challenge for farmers around the world to supply this demand that they have ever faced and that they will ever face. A large part of any solutions for meeting increased global demand for crop product will be to increase the yields of the major grain crops on the currently cultivated land. The rate of increase in yield potential of the major grain crops worldwide has declined from 3 to 4% p.a. in the 1970s and 1980s, to around 1% p.a. 2017. To meet the food demands that will result from population growth and demand for protein rich food of the wealthier future world population, the annual increase in yields of the major grain crops, and quality, will need to be higher than is currently the case. This is where improved crops such as transgenic wheat could come into the picture.

1.1 The Agribusiness Value Chain

The agribusiness segment of an economy is where inputs and outputs of goods and services related to agriculture are produced and consumed. Agribusiness value chains are where the commercial transactions and relationships occur among the individuals and firms involved in producing agricultural output and transforming it to meet consumer needs and wants, of the quality they require, when they want it, and where they want it, anywhere in the world. Large food retailers, processors, and trading companies have numerous domestic value chains and nowadays invest in production and processing facilities worldwide and are part of global value chains. It is in these relatively few, relatively large international agribusiness value chains, that farmers around the world will obtain some of their inputs and supply their output to help to meet the demand that is growing. Like biotechnological science itself, agricultural and food product markets in the agribusiness sector are increasingly coordinated internationally, and in both wealthy and rapidly growing economies. It is within large international agribusiness value chains that the scientific and business activity will take place that will help make possible feeding the near one billion people in the world who are currently underfed and undernourished every day, as well as helping to meet the increased demand for food that will come from the extra two to three billion people who will exist by 2050.

The constant in any agribusiness value chain is that the consumer reigns supreme. The processes of providing what consumers want are called ‘creating value’ in agricultural value chains. The focus of participants in agribusiness value chains is on “continuous value creation for the customer” [1]. Significantly, in the context of farmers growing crops that have been genetically modified, creating value for customers starts with decisions made about the characteristics of the output itself. The characteristic of the farm product—GM or not GM—traverses the whole value chain from producer to consumer. This part of the economy focussed on creating value for customers is where the development, evolution, production and

consumption of genetically modified agricultural products takes place, and where all the GM-related issues for consumers, producers, scientists, policy-makers, and regulators play out. The first customer, the buyer to whom the farmer sells and which takes place much earlier in the value chain than the final consumer, has to be just as aware of the GM status of the farm product as every other participant right through to the final consumer. The attitudes to genetically modified foodstuff of consumers and policy makers, and their consequential actions, ripple throughout the agribusiness value chain.

1.2 The Customers

In the context of consumer acceptance or rejection of GM food, such as transgenic wheat, the question of interest is: What characteristics of goods and services are valued by customers?

The value of an agribusiness good or service along the agribusiness value chain, the combined set of value—traits, which determine ultimately how much a buyer is prepared to pay, is called the "marketing mix." This refers to all the characteristics of the good or service a firm presents to customers. A common description of the elements of this marketing mix is Product, Place (distribution), Promotion and Price.

A central idea in marketing is that this mix of value traits can be optimized for a particular good or service at whatever level of the value chain is relevant to a particular good or service, i.e., what the next customer along the chain values. Meeting the requirements of customers to the maximum requires that the best balance is struck between (1) the needs and capabilities of the firm at that level of the value chain, (2) marketing mixes of competing firms supplying alternative products, and (3) the preferences of the customers [2]. In the specific case of an innovation such as a foodstuff made using a genetically modified component, the parts of the marketing mix of "value traits" that are directly relevant and most significant are the Product and Price characteristics.

1.2.1 Marketing Mix Element—Product

Characteristics of the Marketing Mix element "Product" that is valued by customers can be categorized as:

- Trigger needs: the set of characteristics of the product that help satisfy the basic motivation for wanting to acquire the product, e.g., need for food.
- Experience Characteristics: There are characteristics of an agribusiness product that are not detectable by simple inspection and can only be confirmed by using or consuming the product. These characteristics are Experience Characteristics. The presence or absence of Experience Characteristics may create in the customer's mind the risk of an inappropriate choice with subsequent consequences.

– Credence characteristics: These are characteristics that the customer can never readily confirm personally whether they actually are characteristics of the product or not. Credence Characteristics include claims about production methods, freedom from genetically modified (GM) inputs or other producer behavior. These are also possible sources of perceived risk. For example, someone might consume the product because they think these presumed Credence characteristics are real, and they turn out to be illusory.

While wheat meets the food "Trigger Need" of consumers, the Experience and Credence characteristics of a product, especially for GM wheat, can create questions, doubt and risk for consumers. This is of particular pertinence to GM crops and, potentially, GM foods where consumer Experience and Credence values come to the fore. If Experience and Credence characteristics are of interest to customers, and are significant criteria for choosing between products, then not being able to know for certain about these characteristics at the time of purchase makes the purchase risky. There are various ways customers can alleviate the risk they perceive about Experience and Credence characteristics of a product. One way is to identify the product transparently and rely on a third party as a provider of an implicit or explicit guarantee about the characteristic in doubt. If concerned about food safety, for example, a customer may rely on the credible labeling and a conviction that their supplier only offers safe products, or that "the government" oversees properly such issues. Customers may rely on accrediting authorities as to the healthiness of product, e.g., the GM status of foods.

Changes in consumer taste and technology has enabled products to be designed to more closely than ever meet the specifications desired by consumers. Concomitant with these changes has been improvements in operational efficiency benefiting consumers and producers. These gains, however, do not apply to all agricultural products, farmers, and consumers. Most notably, farmers and consumers who could reap considerable benefits of genetically modified agricultural commodities used as human foods are not given a choice—they are excluded from the markets for food. Education remains key: there is a overwhelming case for the science professions educating populations by exposing the pseudoscience and "false facts" that surround not only the issue of GM foods but many other technical phenomena of the modern world.

1.2.2 Marketing Mix Element—Price

Price refers to the price the customer pays for a product at each level of the value chain. The prices of GM and non-GM crop products in commodity markets reflect the state of supply and demand for those products in the markets. If GM wheat was a perfect substitute in consumption for non-GM wheat, there would be no price

premium for the characteristics of either the GM wheat or the non-GM wheat. A price difference would be the result solely of the two types of grain having different costs of production. The share of each type of grain in world markets would simply be determined by its cost to produce and supply. The wheat with the lower cost of production would dominate the market. However, if GM wheat and non-GM wheat were not perfect substitutes in consumption, then consumers would place a premium or discount on the values of the non-GM characteristics and GM-characteristics of the grains, depending on their view of the worth of the difference in the characteristics of the two grains. If GM wheat was the cheaper grain even after costs of segregating and labeling the GM grain, consumers who preferred a non-GM grain would pay a price premium for the non-GM characteristics of the non-GM grain. The net economic and non-economic benefits of GM crops, described below, warrant scientific narratives to refute misguided anti-GM campaigns.

2 Genetically Modified Agricultural Products

Over the past 20 years, research into and commercialization of genetically modified agricultural products years has been prolific, although barriers to research and commercialization have been profound. Resistance by some consumers and also by some producers of grain has resulted in government intervention to regulate and prevent commercialization of GM crops in parts of the world, and their use in GM foods everywhere.

2.1 Prevalence of GM Agricultural Products

The major commercial genetically modified crops produced around the world are corn (maize), soybean, cotton, and canola. Adoption of these genetically modified grain crops has been rapid and widespread in North and South America, because they are used primarily for animal feed, highly processed vegetable oils or textiles, and not directly used for human food. Cotton has been adopted widely in China and India. Further, modification has made these crops more profitable crops for farmers by reducing costs and facilitating greater yields through more effective pest and weed control, at the same time reducing negative environmental externalities by reducing the use and toxicity of chemicals required to control weeds and pests.

2.2 Regulation of GM Agricultural Products

Public policies about food mirror consumer attitudes. The responses of the public and their elected representatives to the emergence of GM crops for animal feed, industrial, and textile uses has differed in major economies around the world.

Regulation of biotechnology is essential to avoid social costs and make it possible for a GM food crop to be accepted by the

public, but is also costly. Even with successful GM products that have been adopted widely, the considerable time lag between the time GM grain varieties become commercialized in the producing countries and obtaining approval to import to other countries has been a major constraint to world grain trade.

A wide range of approaches to the regulation of GM crops is evident around the world. North America has a relatively laissez faire approach to regulation of GM crops [3]. Canada has a heavily regulated approach and, at the same time, is the largest producer of GM canola, which is exported all over the world. Australia has a stringent regulatory approach to testing, while the States of Australia have autonomy about whether or not farmers are allowed to grow GM crops.

In Australia the Food Standards Code of Australia and New Zealand (FSANZ) assesses GM products prior to them being permitted to enter the food value chain. The approach is based on scientific and technical evidence, using international standard tests, considering risk and uncertainty, underpinned by the principle that regulation must be proportionate to risk. The Productivity Commission Draft Report on Regulation of Australian Agriculture [4] states:

> To date, gene technology has not been shown to introduce any new or altered hazards into the food supply; therefore the potential for long term risks associated with GM foods is considered to be no different to that for conventional foods already in the food supply. As a consequence, FSANZ does not consider that long term studies are generally needed to ensure the safety of GM foods. (p.230)

The Productivity Commission [4] continued (p.231):

> This view is shared by international organisations. For example:
>
> • the US Food and Drug Administration stated that:
> . . . credible evidence has demonstrated that foods from the [genetically engineered] plant varieties marketed to date are as safe as comparable, non-GE foods. (2015a).
>
> • The World Health Organization recently reported that:
> GM foods currently available on the international market have passed safety assessments and are not likely to present risks for human health. In addition, no effects on human health have been shown as a result of the consumption of such foods by the general population in the countries where they have been approved.
>
> • The OECD also said that:
> Worldwide, many people are eating GM foods (especially in North America and China) with no adverse effects on human health having been reported in the peer-reviewed scientific literature.

The least "GM friendly" bloc of economies in the world is the European Union and the UK. Despite the EU approving dozens of traits for food and feed and importation and processing, national governments can "opt out" from EU approved GM cultivars and imports. Insect resistant GM Corn is an approved crop currently

cultivated inside the EU: Spain produces 90% of this crop. The GM Corn grown represents 1.5% of the total area of corn grown in the EU. Genetically modified crops are banned in Austria, Bulgaria, France, Germany, Greece, Hungary, and Luxembourg. This ban applies to GM crops, not GM organisms used in animal feeds, such as soybean meal. It is inordinately costly to aim to remove every grain of a contaminant crop. Thresholds are used instead, ranging from zero contaminant in China to 0.5% in Europe and 5% in Japan. Still, there are several barriers to importation of GMOs to the EU, having to pass the "European Food Safety A" requirements, and then gain approval of individual nation states. The regulatory policies of the European Union are open to criticism as being politicized and characterized by suspicion of science, mistrust of government and industry. The EU and its policies on agricultural production and trade are usually open to opportunities to impose non-tariff barriers. The EU approval process was successfully challenged by the US in 2006 under WTO rules. This led to an EU tolerance of 0.5% unintended presence of GM material not yet approved for import by the EU, but which has been assessed by the European Food safety Authority as being safe for consumption.

2.3 Consumer Attitudes to GM Agricultural Products

A study of consumer attitudes to GM agricultural products [5] identified three categories of consumers according to their attitudes to GM food:

- Anti-GM food or pessimistic.
- Risk-tolerant or information searchers.
- GM accepters or optimistic.

This study concluded that consumers in the USA and Spain and Portugal were more tolerant of GM agricultural products than consumers in France and the Nordic states [5]. British, Nordic, and Germanic consumers were likely to regard the benefits of GM as being insufficient to overcome the perceived risks. In the USA, Spain, and Italy, consumers in general perceived risks but considered the benefits outweighed the risks. This meta-analysis found that in all the papers reviewed and for whatever techniques were used, whether stated preferences, real market behavior or blind tastings, it was found that consumers mainly prefer GM free food—until price comes into it. Preference for GM-free products disappeared when a higher price had to be paid to obtain the GM-free product.

In Australia, the food safety code (FSANZ) makes it mandatory to label foods, ingredients, additives, or processing inputs that contain novel DNA or protein that have been genetically modified, or genetic modification results in altered characteristics [6–9]. Labeling is not required, in the case of canola oils for instance, which does not contain novel DNA or protein and do not have

altered characteristics. The European Union requires that food be labeled if they are made from GM crops or contain GM ingredients, even if there is no GM material in the food. In the USA, it is proposed to introduce a voluntary certification scheme, though some states have GM labeling laws. Canada does not require GM food to be labeled as such. The FSANZ [5] says that the purpose of mandatory GM labeling in Australia is to help consumers to make an informed choice about their purchases of food. As the Australian Productivity Commission Inquiry into Regulation and Australian Agriculture [4] reported:

> Australia's robust gene technology regulatory framework requires that GM organisms and food are assessed for their impact on human health and safety and the environment before they are approved. FSANZ has declared, based on a rigorous assessment process that considers credible scientific evidence, that approved GM foods are as safe and nutritious as similar conventional foods. Internationally, the purpose of GM labeling is also to provide consumers with information, rather than to ensure food safety (European Commission 2016b, for example).

The 2016 Australian Productivity Commission report about Regulating Agriculture in Australia [4] made the argument that, as the safety of foods was dealt with by the FSANZ code, the labeling about GM content was about consumer value, not safety. On these grounds the Inquiry recommended "Australia New Zealand should remove the requirement in the Food Standards Code to label genetically modified foods." With GM labeling a consumer value issue, producers have an incentive to inform their potential consumers about the GM status of their product. Equally, governments and regulatory bodies are obliged to inform consumers fully about the scientifically accurate state of affairs regarding the safety, risks, benefit, costs of GM and non-GM foods. The Productivity Commission Inquiry into Regulation of agriculture in Australia concluded that "Despite Australia's robust gene technology regulatory framework, health and safety concerns about GM foods remain."

As has happened, the focus of most of the GM innovation in agricultural products has been to reduce production costs. This results in lower prices to consumers. However, the risk story is not so transparent. Knowledge is the key. Understanding will be the single most important factor that determines whether GM food will be accepted. Consumers require objective information that enables them to make a choice that is well-informed about the nature and extent of any additional risk associated with consuming GM foods relative to consuming the alternative equivalent non-GM foods. Product labeling in the USA is voluntary whereas in the EU it is mandatory and contains significant detail. Consumers in the USA have access to less information than their European counterparts. Unsurprisingly, the study by Costa [5] also found that attitudes to GM products were closely tied up with the values that

individuals hold regarding issues of environmentalism, conservationism, materialism, and equity. Interestingly, these researchers also found that consumer attitudes to GM products in different countries paralleled belief in the effectiveness of government regulation in the area as well as to some extent their "faith in science." Consumers in the USA were more tolerant of GM products and had more confidence in government oversight and endorsement about whether or not a product is safe. In the EU, greater scepticism was the rule: tolerance and confidence was more likely to be absent.

2.4 The Worth of GM Agricultural Products

The merit of introducing a biotech crop depends on costs and benefits relative to alternative existing technologies, i.e., "Compared with What?" Biotechnology has a good deal to contribute to agricultural profitability and competitiveness, and survival of farm businesses, as well as contributing to improving the environment. Public and private research and development investments have been to develop and sell agricultural products, which have herbicide tolerance and pest resistance. The GM technologies have reduced problems of chemical pest control, enhanced pest control, and lowered costs of pest management. Higher yields become possible for farmers without access to chemicals who would struggle to control weeds and pests. Increased food can be produced without using more land. Less chemical use has the positive externality of reducing the exposure of farmers to chemical with its associated health risks. Environmental side effects of pesticide use are reduced [10, 11].

Economists have estimated the social benefits from biotech crop varieties to be in billions of dollars. These benefits are shared between farmers, consumers, and developers of the biotechnologies. A meta-analysis of the impacts of agronomic and economic impacts of GM crops around the world by Klumper and Qaim [12] found that once GM technology became part of the farm system on average GM technology had reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%. Yield gains and pesticide reductions were larger for insect-resistant crops than for herbicide-tolerant crops. Contrary to popular belief, GM technology benefits consumers more than producers and the developers of the technology, and benefits farmers in developing countries more than farmers in developed countries. The gains in yield and profit of farmers were higher in developing countries than in developed countries [12]. Another estimate is that $1b of the $8b potential annual benefits of GM technology has been realized, with most of the unrealized potential in poor countries [13]. Price premia for non-GM crop products too are hard to find, other than for some niche, certified non-GM grains for a specific purpose [14].

There is much evidence that genetically modified crops can improve welfare of people. There is also evidence that excessive regulation, or high cost of segregation to preserve identity of GM and non-GM foods, adds significantly to costs.

3 Transgenic Wheat

The technique of using recombinant DNA is the basis of the methods used to create transgenic wheat. The term "Transgenic" means transferring characteristics from other source that are new traits and are controlled by one or more genes. The aim is to improve yield, enhance quality, increase insect and disease resistance and other beneficial properties [15]. Potential new GM wheat products developed from research around the world include wheat that has been modified to include traits that improve them in the following ways:

- Tolerant to herbicides.
- Resistance to pests.
- Tolerant of salt.
- Fortified with beneficial elements.
- Tolerant to drought.
- Low-gliadin (for gluten allergy sufferers, celiac disease).

3.1 History of Commercialization of GM Wheat

In 2004, the wheat breeder Monsanto decided not to proceed with the plan to release a herbicide-tolerant GM wheat. This decision was taken because of the mismatch between the highly regulated Canadian GM accreditation system and the relatively unregulated US GM accreditation system. Given the geography and trade involved, it only made sense to Monsanto to release the GM wheat if it could be released in both the USA and Canada. The regulatory burden of Canada meant that the GM wheat was not released at all. Carter et al. [16] studied how much this decision had cost producers and consumers in both countries. They estimated the decision not to release the herbicide tolerant GM wheat had imposed a cost on producers and consumers in the USA of $1.4b p.a. in 2006. This decision also had spill-over effects on the respective wheat industries, driving resources out of wheat growing and into alternative production.

Carter et al. [16] argued that the standard argument against introducing GM crops, the belief that doing so risks contamination of non-GM product and their markets, which will result in loss of major export markets, was flawed. Segregation of GM and non-GM crops is physically and economically feasible, and if the relatively small GM-sensitive markets continued to rely on non-GM product that will become relatively more expensive over time, other less sensitive and equally significant markets will readily import GM wheat. Fear of some consumers in a proportion of all the available export markets ought not to determine what agricultural technology is or is not adopted by farmers.

Genetically modified traits that have been developed for wheat include a Roundup ready (R) trait for the spring planted hard red wheats grown in the USA and Canada. Roundup Ready wheat facilitates enhanced control of weeds, with beneficial effects on yields and costs. Recognizing the consumer resistance to the use of GM crops is strong in the EU which is also a large importer of the spring planted hard red wheat of the USA and Canada, Wilson et al. [17] analyzed how much better off, in dollars of income, people in wheat producing and consuming countries would be if Roundup ready wheat was available for farmers to use. The analysis included costs of segregating GM wheat and reduced supplies of non-GM wheat and investigated the addition to incomes, whether consumers or producers were affected, and in which countries. Wilson et al. [17] found that in a plausible scenario, producer welfare would increase by $300m p.a. and consumer welfare would increase by $250m p.a. Producers of hard red spring wheat gain while producers of hard red winter wheat lose, primarily because of a reduction in wheat prices overall. A wheat market with two segments—GM and non-GM—would have implications for users of both types of wheat in the value chain. Those users requiring non-GM wheat would face keen competition from users willing to use the lower cost GM wheat.

3.2 Drought Tolerance in Wheat

Drought reduces the yield of grain crops. Depending on severity, the reduction in yield can be anything from 30% to 100% below the yields achievable when soil moisture is not limiting a grain crop achieving its potential. Research in transgenic wheat to enable wheat to perform better under conditions of moisture stress is progressing around the world, including in China, the USA, and Australia. The extent to which this research will be effective depends to a considerable degree on the "trait efficiency" of the modified wheat, i.e., how much extra yield is attainable under dry conditions compared with the nonmodified wheat. The Australian work on GM drought resistance in wheat, reported by Wynn [18], found that trait efficiency was difficult to estimate from the field trial results because the trait efficiency of the GM wheat over the non-GM wheat was widely distributed. For example, in the field trials, the GM wheat demonstrated:

- 18–68% trait efficiency with the treatment that received 45% below average rainfall; and
- 7–47% trait efficiency with the treatment that received 96% above average rainfall.

To be worthwhile, drought tolerance traits in wheat have to enable the modified wheat plant to perform better under conditions of moisture stress than nonmodified wheat, while at the same time perform comparably to the nonmodified wheats under all

other conditions. Otherwise the farmer will be better off overall by making the good profits available in the good seasons and suffering the poorer profits of the non-modified wheats in the dry times.

Development of drought tolerance in wheat has been conducted in the USA, Australia, and increasingly in China. Several studies have used real options methods to investigate the potential value of having a wheat plant that is tolerant to drought [18–20]. Wilson et al. [20] estimated the worth of developing and using a drought-tolerant wheat in the USA. The analysis was conducted for growers with a range of attitudes to risk, a random draw of droughts and an assumed adoption profile with 70% adoption peaking at year 7, with the real options valued for 15 years after commercialization, and a technology fee at 30% of the traits value to growers. They found the value of drought-tolerant wheat using GM technology was in-the-money at each phase of development and a developer would likely proceed with the investment [20]. If 20% trait efficiency was achieved (extra yield in drought) without any yield penalty in the good seasons, such an investment was "in the money" at each step along the path of development and commercial use. The expected value of the drought tolerant trait was over $400m.

Research into drought tolerant modification of wheat [21], and a similar real options analysis of the value of the trait, has also been done in Australia [18]. The method was a stochastic binomial model of real options, including options to continue, wait, or abandon the investment. The model used by Wynn [18] to value GM trait development was based on Shakya et al. [19] and Wilson et al. [20], using Australian data. Farm budgets from cropping regions were used to estimate gross margins with and without GM technology. Monte Carlo simulation using @Risk and stochastic efficiency with respect to a function was used to derive a risk premium and value the GM technology based on areas expected to be sown in regions, patterns of adoption and the timing and costs associated with the development of a new GM technology.

Results from this Australian research showed that the potential value to wheat farmers of a new GM drought tolerance trait varied between regions. The trait value for wheat per hectare ranged from –AU$62 in South Australia to AU$54 in Victoria. The negative value of the drought tolerance trait in wheat to South Australian farmers indicated that if farmers do not currently suffer from sufficient volatility of yield and profit, they may not value highly a technology that protects against yield and profit variability. The reasonable probability that volatility of production conditions will increase in coming decades is pertinent here. The average value of the drought tolerant trait in wheat ranged from US$9/Ha in India to US$34/Ha in Australia (Wynn 18). Scenario testing revealed that the largest global markets for the new wheat trait would be Australia, China, and the USA.

The Australian research by Wynn [18] into the options values of developing transgenic wheat with resistance to drought showed that investing in developing and commercializing such wheat had positive expected cash flows at all stages of development and was worthwhile in both Australian and global markets. Such results suggest that a firm would continue the investment. By the last regulatory stage, mean option values for developing transgenic drought resistant wheat were estimated to be AU$386m for wheat in Australian markets; and US$608m for wheat in global markets. Trait efficiency across high and low rainfall levels is necessary for the trait to have market value.

4 Conclusion

Realizing the potential of increases in the supply of wheat to directly increase the supply of food for humans is tied up with wider elements of the GM debate. In particular, the role of science and other participants through the agribusiness value chain is allowed to play in informing regulators and consumers about GM crops for food will be critical. Developing GM products is a slow process. The firm Ausbiotech [22] estimated that in Australia it takes 15–20 years to develop a new GM crop. The effect is that the advantage of rapid breeding which GM techniques have over traditional breeding techniques is lost in practice by the time (and cost) it takes to obtain regulatory approval. The potential of transgenic wheat to help meet growing demands for food from the growing world population over the next 30 years is high; the degree that this potential is realized will depend on all participants—private sector and public—involved with the agribusiness value chain of wheat worldwide.

Scientific and official conclusions are strong that current and next generation biotechnology is safe, and has potentially large benefits in agricultural and industrial production and in feeding the growing global population. There are also positive consequential effects, such as reducing chemical use in agricultural production. The wide resistance of consumers to GM agricultural products is not founded on the science, and the consequential bans and regulatory barriers and costs are imposing immense cost burdens on producers and consumers around the world. Next-generation technology holds even greater promise, including the possibility that over time distinctions across the full array of breeding methods will become indistinguishable, making distinction unnecessary. Successful utilization of next-generation biotechnology will still hinge on the unpolitical assessment of risk and sound approaches to the regulatory tasks including segregation and identification, with the scientific evidence and information trumping pseudoscience and being accepted by consumers.

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Part II

Protocols

Chapter 8

Agrobacterium-Mediated Transformation of Wheat Using Immature Embryos

Goetz Hensel, Cornelia Marthe, and Jochen Kumlehn

Abstract

Methods of the *Agrobacterium*-mediated transformation of bread wheat (*Triticum aestivum* L.) have been improved in recent years so that genetic engineering can be routinely used for functional genomics as well as for wheat breeding. In the protocol described here, immature embryos of the spring-type model genotype Bobwhite SH 98 26 have been used. Preculture and temperature pretreatment of these explants have led to the reproducible generation of transgenic plants at efficiencies between 5 and 15%. Whereas primary transgenic plants regenerated in vitro commonly show reduced fitness and fertility, no apparent variations with regard to morphology and grain set in their transgenic progeny as compared to wild-type counterparts were observed.

Key words Gene transfer, Hygromycin selection, Hypervirulent, Transgenic

1 Introduction

Wheat is one of the most important crop plants and used by millions of people as a primary calorie source. To cope with increasing demand and various local and environmental conditions, breeding of wheat has been intensified in recent years. In this context, genetic transformation using biolistic and *Agrobacterium*-mediated methods were remarkably improved. In addition, new sequencing technologies combined with sophisticated chromosome sorting have resulted in the decoding of the genome [The International Wheat Genome Sequencing Consortium 2014], which greatly facilitates the identification and cloning of agronomically important genes.

The capability of efficiently forming shoots that originate from single totipotent cells is indispensable for a successful genetic transformation of plants. In contrast to dicotyledonous plants, grasses are hardly able to regenerate plants from leaf tissue. However, alternative explants, e.g., immature embryos [1], embryogenic

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pollen cultures [2], and isolated ovules [3], proved useful for the production of transgenic plants in Triticeae cereals.

A careful choice of the binary vectors for cereal transformation is necessary, as most of the binary vectors have been developed for dicot species and found to be unsuitable for cereals, especially promoters and selectable marker genes. Further, a high stability of the plasmids in *Agrobacterium* is essential throughout the cocultivation period. In this regard, pVS1-based vector backbones proved particularly valuable [4]. In addition, cereal transformation has been particularly efficient when hypervirulent *Agrobacterium* strains such as AGL1 were used. The significantly improved transformation capability of those strains relies on additional copies of virulence (*Vir*) genes [5].

Biolistic gene transfer was the method by which transgenic wheat was produced [6]. Soon after, *Agrobacterium*-mediated transformation was successful using immature [7] or more recently mature embryos [8]. Besides these classical target tissues, anther culture-derived haploid embryos were used for the generation of drought-tolerant bread wheat [9]. The selection of transgenic tissue has been shown to be effective using phosphinothricin (Ppt) or BASTA for the *BAR* gene [10], hygromycin for the *HYGROMYCIN PHOSPHOTRANSFERASE* (*Hpt*) gene [1] or G418 for the *NEOMYCIN PHOSPHOTRANSFERASE II* (*NptII*) gene [11].

The method described here depends on the use of immature embryo-derived callus from genotype Bobwhite SH 98 26 from CYMMIT and on gene transfer effected by *Agrobacterium* strain AGL1 containing the plasmid pGH215 which carries the *Hpt* hygromycin resistance gene under the control of an enhanced *CaMV 35S* promoter for the selection of transgenic tissue. To facilitate the establishment of the method and an evaluation of various treatments, the T-DNA additionally harbors a synthetic *sGfp* (S65T) gene under the control of the maize *UBIQUITIN-1* promoter (Fig. 1).

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless specified otherwise).

2.1 Materials for the Generation of Transgenic Plants

1. Forceps, scalpel, preparation needles.
2. Preparative microscope.
3. 5.5 and 9.0-cm diameter Petri dishes with lid.
4. Plastic boxes with a lid (107 × 94 × 96 cm).
5. Two milliliter Eppendorf tubes.

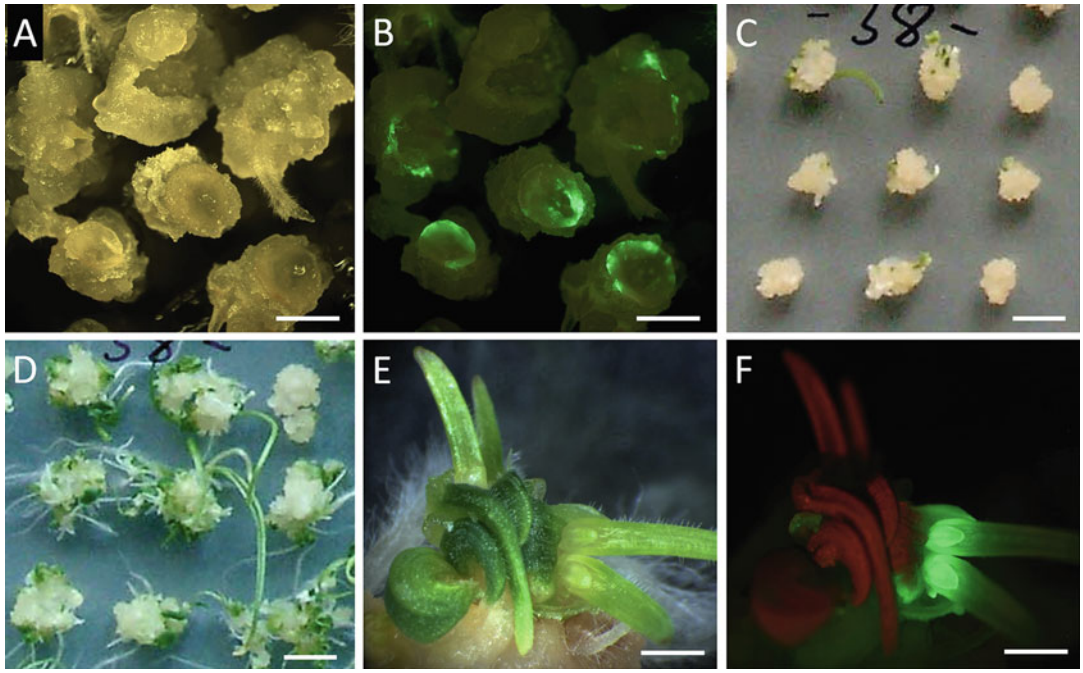

Fig. 1 *Agrobacterium*-mediated transformation of wheat. Immature embryo-derived calli 2 days after coculture with *Agrobacterium* shown under brightfield illumination (**A**) in comparison to UV light with GFP filter set revealing the sites of successful T-DNA transfer (**B**); shoot formation in the presence of hygromycin three (**C**) and 5 weeks after coculture showing the very same explants (**D**); multiple shoot formation shown under brightfield illumination (**E**) in comparison to UV light with GFP filter set indicating mosaicism of regenerants (**F**), which may entail non-Mendelian transgene segregation in the T1 generation. Scale bars: 2 mm (**A**, **B**, **E**, **F**) and 5 mm (**C**, **D**)

6. Pipettes and disposable autoclaved filter tips (200–1000 μL).
7. Filter paper disks (7 cm diameter, ash-free, autoclaved).
8. Thermomixer.
9. Magnetic stirrer.
10. Erlenmeyer flasks (100 mL), with chicane.

2.2 Agrobacterium Strain and Binary Vector

1. *A. tumefaciens* AGL1 [12] (*see* **Note 1**) is a succinamopine strain with a C58 background. It carries chromosomal marker genes for rifampicin and carbenicillin resistance and harbors a disarmed Ti plasmid (pTiBo542ΔT), which includes a duplicated DNA fragment bearing additional *Vir* gene copies, rendering the strain hypervirulent.
2. The binary vector pGH215 harbors the *HYGROMYCIN PHOSPHOTRANSFERASE* (*Hpt*) selectable marker gene, driven by the doubled-enhanced *CaMV 35S* promoter; the *sGfp* (S65T) reporter gene, driven by the maize *UBIQUITIN-1* promoter including its first intron; and T-DNA borders

derived from a nopaline Ti plasmid [13] (*see* **Note 2**). The binary vector was introduced into AGL1 by electroporation. Derived vectors carrying an intron in the *Hpt*-coding sequence have also been used, rendering the bacterium fully susceptible to the selective agent; as a result, treatment with hygromycin highly effectively prevents the persistence of *Agrobacterium* after cocultivation.

2.3 Stock Solutions and Nutrient Media

2.3.1 Mineral Salts

1. K4N macro minerals (20×): 6.4 g/L NH_4NO_3, 72.8 g/L KNO_3, 6.8 g/L KH_2PO_4, 8.82 g/L $CaCl_2{\cdot}2H_2O$, 4.92 g/L $MgSO_4{\cdot}7H_2O$, filter-sterilized and stored at room temperature (RT).
2. K micro minerals (1000×): 8.4 g/L $MnSO_4{\cdot}H_2O$, 3.1 g/L H_3BO_3, 7.2 g/L $ZnSO_4{\cdot}7H_2O$, 120 mg/L $Na_2MoO_4{\cdot}2H_2O$, 25 mg/L $CuSO_4{\cdot}5H_2O$, 24 mg/L $CoCl_2{\cdot}6H_2O$, 170 mg/L KI, filter-sterilized and stored at 4 °C.
3. Copper sulfate (10 mM): 2.5 g/L $CuSO_4{\cdot}5H_2O$, filter-sterilized and stored at 4 °C.
4. Ethylenediamine-tetraacetic acid, ferric-sodium salt (NaFe-EDTA, 75 mM): 27.53 g/L NaFe-EDTA, filter-sterilized and stored at 4 °C.

2.3.2 Carbohydrates

1. Maltose (1 M, standard quality): 360 g/L maltose·H_2O, filter-sterilized and stored at RT.

2.3.3 Vitamins

1. Gamborg B5 vitamins (1000×): 112 g/L ready-to-use product (Duchefa, no. G 0415.0100), filter-sterilized and stored at −20 °C.
2. MS vitamins (1000×): 100 mL ready-to-use product (Duchefa, no. M 0409.0100) filter-sterilized and stored at −20 °C.
3. Biotin (0.4 mM): 0.1 g/L, filter-sterilized and stored at 4 °C.

2.3.4 Growth Regulators

1. 6-benzylaminopurine (6-BAP, 1 mM): 0.225 g/L, dissolved in a few drops of 1 M NaOH, then made up to the final volume in H_2O, filter-sterilized and stored at 4 °C.
2. Dicamba (36 mM): 8 g/L dissolved in a few drops of 96% heated ethanol, then made up to the final volume using hot H_2O, filter-sterilized and stored at 4 °C.
3. 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mM): 221 mg/L dissolved in few drops of 50% ethanol by heating gently, made up to the final volume with hot H_2O, filter-sterilized and stored at 4 °C.

2.3.5 Amino Acids

1. L-glutamine (0.25 M): 36.6 g/L, dissolved in a few drops 0.1 M KOH, then made up to the final volume using warmed H_2O, filter-sterilized and stored at −20 °C.

2.3.6 Selective Agents

1. Carbenicillin (237 μM): 100 mg/L, filter-sterilized and stored at −20 °C.
2. Hygromycin (10 μM): 50 mg/L, ready-to-use product (Roche, no. 10843555001), stored at 4 °C.
3. Rifampicin (6 μM): 50 mg/L, filter-sterilized and stored at −20 °C.
4. Spectinomycin (0.3 mM): 100 mg/L, filter-sterilized and stored at −20 °C.
5. Timentin (0.2 mM): 150 mg/L, filter-sterilized and stored at −20 °C.

2.3.7 Gelling Agent

1. Phytagel (0.35% w/v): 3.5 g/L dissolved in cold water, autoclaved, the temperature of this stock as well as the remaining components of the medium are set to ca. 50 °C before mixing.
2. Phytagel (2×): 6 g/L suspended in cold water (at best 1.5 g Phytagel per 250 mL unit), autoclaved and stored at room temperature.

2.3.8 Other Additives and Solutions

1. Acetosyringone (1 M): 196.2 g/L dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C.
2. Ethanol (70%): 729.2 mL/L of 96% ethanol, stored at RT.
3. Glycerol (15%): 150 g/L glycerol, autoclaved and stored at RT.
4. Sodium hypochlorite (2.4% w/v): 200 mL/L of 12% (w/v) NaOCl, to which is added 0.1% (v/v) Tween 20. This solution should be freshly prepared.

2.3.9 Medium for A. tumefaciens

1. MG/L medium: 250 mg/L KH_2PO_4, 100 mg/L NaCl, 100 mg/L $MgSO_4 \cdot 7H_2O$, 1 g/L L-glutamic acid, 5 g/L mannitol, 5 g/L tryptone, 2.5 g/L yeast extract. The pH is adjusted to 7.0, the solution is autoclaved and cooled, and then 10 μL/L biotin stock is added. For solidification of MG/L medium, 1.2% (w/v) agar is added.

2.3.10 Media for Plant Transformation

1. Solid precultivation medium (WPCM): The medium (1 L) contains 4.3 g/L MS mineral salts (ready-to-use product, Duchefa No. M 0221), MS vitamins (ready-to-use product, Duchefa No. M 0409.0100) (*see* **Note 3**), 0.5 mL/L $CuSO_4 \cdot 5H_2O$ stock, 0.5 g/L L-glutamine, 0.1 g/L casein hydrolysate, 100 mL/L maltose stock, 1 mL/L dicamba stock.
2. Liquid inoculation medium (WIM): The medium (1 L) contains 2.6 g/L MS Modification No. 4 mineral salts (ready-to-use product, Duchefa No. M 0238), 0.165 g/L NH_4NO_3, MS vitamins (ready-to-use product, Duchefa No. M 0409.0100), 0.5 mL/L $CuSO_4 \cdot 5H_2O$ stock, 0.5 g/L L-glutamine, 0.1 g/L casein hydrolysate, 60 g/L maltose·H_2O, 10 mL/L 2,4-D

stock, 2 g/L morpholinoethanesulfonic acid (MES), 800 mg/L L-cysteine (*see* **Note 4**). The pH is adjusted to 5.8, the solution is filter-sterilized, and then 0.5 mL/L acetosyringone stock (*see* **Note 5**) is added.

3. Solid callus induction medium 1 (WCIM 1): The medium (1 L) contains 4.3 g/L MS mineral salts (ready-to-use product, Duchefa No. M 0221), MS vitamins (ready-to-use product, Duchefa No. M 0409.0100), 0.5 mL/L $CuSO_4 \cdot 5H_2O$ stock, 0.5 g/L L-glutamine, 0.1 g/L casein hydrolysate, 100 mL/L maltose stock, 10 mL/L 2,4-D stock, 0.5 g/L morpholinoethanesulfonic acid (MES), 0.4 mL/L hygromycin stock as well as 1 mL/L Timentin stock is added. The solution is filter-sterilized, and one volume is mixed with three volumes of Phytagel stock.
4. Solid callus induction medium 2 (WCIM 2): The same as WCIM 1, but with the hygromycin content being increased to 1 mL/L.
5. Solid shoot induction medium (WSIM): The medium (1 L) contains 50 mL/L K4N macro minerals, 1 mL/L each of the NaFeEDTA, K micro minerals, vitamin B5 and 6-BAP stocks, 4 mL/L L-glutamine stock, 100 mL/L maltose stock, 0.49 mL/L $CuSO_4 \cdot 5H_2O$ stock and the pH is adjusted to 5.8. For selection 0.5 mL/L hygromycin stock as well as 1 mL/L Timentin stock is added, the solution is filter-sterilized, and one volume is mixed with three volumes of 2× Phytagel stock.
6. Solid rooting medium (WRM): The medium (1 L) contains 33.3 mL/L K4N macro minerals, 0.67 mL/L each of the NaFeEDTA, K micro minerals, vitamin B5 stocks, 2.67 mL/L L-glutamine stock, 66.7 mL/L maltose stock, 0.33 mL/L $CuSO_4 \cdot 5H_2O$ stock and the pH is adjusted to 5.8. For selection 0.4 mL/L hygromycin stock as well as 0.67 mL/L Timentin stock is added, the solution is filter-sterilized, and one volume is mixed with three volumes of Phytagel stock (*see* **Note 6**).

3 Methods

3.1 Generation of Transgenic Plants

The production of transgenic wheat via gene transfer to immature embryos comprises six major steps: growth of donor material, dissection and preculture of immature embryos, pretreatment and coculture of immature embryos with *Agrobacterium*, callus induction, plantlet regeneration, and establishment of plants in soil.

3.1.1 Growth of Donor Plants

1. Germinate grains of spring-type cultivar Bobwhite SH 98 26 [14] (*see* **Note 7**) in trays filled with soil substrate (*see* **Note 8**) in a growth chamber set to provide a 12-h photoperiod (136 μmol/m^2/s photon flux density) and a light/dark temperature regime of 14/12 °C. Ten to twenty grains are germinated at fortnightly intervals to ensure a continuous supply of explant material.
2. Transfer seedlings after 3 weeks into 2 L pots**.** At the tillering stage (BBCH code 29/30), 15 g Osmocote (Scotts, Netherlands) is given to each pot (*see* **Note 9**). Transfer the plants at the tiller elongation stage (BBCH code 39) to a glasshouse maintained at 18/16 °C with a 16 h photoperiod (170 μmol/m^2/s photon flux density).

3.1.2 Preparation and Culture of Agrobacterium Strain

1. Introduce the binary vector into chemically or electrocompetent AGL1 cells and streak the cells on solid MG/L medium containing 100 mg/L carbenicillin and 50 mg/L rifampicin, as well as the selectable agent for the binary vector (typically 100 mg/L spectinomycin). Incubate the plates at 28 °C in the dark for 2–3 days.
2. Sample at least two single colonies with a toothpick and inoculate each colony into 3 mL liquid MG/L containing antibiotics. Incubate overnight at 28 °C on a rotary shaker running at 180 rpm.
3. Confirm the presence of the binary vector by plasmid isolation and restriction digestion.
4. Start a fresh overnight culture by adding 20 μL of the overnight culture to 5 mL fresh MG/L medium containing antibiotics, and incubate for a further 24 h as above until an OD_{600} of 2–3 has been reached.
5. Prepare glycerol stocks by mixing 200 μL of the overnight culture with 200 μL glycerol solution, and maintaining at RT for 2–3 h. Store in a −80 °C freezer until required (*see* **Note 10**).
6. Thaw a tube of glycerol stock, add the contents to 10 mL antibiotic-free MG/L medium [12] in a 100 mL Erlenmeyer flask, and incubate overnight at 28 °C on a rotary shaker running at 180 rpm (*see* **Note 11**).

3.1.3 Preparation and Preculture of Immature Embryos

1. Harvest immature caryopses ca. 12 to 14 days after anthesis, when the embryos turn from glassy to whitish appearance owing to cellular starch accumulation. Remove the awns and place the caryopses in a 500 mL bottle standing on ice.
2. In a laminar airflow hood, immerse the caryopses for 30 s in 70% ethanol, wash once with sterile distilled water, then in NaOCl solution for 15 min on a shaker. Wash five times in sterile, distilled water.

3. Excise the embryos from the caryopses using forceps and preparation needle. This is achieved by slitting the caryopsis down the middle, right above the embryo. Then, the embryo can be readily released from the caryopsis using the preparation needle.
4. Place the embryo scutellum facing up on WPCM and incubate at 24 °C in the dark for 5 days (*see* **Note 12**).

3.1.4 Pretreatment and Coculture of Precultured Immature Embryos

1. Immerse 30–50 embryos in a 2-mL Eppendorf tube by adding 1 mL freshly prepared liquid WIM.
2. Incubate for 5 min at 42 °C without agitation.
3. Remove the WIM from the Eppendorf tube containing the embryos and replace with 600 μL *Agrobacterium* culture ($OD_{600} = 2.0$).
4. Keep the tube inside a laminar flow hood for 30 min without any agitation, and then wash the embryos in 1.5 mL WIM.
5. Add 300 μL WIM to a filter paper disk in a 5.5-cm Petri dish and place immature embryos as one stack.
6. Incubate at 21 °C in the dark for 60–72 h.

3.1.5 Callus Induction

1. Place the embryos with their scutellum side facing up on solid WCIM 1; use 15–20 embryos per 9-cm Petri dish.
2. After sealing, incubate the plates at 24 °C in the dark for 10 days, and then transfer the material to fresh solid WCIM 2 for another week.

3.1.6 Shoot Induction, Rooting and Transfer into Soil

1. Transfer the callusing embryos onto WSIM and incubate at 24 °C under 136 μmol/m^2/s photon flux density for 10 h per day.
2. Transfer the material to fresh WSIM every 2 weeks until shoots emerge.
3. Once their leaf length has reached 2–3 cm, transfer the shoots into a plastic boxes containing 80 mL WRM; use up to 12 plantlets per box (*see* **Note 13**).
4. As soon as the plantlets have developed roots, they can be transferred to soil. Ensure high humidity for 7–10 days of acclimation by covering with a plastic hood. Grow the primary transgenic plants to maturity as described for donor material (**step 2**) (*see* **Note 14**).

3.2 Validation of Transgenic Plants

After successful generation of primary transgenic plants, the presence of the T-DNA has to be confirmed.

1. Snap-freeze a 200–400 mg sample of fresh leaf tissue in liquid nitrogen. Isolate genomic DNA by kit or as previously described [15].
2. Confirm the presence of T-DNA elements using gene-specific primers. For the junction between *ZmUBI1* promoter and the

sGfp gene use Ubi-F1 (5′-TTCCGCAGACGGGATCGATC-TAGG-3′) and GFP-R1 (5′-GGTCACGAACTCCAG-CAGGA-3′), whereas for the CaMV *35S* promoter and *Hpt*, 35S-F2-Catrin (5′-CATGGTGGAGCACGACACTCTC-3′) along with GH-HYG-R5 (5′-GATTCCTTGCGGTCC-GAATG-3′) are used. Run 0.8% agarose gel in 0.5× TBE buffer at 200 V for 45 min to confirm the expected fragment sizes (*see* **Notes 15** and **16**).

4 Notes

1. Other hypervirulent *A. tumefaciens* strains such as LBA4404/pSB1 [16] have also been successfully used for stable wheat transformation.
2. Several promoters have proven to be effective in driving the selectable marker gene or the transgene of interest. The most commonly used ones for constitutive expression are the maize *UBIQUITIN-1* promoter including the gene's first intron (p6U, www.dna-cloning.com), the *CaMV 35S* promoter (pLH6000, www.dna-cloning.com), and its doubled enhanced derivative (pCAMBIA vectors).
3. Comparative studies using either MS or Gamborg B5 vitamins showed no significant differences.
4. L-cysteine supplementation of the cocultivation medium has been reported to prevent tissue browning in soybean induced by the presence of *A. tumefaciens*, and also to increase transformation efficiency [17]. A comparison of concentrations in barley cocultivation experiments suggests that the optimum L-cysteine concentration is 800 mg/L (unpublished data). Adjustment of the pH to 5.8 before the addition of L–cysteine results in a final pH of ca. 5.5.
5. Contradictory results have been published regarding the effect of acetosyringone on the agroinfection of immature barley embryos [18–20]. The addition of acetosyringone at the concentration specified is optimal with respect to transformation efficiency under the conditions described here (unpublished results).
6. In several species ½-strength media are reported for rooting medium. According to several independent experiments such a reduction was beneficial for wheat in our laboratory.
7. The protocol described was also successfully used to transform the German winter type cultivar Certo [1]. In this case, the efficiency varied between 2% and 10%.

8. Klasmann Substrate 2 consists of black and white peat. After germination plants are transferred to a substrate mix (compost, sand, and white peat).
9. Osmocote is a commercially available fertilizer formulated to contain 19% N, 6% P, and 12% K.
10. Most protocols recommend snap-freezing glycerol stocks in liquid nitrogen, but in our hands gradual freezing is not deleterious: stocks generated as described have remained fully viable for several months.
11. There is a documented risk that the *A. tumefaciens* cells delete nonessential plasmids. However, in the present protocol, binary vectors carrying the pVS1 origin of replication appear to be very stable even in the absence of antibiotic selection. The advantage of growing *A. tumefaciens* without antibiotics prior to inoculation is that the cell suspension can be used directly, and the recipient plant cells are not exposed to any antibiotic challenge.
12. Usually 25–50 immature embryos are precultured per 9-cm Petri dish.
13. Alternatively, up to 16 plantlets can be grown per box on WRM. If desired, individual plantlets can also be raised in a glass tube, which minimizes the risk of cross-contamination, e.g., by persisting *Agrobacterium*.
14. The regeneration in vitro via callus formation causes a reduced fitness of the plants as compared to regular germination from mature grains. Nonetheless, primary transgenic plants commonly produce more than 50 grains.
15. For the primer combinations given, the expected fragment sizes are 1027 bp for *ZmUBI1::sGfp* and 900 and 1200 bp for *35S::Hpt*.
16. When establishing a new method of stable transformation in the lab, it is strongly recommended to confirm the integration of the T-DNA in the plant genome by means of DNA gel-blot analysis (Southern blot).

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Chapter 9

Biolistic Transformation of Wheat

Caroline Tassy and Pierre Barret

Abstract

The wheat genome encodes some 100,000 genes. To understand how the expression of these genes is regulated it will be necessary to carry out many genetic transformation experiments. Robust protocols that allow scientists to transform a wide range of wheat genotypes are therefore required. In this chapter, we describe a protocol for biolistic transformation of wheat that uses immature embryos and small quantities of DNA cassettes. An original method for DNA cassette purification is also described. This protocol can be used to transform a wide range of wheat genotypes and other related species.

Key words Biolistic transformation, Gene cassette, Wheat

1 Introduction

More than 95% of the wheat grown today is hexaploid wheat, also named bread wheat. Bread wheat is an abundant source of energy and protein for the world population. One of the major agricultural challenges for the next 20 years to feed the growing global population is to improve important agronomic characteristics of bread wheat, such as tolerance of high salinity and drought, disease resistance, and yield. For this, all available tools have to be mobilized, both classical breeding tools like genetics, marker-assisted selection, quantitative trait loci mapping and management, and new biotechnological tools including production of pure doubled haploid lines, high throughput generation of genetic variability using mutagenesis, and introduction of new characteristics in the species by transgenesis or gene editing. Some methods, especially transgenesis, have raised ethical or environmental concerns from farmers, trade organizations, and state governments and this has undoubtedly had the effect of delaying the development of biotechnology tools for wheat improvement. However, it seems that this time is over as the global challenge for food production becomes more urgent [1].

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Wheat has been genetically transformed by *Agrobacterium* mediated [2] and biolistic methods [3]. In this chapter, we describe a detailed protocol for the production of transgenic wheat lines by biolistic technology.

Methods for stable transfer of genetic information into plants have been developed since the end of the 1980s. The process can be defined as the transfer of genetic information into the nucleus with the recovery of fertile, transgenic plants. The advantages of transforming plants are twofold. First, the stable transfer of a characteristic to a fertile plant that can be propagated and cultivated in multiple environments makes it possible to validate the functions of candidate genes for fundamental and applied research. Second, the ability to transfer the favorable characteristic by backcrossing to elite accessions facilitates the creation of new varieties for large-scale breeding. Moreover, the transgene can be patented and tracked which can aid in the identification, protection, and commercialization of a new wheat variety. The first successful transformation of long-term callus-type wheat tissue using a biolistic protocol followed by herbicide resistance selection was reported in 1992 [3]. As this type of tissue was difficult to obtain and to maintain in culture, often due to genotypic factors, another protocol using immature embryos was developed using the highly embryogenic wheat cultivar Bobwhite, the *bar* gene as selection system and the *iudA* (*gus*) gene as a reporter gene [4]. To improve the quality and the efficiency of transformation, numerous parameters were tested, including the ammonium nitrate concentration of buffer [5], physical parameters of the gun [6], and a wide range of molecular, physical and tissue culture parameters [7]. An optimized protocol for wheat biolistic transformation was proposed. Other improvements of the protocol came from working with other plants. For example, to minimize damage to plant tissues, a low-pressure gene gun was proposed for transforming maize [8]. DNA fixation onto particles was optimized by the use of cationic gold microparticles [9]. The coating of DNA onto microparticles was improved by using protamine instead of spermidine, which led to a 3.3-fold improvement in stable transformation efficiency of rice suspension cells [10].

Another advantage of microprojectile bombardment is that elite wheat genotypes are amenable to transformation, eliminating the work-intensive and time-consuming necessity to backcross the trait [11–13]. Indeed highly transformable wheat genotypes can be selected for medium-throughput transformation [14]. Another aspect of the method which is of great applied interest is that the DNA to be transferred can be processed, mixed, multiplexed, cut by restriction enzymes, or purified before transformation. For example, gene cassettes and multiplex transformation can be carried out to produce simple integration patterns in the wheat genome [15] or high expression levels in rice [16], single-stranded DNA

can be used to transform wheat [17], and co-bombardment with different gene cassettes can improve transformation frequency [18].

It is generally admitted that biolistic transformation leads to complex patterns of integration with unstable expression, but exhaustive reviews of available data have not upheld such conclusions [19]. Initial results in papaya indicated that transgene structure and expression level were stable from generation to generation [20, 21]. Transgene integration sites were precisely analyzed in rice [22], wheat [23], and oat [24]. In rice, a two-phase integration mechanism is thought to occur: a pre-integration phase leads to a rearranged transgenic sequence free of host DNA with subsequent integration of these molecules into the host genome. Results also suggest that the first integration site acts as a hot spot for subsequent integration, leading to multiple copies of the transgenic DNA integrating at the same locus, interspaced with stretches of host DNA [22]. In oat, multiple integration of sometimes very small (<200 bp) DNA fragments was observed, indicating that transgene locus formation involves the concerted action of several DNA break-repair mechanisms [24]. These results were confirmed in wheat [23] as large tandem integrations interspersed with unknown DNA were detected by fluorescence in situ hybridization on extended DNA fibers (fiber-FISH). Results from the same study indicated that the type of promoter used, rather than the chromosomal site of transgene integration, was most critical for transgene expression. Another FISH study of wheat revealed that multiple transgene integrations were separated by considerable lengths of DNA (>1 Mbp), but these sites of integration lay in close physical proximity in the interphase nuclei [25].

Regarding transgene expression, two studies in wheat showed a link between cytosine methylation at the transgene locus and loss of transgene expression [26, 27]. Moreover, results previously obtained in rice using three selection or reporter genes indicated that methylation can be gene specific, with no evidence for spreading of silenced states or methylation patterns from one transgene to another [28].

Taken together, these results indicate that particle bombardment is a method of choice for the production of genetically modified wheats. In this chapter, we describe the different steps of a biolistic protocol for wheat transformation, including specific tips like using multisite GATEWAY® based tools for building DNA constructs and a protocol for gene cassette recovery and purification.

2 Materials

2.1 Plant Materials

The tissue used in this transformation protocol is immature embryos from *T. aestivum*. Transformation efficiency for bread wheat genotypes is presented in Table 1. The protocol described has allowed us to transform a large panel of species related to wheat like *Triticum monococcum*, *Triticum durum*, *Triticum diccocoides*, and *Secale cereale*. The regeneration stage and *bar* selection are performed according to the protocol described by Pellegrineschi et al. [14], and the *NptII*/gentamicin selection is performed as described by Harsh [29].

2.2 DNA Used for Shooting

Plasmid DNA is used in many protocols, but we show [15] that the use of cassettes increases the rate of simple events without any trace of plasmid backbone. Extract and purify plasmids using a Maxiprep kit (Qiagen) following the instructions of the manufacturer.

2.3 Stock Solutions

1. 2-4-Dichloro-phenoxyacetic solution (2,4-D, 1 mg/mL): made by dissolving 100 mg of 2-4D powder in 80 mL water. Add droplets of 4 N NaOH solution until complete 2-4-D dissolution, and then adjust the final volume to 100 mL. Sterilize the 2-4D solution by filtration using a nylon syringe filter (0.2 μm). Aliquot stock and freeze at −20 °C.
2. Indole acetic acid solution (IAA, 1 mg/mL): prepared by dissolving 100 mg of IAA in 80 mL water. Add droplets of 4 N HCl solution until complete IAA dissolution, and then adjust the final volume to 100 mL. Sterilize by filtration using a nylon syringe filter (0.2 μm). Aliquot stock and freeze at −20 °C.
3. 6-benzylaminopurine solution (BAP, 1 mg/mL): prepared by dissolving 100 mg of BAP in 80 mL water. Add droplets of a

Table 1
Transformation efficiency for bread wheat genotypes

Accession	Origin	Growth class	Fertile plants/Immature embryos (%)
BobWhite S26	USA	Spring	2.5
Apogee	USA	Spring/rapid cycling	1.9
Courtot	France	Spring/agronomic	1.5
Recital	France	Winter/agronomic	2.3
Oligoculm	Israel	Winter	1.2
Mexipak	Pakistan	Spring	1
Docteur Mazet	France	Spring	1
Thatcher	USA	Spring	0.9

4 N HCl solution until complete BAP dissolution, and then adjust the final volume to 100 mL. Sterilize the BAP solution by filtration using a nylon syringe filter (0.2 μm). Aliquot stock and freeze at −20 °C.

4. D-Mannose solution (1 g/mL): dissolve 600 g of mannose in 600 mL water, and filter the solution using a sterile nylon syringe (0.2 μm). Aliquot stock and freeze at 4 °C.
5. Na acetate 3 M pH 5.2: prepared by dissolving 24.6 g sodium acetate in 90 mL of water. Adjust pH to 5.7 with glacial acetic acid. Complete the solution to 100 mL and to pH 5.2. Autoclave for 45 min using a liquid cycle.
6. Bleach (5.25% NaOCl).
7. Spermidine solution (0.1 M): Prepared in sterile water. Aliquot stock and freeze at −20 °C less than 3 months.
8. CaCl2 (2.5 M): prepared in water and filter-sterilized. Aliquot stock and freeze at −20 °C.

2.4 Media

1. Plasmolyzing medium: per L, add 4.4 g Murashige and Skoog medium including vitamins, 100 g maltose, 0.150 g asparagine, 0.04 g thiamine HCl, and 8 g agar. Adjust medium to pH 5.7 with 1 M KOH and add water to 1 L before sterilization in an autoclave. Add 2.5 mL of filter-sterilized (0.2-μm mesh) 2-4-D solution (1 mg/mL) to the autoclaved medium.
2. MSE3 medium: per L, add 4.4 g Murashige and Skoog medium including vitamins, 60 g sucrose, 0.150 g asparagine, 0.04 g thiamine HCl, and 8 g agar. Adjust pH to 5.7 with 1 M KOH and the volume to 1 L before sterilization by autoclaving. Add 2.5 mL of filter-sterilized 2-4-D solution (1 mg/mL, 0.2-μm mesh) to the autoclaved medium.
3. PMI media: per L, make three PMI media differing in their sucrose and mannose concentrations.

   PMI 1, add 4.4 g Murashige and Skoog medium including vitamins, 5 g sucrose. PMI 2, add 4.4 g Murashige and Skoog medium including vitamins, 20 g sucrose. PMI 3, add 4.4 g Murashige and Skoog medium including vitamins only (no sucrose). Add 0.150 g asparagine, 0.04 g thiamine–HCl, and 8 g agar to all the three media, adjust pH to 5.7 with 1 M KOH and the volume to 1 L before sterilization by autoclaving. Add filter-sterilized (0.2-μm mesh) 0.5 mL of indole acetic acid (IAA, 1 mg/mL), 1 mL of 6-benzylaminopurine (1 mg/mL) to the autoclaved media. In addition, add 10, 5, and 15 mL of filter-sterilized mannose solution (1 g/mL, 0.2-μm mesh) to PMI 1, PMI 2, and PMI 3 media respectively.
4. MS0 PMI medium: per L, add 2.2 g Murashige and Skoog medium containing of vitamins and 8 g agar. Adjust pH to 5.7 with 1 M KOH and the volume to 1 L before sterilization by

autoclaving. Add filter-sterilized (0.2-μm mesh) 1 mL of indole acetic acid (1 mg/mL) and 15 mL of mannose (1 g/mL) solutions to the autoclaved medium.

2.5 Other Solutions, Reagents, and Supplies

1. Dissection blades: Swann Morton n° 11 (ref 0203).
2. 0.2 μm nylon syringe filters.
3. Grade P4 7.0 cm Whatman circular filter paper: Autoclave on a liquid cycle for 45 min in glass petri dishes and allow desiccating overnight at 75 °C.
4. Magnifying glass for dissecting immature embryos.
5. Laminar flow hood.
6. Soil for growing *T. aestivum*: "Substrat SP Fleurissement" Klasmann/Deilmann ref. 384. Before planting, mix into the soil the appropriate amount of a slow-release fertilizer (NUTRICOTE® 13/11/11+oligoelements Ref 173100; 10 g/ 4 L pot).
7. Growth chamber to maintain a constant 28 °C without lighting.
8. Growth chamber or greenhouse suitable for growing *T. aestivum*: under controlled conditions with 16-h days at 18 °C and 8-h nights at 15 °C (maximum control temperature 28 °C). Lighting to 65 μEm/m^2/s.

3 Methods

3.1 Preparation of DNA for Shooting

1. Make DNA constructs for transgene overexpression using the Multisite Gateway system (Invitrogen). This system allows combining three oriented DNA fragments, typically a promoter in position one, an open reading frame (ORF) in position two and a terminator and a selection cassette in position three. The promotor (whether ubiquitous, tissue specific or inducible) is in the pDONR P4-P1R plasmid; the gene of interest is in the pDONR 221 plasmid and the terminator followed by a cassette for selection is provided in pDONR P4-P1R plasmid. Recombine the three entry plasmids using the Gateway system into one destination plasmid pDESTR4-R3.
2. Protocol to recover and purify agarose-free DNA fragments was developed in our laboratory. For this, digest plasmid DNA with the appropriate enzyme to release the cassette (1 h, 1 U of enzyme per μg of plasmid DNA). Separate linear DNA fragments by agarose gel electrophoresis (0.5% agarose in 1× TAE including 0.06% ethidium bromide, 5 V/cm) in a gel which has two wells in a single lane, one for loading the sample and one for recovering the cassette fragment. Monitor DNA migration using a hand-held UV lamp, and when the DNA

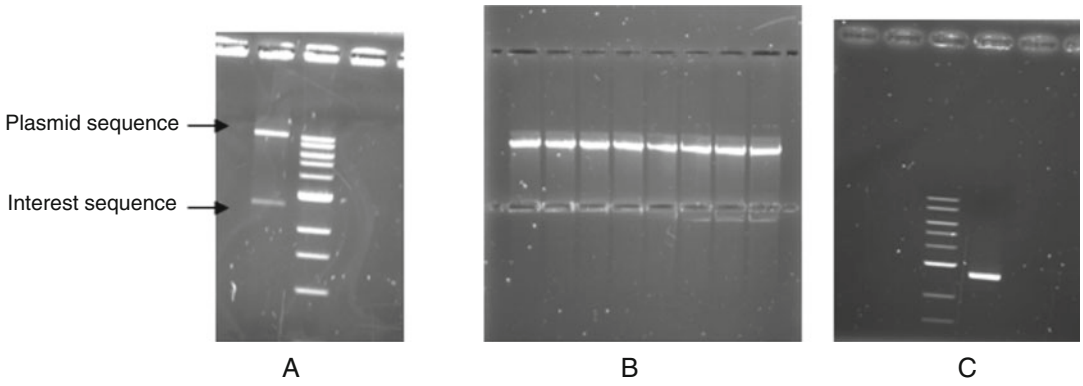

Fig. 1 Recovery of DNA cassette during agarose gel electrophoresis. After enzymatic digestion using the appropriate restriction endonuclease, electrophoresis of DNA fragments was performed on a standard agarose gel, except that two lines of wells were created in the same lane, one for loading samples and one for cassette fragment recovery. The migration was monitored using ethidium bromide and UV light. (**A**) Correct digestion of the plasmid check by agarose gel electrophoresis. (**B**) Recovery of the DNA fragment corresponding to the cassette after arrival at the well. (**C**) Purity of the cassette DNA fragment check by agarose gel electrophoresis

arrives in the second well, collect the DNA by aspiration (Fig. 1). Extract the DNA solution twice with butanol (v/v) to eliminate the ethidium bromide, and once with ether (v/v) to eliminate the butanol. Concentrate DNA by ethanol precipitation (1/10 vol 3M Na acetate pH 5.2 and 3 vol EtOH) and followed by washing with 70% EtOH. Dry the DNA pellets before suspending in TE (10 mM Tris–HCl and 1 mM EDTA).

3. Make constructs to knock-out gene function using RNAi using the pStargate plasmid (CSIRO®) and the Gateway technology (Invitrogen®). In this strategy the sequence of interest (typically 300 bp of the coding sequence of the gene) is inserted in sense and antisense directions under the control of the maize ubiquitin promoter. The sequence of interest, flanked by the two 25-bp Gateway recombination sequences, is produced by DNA synthesis. Plasmid extraction, purification, and cassette recovery are performed as previously described.
4. Prepare the DNA samples for shooting by mixing equimolar amounts of the RNAi construct and the selection cassette.

3.2 Production and Preparation of Immature Embryos for Shooting

1. Grow donor plants for immature embryo production and transgenic plants in a greenhouse under controlled conditions with 16-h days at 18 °C and 8-h nights at 15 °C. Grow plants under the best possible conditions without biotic or abiotic stress (*see* **Notes 1–3**).
2. Harvest immature seeds 12–14 days post anthesis.
3. Sterilize the seeds in a sodium hypochlorite solution (20% v/v) containing 1 drop of Tween® 20 per 200 mL for 15 min by shaking in a sterile environment at room temperature.

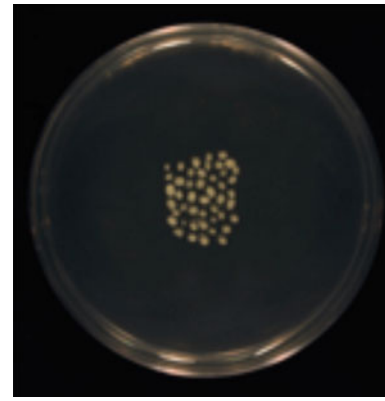

Fig. 2 Immature wheat embryos ready for particle gun shooting. Recovered embryos from green seeds 14 days after pollination

Table 2
Transformation efficiency using varying amounts of DNA

DNA linear quantity	Number of embryos	Fertile plants/Immature embryos
500	354	2.3
50	696	1.9
5	388	4.0

4. Isolate immature embryos (*see* **Note 2**) in a laminar flow hood with the aid of a microscope. Remove the embryo axis to prevent precocious germination during in vitro culture.
5. Transfer the embryos to plasmolysis medium with the side of the embryonic axis touching the culture medium. For a standard one-shoot experiment, place 50 embryos in the middle of a petri dish (55 mm diameter). Leave the samples for at least 2 h in the dark at 28 °C (Fig. 2) before transformation.

3.3 Shooting

Embryos are transformed by particle bombardment using a BioRad PDS 1000 He device [30] with 900 psi rupture discs at a target distance of 7 cm.

1. Suspend Gold microprojectiles, 0.6 μm in diameter (BioRad) in ethanol (1 mg/20 μL), sonicate for 1 min and add 10 μL of DNA solution. We tested different DNA concentrations, ranging from 500 to 5 ng/μL, and we did not observe any significant difference in transformation efficiency (Table 2).
2. Mix 20 μL of 0.1 M spermidine and 50 μL of 2.5 M $CaCl_2$ first before adding to the particles coated with DNA. At this stage, total volume of the sample is 100 μL.

3. Incubate the sample for 15 min at room temperature with gentle agitation.
4. Centrifuge (6000 × *g*) for 1 min at room temperature. Remove the supernatant and wash the pellet with 99.8% ethanol.
5. After a second pulse of centrifugation (1 min, 6000 × *g*), suspend the gold bullets in 99.8% ethanol (100 μL).
6. Use 10 μL of suspension for each shot.

3.4 In Vitro Culture and Selective Mediums

The selectable markers (for review on plants, [31]) used for wheat transformation are widely used genes like *bar* [3], which confers resistance to the herbicide *basta*, *nptII*, which confers resistance to the antibiotic kanamycin [2], and a combination of a 3-enolpyruvylshikimate-5-phosphate (EPSPS) from the *Agrobacterium* strain CP4, which is glyphosate tolerant, and the glyphosate oxidoreductase (GOX) gene that degrades glyphosate, together conferring resistance to the herbicide glyphosate [32]. More recently, a mutated rice acetolactate synthase gene was introduced into wheat as a system of selection using the herbicide bispyribac sodium [33], and is the first selectable marker of plant origin to be used in wheat. These types of selection involve toxic selection agents that induce necrosis of non-transformed tissues, which can also be deleterious to the development of transformed tissues. Another type of selection was developed for maize and wheat that involves using the *E. coli manA* (*pmi*) gene and mannose as the selective agent [34]. The activity of the gene in transformed cells allows mannose to become a carbon source.

For routine wheat transformation in our lab the *pmi*/mannose selection system is used [34]. In our hands, compared to *bar*/basta and *nptII*/gentamycin, this protocol gives a better percentage of transformed plants after in vitro culture selection, typically 98%.

1. Incubate bombarded embryos overnight at 28 °C in the dark on osmotic medium.
2. Transferred the embryos to MSE3 medium for 2 weeks at 28 °C in the dark for callus induction (Fig. 3).
3. Apply selection by including mannose in the culture medium. Calli are placed on PMI1 medium (containing 1/3 sucrose and 2/3 mannose as sugar sources) for 2 weeks in the light at 24 °C.
4. Transfer calli with green spots to PMI2 medium (4/5 sucrose and 1/5 mannose) to induce shoot initiation.
5. When shoots on PMI2 develop small leaves, place the shoots on PMI3 (100% mannose).
6. After about 2 weeks, transfer microplantlets harboring vigorous shoots to larger containers containing MS0 PMI medium to produce roots.
7. Transfer well-developed plantlets with leaves and roots to a containment greenhouse for genetically modified organisms.

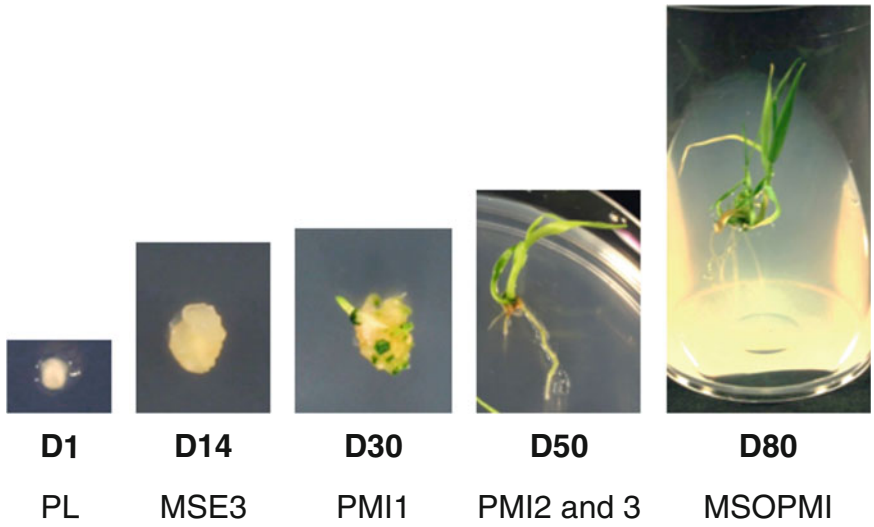

Fig. 3 In vitro culture of immature wheat embryos after shooting and regeneration of green plants. Day 1: Immature wheat embryos after shooting. Day 14: Callus development. Day 30: Green spots are visible and morphogenesis is induced. Day 50: Leaves develop and roots are induced. Day 80: Plantlet development. *PL* plasmolyzing medium

3.5 Analysis of Transgenic Plants

1. Isolate genomic DNA from young wheat leaves using a modified cetyltrimethylammonium bromide (CTAB) method [35].
2. Perform Southern hybridization as described by Barret et al. [36].
3. For PCR detection, amplify the *pmi* gene by using 50 ng of total genomic DNA as template and Taq DNA polymerase and the primer combination UP[5′-GCA CAG CCA CTC TCC ATT CA-3′] and LP 50[5′-ATC GGA GTT TGC CAT CAC TTC-3′].

   The PCR reaction mixture (50 μL) contains 1× Taq polymerase buffer (Qiagen), 1.5 mM $MgCl_2$, 150 μM of each dNTP, 20 pmol of each primer, and 1 unit of Taq polymerase.
4. Performed PCR using a PCR thermocycler with 1 cycle at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension step of 72 °C for 5 min.
5. Detect the amplified transgene product is by electrophoresis on a 2% agarose gel and visualize by ethidium bromide staining.

4 Notes

1. Plants, which suffer from stress during development, may produce embryos with low fitness that are unable to produce calli and to regenerate in vitro. Low fitness embryos cannot be

differentiated from standard embryos. Biotic and abiotic stress including drought, heat, chemical treatments, and low or high fertilization can induce changes.

2. The embryos should measure about 1 mm. Smaller embryos and transparent are too young and difficult to handle, so are dramatically impacted by in vitro culture. Larger embryos are easily handled and give rise to nice callus structures until the "green spots" phase, but they do not start organogenesis.
3. Thrips are common pests in greenhouses that damage plants by sucking their sap. They are sometimes difficult to detect. The presence of thrips on donor plants is not directly deleterious to plant fitness, but thrips may bite the immature embryo and induce bacterial contamination during in vitro culture.

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Chapter 10

Wheat Genetic Transformation Using Mature Embryos as Explants

Harsh Chauhan and Paramjit Khurana

Abstract

Feeding the growing population utilizing the limited agricultural resources remains a great challenge. Plant biotechnology plays a vital role in crop improvement by incorporating desired quality traits, tolerance to abiotic stresses and resistance to biotic stresses, which are otherwise tough to achieve by conventional plant breeding methodologies. Genetic engineering is a powerful tool to develop desired traits in selected crops to make the crops suitable for future demand and environment. For genetic engineering of crops like wheat, the development of efficient transformation and regeneration systems has always been a prime requirement. Immature embryo cultures have been used largely for genetic engineering purposes in wheat, but the availability of healthy immature embryos as explant throughout the year is difficult. In contrast, mature embryos are readily available throughout the year. Therefore, it is essential to develop an efficient transformation and regeneration system employing mature embryos as explant. Here, we summarize the recent developments in wheat tissue culture using mature embryos as explants and its use in genetic transformation.

Key words Transformation, Mature embryos, Regeneration, Zeatin, Agrobacterium

1 Introduction

To meet the increasing global demand and for ensuring the food security, it is essential to raise the productivity of staple food crops, like wheat. Further, the adverse effects of abiotic and biotic stresses cause deterioration of growth and productivity, leading to a negative impact on both yield and quality. By incorporating novel and diverse sources of resistance, plant biotechnology can contribute toward mitigating the adverse impact of stresses.

Being hexaploid, wheat possesses a large genome and shows a low rate of regeneration and transformation [1, 2]. For successful genetic engineering in crop plants, well-defined tissue culture procedures are needed for callus induction and regeneration. Callus induction and regeneration frequencies largely depend upon selected explant. In the case of wheat, the commonly used explant

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sources are immature embryos, inflorescence, mature embryos, seeds and mature seedling. Among the various explants, immature embryos are the most efficient due to their high transformation and regeneration frequencies. However, in the case of wheat the immature embryos are not readily available throughout the year for tissue culture, and moreover, the stage of the immature embryo is very specific for tissue culture [3]. When mature embryos are used as explant, the regeneration frequency is low in comparison to immature embryos but due to their availability throughout the year, mature embryos are also the preferred explants. Recently, in wheat, many studies were undertaken to optimize crucial tissue culture parameters such as type and concentration of growth regulator in culture media, type of inoculation, duration and conditions for preculture to overcome low transformation, and regeneration rate in case of mature embryos [1, 4, 5].

1.1 Wheat Genetic Engineering

Genetic engineering makes it possible to introduce a gene of interest into a target cultivar from any source, overcoming the limitation of relatedness associated with conventional breeding methodologies. The process to genetically modify a plant involves callus formation, then the introduction of the gene of interest to callus tissue (transformation) and regeneration of plantlets from the transformed callus. Particle bombardment and *Agrobacterium*-mediated transformation are the two major methods of transformation.

Particle bombardment (also called biolistic) and *Agrobacterium*-mediated transformation methodologies have been used largely for the introduction of the transgene to wheat. Vasil et al. (1992) for the first time developed the herbicide resistant wheat plant by particle bombardment on calli [6]. Biolistics has the limitations of being costly, insertion of multiple copies of gene and fragmentation of target DNA during the process of bombardment. Apart from less expensive, *Agrobacterium*-mediated transformation events have lesser number of partial fragments in comparison to particle bombardment method; this made *Agrobacterium*-mediated transformation system the preferred one for wheat [7]. Sarker and Biswas (2002) found that transformation efficiency in wheat also depends on the type of explant [8]. Four explants namely mature and immature embryo and calli developed from them were experimented to analyze their suitability for transformation. Immature embryo-derived calli showed highest transformation efficiency (85%).

1.2 Mature Embryo as Explants

Contrary to immature embryos, mature wheat embryos are readily available, throughout the year. Thus, using mature embryos as explant source bypasses the hectic and time-consuming process to grow plants in controlled conditions to collect a large number of immature embryos of the desired stage.

For establishing mature embryos as suitable explants for wheat tissue culture, Bartok and Sagi (1990) developed a rapid method of callus induction supported with endosperm as a natural nutrient source [9]. For this purpose mature seeds of *Triticum aestivum* L. cv. GK Kincso were used as explants, and for callus formation all the germ tissue except mesocotyl was removed using a scalpel to avoid germination. It was found that in the presence of nutrients of endosperm, callus formation from mesocotyl cells was early and faster in comparison to callus induction on artificial media.

High expression of germin-like *oxalate oxidase* gene has been observed on the surface of embryonic calli as oxalate oxidase promotes the localized H_2O_2 generation which increases the peroxidative cross-linking in cell wall constituents to inhibit the expansion of cell, thereby maintaining the cells in an embryogenically competent state [10]. Chen et al. (2006) conducted a study with eight winter wheat varieties (Jimai 1, Guomai 1, Guoyou 1, Yumai 18, Yumai 34, Yumai 36, Xinmai 13, and Neixiang 188) using non-endosperm-supported and endosperm-supported media, and also having different 2,4-D concentrations [11]. Average callus induction percentage was higher in the case of mature embryos in non-endosperm-supported media. However, the percentage of embryogenic callus was higher for endosperm-supported mature embryos, and the regeneration percentage was also significantly higher for endosperm-supported system. The activity of oxalate oxidase was also found significantly higher for endosperm-supported calli than non-endosperm-supported calli.

Ozgen et al. (1998) used 12 commonly grown winter wheat cultivars, namely, Gerek 79, Haymana 79, Bezostaja 1, Bolal 2973, Basak 95, Sadova 1, Tosun 21, Yayla 305, Yektay 406, Sivas 111/33, Kirac 66, and T-115, to conduct a comparative study of callus induction and regeneration by using immature and mature embryos [12]. Instead of excising from seeds, mature embryos were only moved slightly using scalpel, and intact seed was placed on media for callus induction. In this study the dependence of culture responses on genotype was observed as all 12 cultivars performed differentially both in the case of immature and mature embryos. Interestingly, they found that average callus induction frequency and regeneration capacity were higher in case of mature embryos (90.6% and 96.1%, respectively) than that of immature embryos (80.4% and 70.9%, respectively).

Delporte et al. (2001) developed an efficient method for transformation by employing thin fragments of mature embryos as explant from one winter (Odeon) and one spring (Minaret) wheat cultivars [13]. Using 100 mature embryos, 500–600 thin fragments of desired sizes were obtained. In this method regeneration frequency of 25–30 plants/100 mature embryos was achieved, which is equivalent to previously reported regeneration frequencies using immature embryos.

As previously summarized, Sarker and Biswas (2002) assessed the callus induction, regeneration as well as *Agrobacterium*-mediated transformation efficiencies of various explants such as immature and mature embryos, shoot bases, root tip, leaf tissue, and endosperm from four wheat cultivars, namely, Sourav, Gourav, Kanchan, and Protiva, for callus formation [8]. No callus formation was observed when leaf tissue was used as explants. A higher callus formation was observed for immature embryos on callus induction media supplemented with 5.5 mgL^{-1} 2,4-D for these cultivars (Sourav-95%, Gourav-93.7%, Kanchan-93.3%, and Protiva-92%). On the other hand, mature embryos provided better response for callusing on media containing 6.0 mgL^{-1} 2,4-D (Sourav-83.3%, Gourav-66.6%, Kanchan-80.0%, and Protiva-84.0). Higher percentage of transformation was found in case of immature embryo (Sourav-68.7, Gourav-63.7, Kanchan-65.3, and Protiva-62.6) than mature embryo (Sourav-31.8, Gourav-30.6, Kanchan-27.5, and Protiva-26.6) for all four selected wheat cultivars. Two strains of *Agrobacterium*, namely, LBA4404 and EHA105 harboring *pBI121* and *pCAMBIA1301* plasmids respectively were used for transformation experiments, but only EHA105 showed the positive results regarding successful transformation. Thus *Agrobacterium* strain EHA105 was found compatible for transformation purpose.

Mendoza et al. (2002) studied the effect of different types and levels of auxin and sugar, independently and in various combinations on frequencies of callus induction and regeneration from mature embryo of Bobwhite cultivar of wheat [14]. Dicamba, picloram, 2-MCPP, and 2,4-D were the four studied auxins at different concentrations. Effect of combination of maltose or sucrose, with all four auxins and the effect of sterilization techniques (autoclaving and filter sterilization), was also studied. The highest callus induction rate was observed in media containing filter sterilized sucrose and 9 μM picloram. However, highest regeneration occurred in case of callus developed on media having 18 μM dicamba with filter sterilized sucrose. Filippov et al. (2006) also studied the effect of different concentration of growth regulators (2,4-D, 2,4,5-T, and Dicamba) independently and supplemented with IAA, IBA, or NAA [15]. Dicamba was found to provide a more positive effect on embryogenic callus induction and regeneration when supplemented IAA.

Transgenic tetraploid wheat (*Triticum dicoccum* Schuble) plants were developed from mature embryo-derived calli using *Agrobacterium* LBA4404 by Khurana et al. (2002) [16]. Regeneration efficiencies of immature and mature embryo-derived calli were compared, and interestingly, mature embryo-derived calli showed a 10–12% better regeneration response. Moreover, GUS expression also supported the suitability of mature embryo for transformation experiments. In case of cultivar, DDK1001 GUS expression for mature embryos and mature embryo derived calli

was observed to be 83% and 100% respectively. Subsequently, Patnaik and Khurana (2003) obtained success in transferring *bar* and *HVA1*genes into *T. durum*. The biolistic method was successfully used for transformation of mature embryo-derived calli of *T. durum* (cultivar PDW215) and *T. aestivum* (cultivar CPAN1676) [17].

Chauhan et al. (2007) assessed the impact of 1-Phenyl-3-(1,2,3-Thia-Diazol-5-YL) urea (Thidiazuron, TDZ), altering the dose and duration of exposure on immature inflorescence, immature and mature embryo explants cultures of *Triticum aestivum* (CPAN1676, HD2329, and PBW343), *Triticum durum* (PDW215, PDW233, and WH896) and *Triticum dicoccu*m (DDK1001, DDK1025, and DDK1029) [5]. It was observed that the process of regeneration was promoted by low concentration of TDZ and for obtaining high regeneration efficiencies from mature embryo derived calli, a combination of TDZ and cytokinin in regeneration media was found to be helpful. These findings could be of great use in developing transgenic wheat involving commercial cultivars and mature embryo as explant source.

To formulate a high throughput regeneration system Yin Guixiang et al. (2011) tested media having different composition and supplemented with different concentrations of ascorbic acid, cystein, dicamba, glutamine, silver nitrate over 20 wheat genotypes (Yangmai6, Yangmai 12, Yangmai 15, Jingdong 17, Jinghua 9, Jingdong 23, Lunxuan 987, Lunxuan 518, CA8694, Han 6172, Zhen 9023, Shi 4185, Yumai 66, Jimai 20, Kenong 199, Jing 411, Bobwhite, Xinchun 9, Ningchun 4, and CB037) [18]. As explant, excised intact mature embryos and fully scrapped pieces of mature embryos were used. Ascorbic acid was used to overcome the problem of oxidative species generation in transformed cells and dicamba was used as growth regulator. Using this new regeneration system, significant improvement in regeneration efficiency was observed among all the cultivars, and China Innovation Patent (open number: 101265481) was acquired for this new regeneration system. Among the 20 genotypes investigated, CB 037 and Lunxuan 987 were recommended for transformation programs because of higher regeneration frequency of 85.6% and 60.1%, respectively.

To find the best method, Aydin et al. (2011) studied response of four wheat genotypes namely Kirik, Dogu 88, Bezostaja 1, and Kate A-1 12 in twelve culture media with different composition, separately in endosperm-supported and non-endosperm-supported conditions for callus formation and plant regeneration [19]. They found a significantly higher regeneration in case of endosperm-supported media. Results verified that concentration and type of growth regulators play important role in regeneration and the higher auxin concentrations favor the process of regeneration.

Wang et al. (2009) performed transformation using *Agrobacterium* strain C58C1 and mature embryo of three different cultivars of wheat, Yumai66, Lunxuan208, and Bobwhite [20]. The results showed cultivar specific transformation frequency, Yumai66–0.06%, Lunxuan208–0.67%, and Bobwhite-0.89%.

Ding et al. (2009) also optimized transformation of mature embryo of wheat cultivar EM12 using *Agrobacterium*-mediated transformation [4]. For optimization of methodology, four factors were kept variable, namely, duration of preculture, inoculation type, inoculation length, and condition of coculture. Variability in factors were as follows: preculture at 25 °C for 5 days or 14 days; type of inoculation suspension—suspension with full strength MS salts or one-tenth strength; duration of inoculation—0.5, 1.0, 3.0, or 5.0 h; and coculture condition—co-culture on fresh cultivation media or under desiccation condition. Higher transformation percentage (89.5%) was observed with a combination of 14 days preculture, inoculation suspension with full strength MS salts, 3-h inoculation duration, and coculture under desiccating condition. Thus, the study resulted in an efficient transformation method for mature embryos of wheat.

The requirement for screening of commercial wheat cultivars to identify the better performing genotypes was noticed due to the differential genotypic response of wheat for tissue culture and genetic engineering processes. Abid et al. (2014) screened 13 commercial winter wheat varieties (AARI-11, Aas-11, Dhurabi-11, Faisalabad-2008, Lasani-08, Millat-11, Pak-81, Punjab-11, Sahar-2006, Shafaq, V-07096, V-08203, and VIII-83) for mature embryo explants based processes of callus induction, transformation, and fertile plant regeneration [21]. Callus formation was observed ranging from a minimum of 60% (Cv. Millat) to 100% (Cv. AARI-11, Aas-11, Dhurabi-11, Faisalabad-2008, Pak-81, Punjab-11, Sahar-2006, Shafaq, V-07096, V-08203, and VIII-83). However, the capability of somatic embryogenesis of callus was independent of callus induction response among all varieties. In Pak-81 callus induction was 100%, but 70% of these were nonembryogenic, while in the case of Millat-11, callus formation occurred at the rate of 60%, but most of the calli were highly embryogenic. The transformation was done by using *Agrobacterium* strain AGL1 harboring *pGA482* binary plasmid. Variations were also observed in percentage of transient expression and regeneration in a cultivar-specific manner. Based on this study, three cultivars Aas-11, V-III-83, and Faisalabad-2008 were found better for genetic engineering purposes using mature embryos as explants.

It is clear from above discussion that in different studies researchers have proposed different methods and altered composition of media for raising transgenics by using wheat mature embryo (summarized in Tables 1 and 2). Below we present a simplified and working protocol developed and utilized in our lab using both

Table 1
Present status of wheat genetic engineering using mature embryo as explant

Species/genotype	Explant	*Agrobacterium* strain	Binary vector	Promoter/gene	Transformation efficiency (%)	References
T. aestivum (Bread wheat)						
Sourav, Gourav, Kanchan, Protiva	Immature and mature embryo	LBA4404, EHA105	*pBI121, pCAMBIA1301*	*CaMV35S:gus, Nos:nptII*	NR	Sarker and Biswas, 2002 [8]
CPAN1676	Mature embryo derived calli		*pDM302, pAct-F, pBY520*	*CaMV35S:bar, Act1:gus, Act1: HVA*1	8.56	Patnaik and Khurana, 2003 [17]
Keumkangmil, Alchanmil,Bobwhite	Mature embryo	KYRT1	*pCAMBIA1305.1*	*CaMV35S:hpt*	NR	Han et al., 2006 [22]
HD2329, CPAN1676, PBW343	Mature embryo	LBA4404	*pBI101, pCAMBIA3303*	*Act1:gus, CaMV35S:gus, Nos:nptII, CaMV35S:bar*	1.77	Patnaik et al., 2006 [23]
EM12	Precultured mature embryo	LBA4404	*pBI121*	*CaMV35S:gus, CaMV35S:nptII*	0.025	Ding et al., 2009 [4]
Yumai66, Lunxuan208, Bobwhite	Mature embryo, precultured mature embryo	C58C1	*pUbiGN*	*Nos:nptII, Ubi:gus*	0.06 (Yumai66), 0.67 (Lunxuan208), 0.89(Bobwhite)	Wang et al., 2009 [20]
Gemmiza9, Gemmiza 10	Mature embryo	LBA4404	*pBI121*	*CaMV35S:gus, CaMV35S:nptII*	6.9(Gemmiza9), 8.7 (Gemmiza10)	Moghaieb et al.,2010 [24]
Chakwal97, Inqilab91, Tartara2000	Mature embryo	EHA101	*pTCL5*	*CaMV35S:gus, CaMV35S: Xa21, CaMV35S:hpt*	NR	Raja et al., 2010 [25]

(continued)

Table 1
(continued)

Species/genotype	Explant	*Agrobacterium* strain	Binary vector	Promoter/gene	Transformation efficiency (%)	References
Inqilab91	Mature embryo		*pIG121Hm*	*CaMV35S:gus, Nos:nptII, CaMV35S:hpt*	6.25–15.62	Rashid et al., 2010 [26]
AARI-11, Aas-11, Dhurabi-11, Faisalabad-2008, Lasani-08, Millat-11, Pak-81, Punjab-11, Sahar-2006, Shafaq, V-07096, V-08203, VIII-83	Mature embryo	AGL1	*pGA482*	*gus, nptII*	NR	Abid et al., 2014 [21]
T. dicoccum (Emmer wheat)						
DDK1001, DDK1009	Mature and immature embryo	LBA4404	*p35SGUSINT, pBI101*	*CaMV35S:gus, Act1:gus, Nos:nptII*	NR	Khuranna et al., 2002 [16]
T. durum (Pasta wheat)						
PDW215, PDW233, WH896	Mature embryo	LBA4404	*pBI101*	*Act1:gus, CaMV35S:gus, Nos:nptII, CaMV35S:bar*	1.28	Patnaik et al., 2006 [23]
PDW215 (Pasta)	Mature embryo		*pCAMBIA3301*	*CaMV35S:gus, CaMV35S:bar*	3	Vishnudasan et al., 2005 [27]
Om Rabia3	Precultured mature embryo	GV3101	*pCAMBIA1391Z*	*PrDHN-5:gus, Ubi1:gus, Ubi1:bar*	NR	Amar et al., 2013 [28]
PDW215	Mature embryo-derived calli		*pDM302, pAct-F, pBY520*	*CaMV35S:bar, Act1:gus, Act1:HVA1*	10.0	Patnaik and Khurana, 2003 [17]

Table 2
Explant—mature seedling and mature seed-derived calli

Species/ genotype	Explant	*Agrobacterium* strain	Binary vector	Promoter/gene	Transformation efficiency (%)	References
T. aestivum (Bread wheat)						
Bobwhite	Mature seedling	EHA105	*pIG121Hm*	*CaMV35S:gus, Nos: nptII,CaMV35S:hpt*	NR	Trick and Finer, 1997 [29]
Chinese spring	Mature seed and immature embryo-derived calli	GV3101	*pMVTBP, pNFHK1, p35SGUSINT*	*CaMV35S:gus, CaMV35S:nptII*	1.2–2.2	Peters et al., 1999 [30]
CPAN1676, PBW343	Mature seed-derived calli	LBA4404	*pCAMBIA3301*	*CaMV35S:gus, CaMV35S:bar*	6.7–8.7	Chugh and Khurana 2003 [31]
Shiranekomugi	Mature seedling	M-21, LBA4404	*pIG121Hm, pBI-res, pBI-res2*	*CaMV35S:gus, Nos: nptII, CaMV35S:hpt*	NR	Supartana et al., 2006 [32]
Yan361, Yan2801, H11	Mature seedling		*pBLG*	*CaMV35S:nptII*	9.82	Zhao et al., 2006 [33]
Een1	Mature seedling	LBA4404	*NR*	*CaMV35S:gus, CaMV35S:nptII*	3 to 31	Yang et al., 2008 [34]
HD2329	Mature seedling	GV2260	*p35SGUSINT*	*CaMV35S:gus, CaMV35S:nptII*	1.16	Chugh et al., 2012 [35]
T. durum (Pasta wheat)						
PDW215	Mature seedling		*pCAMBIA3301*	*CaMV35S:bar*	0.84	Chugh et al., 2012 [35]

immature and mature embryo as explants for genetic transformation of wheat.

2 Materials

1. Equipment: Laminar flow cabinet, scalpel and blades, carved forceps, centrifuge, petri dishes (90 mm), tissue culture tubes [Sigma- C1048], micro pipettes, vaccum pump, tissue culture racks, laboratory autoclave, syringe and bottle top filters (0.22 μM), ultraprecision weighing balance, magnetic stirrers, pH meter, and flame sterilizer.
2. Wheat seeds.
3. Flask, sodium hypochlorite.
4. YEM medium.
5. *Agrobacterium strain* (EHA105, AGL1).
6. 2,4-D-2 mg/L [Sigma-D8407].
7. Zeatin [Sigma-Z0164] (2 mg/L).
8. Callus induction media (CIM); MS media [Sigma- M5524] supplemented with 2 mg/L 2,4-D, 1 gm/L casein hydrolysate [Sigma, 22090], 250 mg/L myo-inositol [Sigma, I5125], 700 mg/L proline [Sigma, P0380] and 30 gm/L maltose [Sigma, M5895] (*see* **Notes 1** and **2**).
9. CIM-As; CIM with acetosyringone [Sigma, D134406] 0.5 mM.
10. CIM-Cef250; CIM with 250 mg/L cefotaxime [Sigma, C7912].
11. CIM-Cef250S; CIM-Cef250 with appropriate selection agent (for example 50 mg/L hygromycin [Sigma, H3274]).
12. MSR-S: MS media supplemented with sucrose (30 gm/L), zeatin (2 mg/L), agar (8 gm/L) or Phytagel (3.5 gm/L) and appropriate plant selection agent.

3 Methods

A simple protocol using mature embryo as explants is described below.

3.1 Explant (Seed) Sterilization

1. Take the required amount of seeds in a flask and wash seeds with liquid detergent in running tap water for 1 min.

   (After this, all the steps are done in laminar air flow hood.)
2. Treat with absolute ethanol for 30 s and wash with sterile RO water. Sterilize seeds with 2–4% sodium hypochlorite for 30 min with constant shaking. Finally wash seeds 3–5 times with sterile RO water.

3.2 Embryo Excision

Excise embryo from surface-sterilized seeds by removing endosperm in a laminar flow. (*Sterile blade is used for dissecting out the embryo.*)

3.3 Precallus Induction

1. Place excised embryo, axis side facing down ward on CIM supplemented with 2 mg/L 2,4-D [Sigma, D7299] (*see* **Note 3**).
2. Incubate at 20 °C temperature till the calli obtain appropriate size.
3. Simultaneously initiate *Agrobacterium* culture.

3.4 Bacterial Culture Initiation:

1. Take 50 µl glycerol stock or a single colony of *Agrobacterium* and start culture in YEM medium with appropriate antibiotic selection.
2. Incubate at 28 °C/200 rpm till A_{600} reaches 0.5 to 1.0. (*The growth of Agrobacterium can be synchronized with the mature embryo excision.*)

3.5 Transformation and Cocultivation

1. Immerse the excised embryo or embryo derived calli (Fig. 1a, b) in bacterial suspension for 2–3 h.
2. Excess of bacteria should be removed from the surface of calli by blotting on sterilized blotting sheets.
3. Then transfer excised embryo/callus on CIM-As and incubate at 28 °C in dark for 3 days.

3.6 Washing for Removal of Agrobacterium and Subsequent Callus Induction

1. After co-cultivation, wash explants (3–4 times) with liquid CIM-Cef250 with very gentle agitation to remove *Agrobacterium*. Alternatively, Timentin [Sigma- T5639] at the same concentration can also be used to wash and remove *Agrobacterium*.
2. Remove extra moisture by placing the explants briefly on sterile Whatman filter discs or blotting sheet.
3. Explants are now transferred to CIM-Cef250S (with selection agent) at 20 °C in dark for further growth of callus for 3 × 10 days with subculturing on fresh CIM-Cef250S media after every 10 days. If there is some occasional growth of *Agrobacterium* on some explants, these explants are discarded.

3.7 Regeneration and Shoot Formation:

1. After callusing for about 4 weeks, explants need to be transferred on regeneration media MSR-S supplemented with zeatin [Sigma-Z0164] (2 mg/L)[3] and selection agent with one subculture on the same media after 10 days. At this stage explants in petri plates are transferred in light. (Usually light by four cool fluorescent tube lights of 35 W each in a culture rack measuring 18 in. width and 18 in. height is sufficient.)
2. In about 2 weeks tiny green shoots starts to emerge (Fig. 1c, d).

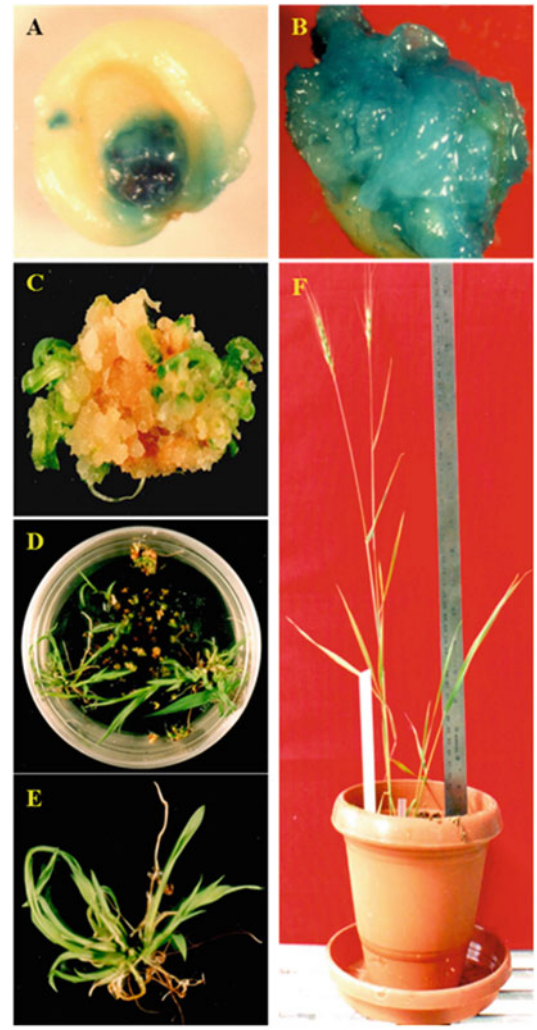

Fig. 1 Different stages in tissue culture protocol using mature embryo as explant from bread wheat cultivar CPAN1676.(**a, b**) Gus histochemical assay in freshly transformed mature embryo and mature embryo derived callus respectively.(**c**) Embryogenic callus derived from mature embryo producing multiple shoots. (**d**) Regenerating shoots on selection media (MSR-S). (**e**) Plantlet on shoot elongation medium. (**f**) A regenerated plant growing in potted soil and producing seeds

3.8 Shoot Elongation and Root Formation

1. Regenerated plantlets should be transferred to half-strength MS media supplemented with selection agent in tissue culture tubes [Sigma- C1048]. Subculturing is done every 2 weeks, and it takes around 4 weeks to have sufficient roots to transfer the plantlets in soil (Fig. 1e).

3.9 Transfer to Soil

1. Roots of plantlets should be cleaned thoroughly to remove any media for avoiding infection.
2. Rooted plantlets with well-grown roots are then transferred to pots containing Soilrite or any other similar soil less media and garden soil (1:1) and grown to maturity in growth chamber Fig. 1f).
3. When plants are at 4–5 leaf stage, we isolate DNA by cutting leaf discs from putative transgenic for PCR analysis of transgene(s).

4 Notes

1. All the components can be autoclaved except maltose, growth hormones, and antibiotics.
2. For filter sterilization of media we make a 2× concentration of all the components except the gelling agent and filter sterilize with 0.45 μm bottle top filters (Corning Cat No. 430512). Separately, the gelling agent in 2× concentration is autoclaved and then both the solutions are mixed in Laminar flow hood to make final 1× media prior to pouring in plates or tubes. For solidification plant tissue culture grade agar [Sigma- A7921] (0.8%) or Phytagel [Sigma, P8169] (0.35%) can be used.
3. Stock solution of 2,4-D and zeatin are made in 1000× concentration (i.e., 2 mg/ml). Hormones and antibiotics are added in the laminar flow prior to pouring of media.

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Chapter 11

Targeted Mutagenesis in Hexaploid Bread Wheat Using the TALEN and CRISPR/Cas Systems

Yanpeng Wang, Yuan Zong, and Caixia Gao

Abstract

The use of sequence-specific transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats-associated system (CRISPR/Cas9) have provided powerful reverse genetic approaches to the targeted modification of genomes in numerous organisms. Both systems have been employed to generate loss-of-function alleles in bread wheat, by targeting multiple and single copies of genes. Here we present protocols for modifying the wheat genome using the two systems. The protocols include the design of TALEN and CRISPR/Cas9 target sites and their construction, evaluation of their activities in protoplasts, transformation of plants, and mutation screening.

Key words Wheat, Genome editing, TALEN, CRISPR/Cas9, Wheat protoplasts, Targeted mutagenesis

1 Introduction

Bread wheat is an allohexaploid species (*Triticum aestivum*; 2n = 42, AABBDD), and the major staple crop worldwide [1]. New traits have constantly been introduced to improve production, quality, and adaptation to biotic and abiotic stresses, mostly through classical breeding. Standard mutagenesis approaches such as insertion of T-DNA or transposable elements [2], chemical mutagenesis with ethyl methanesulfonate (EMS) for TILLING (Targeting induced local lesions in genomes, TILLING) [3], and radiation mutagenesis [4] have been widely used and have yielded many mutants. However, these methods cannot target specific genes and require laborious work to identify the desired phenotype. As genetic analysis in wheat is very challenging due to its complex allohexaploid genome, new technologies are needed to maintain the rate of improvement in productivity and guarantee world food supply.

Recently, sequence-specific nucleases (SSNs) including zinc finger nucleases (ZFNs) [5], transcription activator-like effector

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nucleases (TALENs) [6, 7], and the RNA-guided nuclease Cas9 (CRISPR/Cas9) [8–10] (Fig. 1a, b), have been developed to generate targeted DNA double-strand breaks (DSBs), which are then repaired mainly by either error-prone nonhomologous end joining (NHEJ) or high-fidelity homologous recombination (HR) [11] (Fig. 1c). These SSNs have already been widely used to perform precise genome editing in a range of organisms [12, 13].

ZFNs were the first custom-designed nucleases engineered to cleave at specific DNA sequences; they were based on the Cys2His2 zinc finger domain [5], and bind DNA via a variety of engineered zinc finger proteins fused to the FokI cleavage domain. Because of difficulty in designing the constructs, the limited numbers of repeat units, and frequent off-target effects, this technology has not been widely used. TALEN proteins were first reported in 2009 and had a new modular DNA-binding domain that was more easily reprogrammed than ZFNs to target sequences. Because of the simplicity of their DNA recognition codes, TALENs have been used to engineer genomic loci in a variety of organisms. The CRISPR/Cas9 system employs an RNA-guided Cas9 nuclease to recognize and cleave target DNA [9]. As it is easy to design and use, can target wide range of genome sites, has high cleavage activity, and can perform multiplex gene editing, it has great advantages for genome editing.

1.1 Transcription Activator-Like Effector Nucleases (TALENs)

TALENs are composed of the engineered specific DNA binding domain of a TALE fused to the nonspecific FokI cleavage domain (Fig. 1a). TALEs are natural bacterial effector proteins used by *Xanthomonas* sp. to modulate gene transcription in host plants and facilitate colonization [14]. The specificity of a TALEN is determined by a central tandem domain comprising 13–28 copies of a typically 34 amino acid repeat. Although the sequences of the repeats are highly conserved, they differ primarily in two positions termed the repeat variable diresidues (RVDs, 12th and 13th positions); the 12th amino acid is used to stabilize the hairpin-like structure, while the 13th amino acid makes a base-specific contact in the major groove of DNA [15]. The identity of these two residues determines the nucleotide-binding specificity of each TALE repeat and a simple cipher specifies the target base of each RVD (NI = A, HD = C, NG = T, NN = G or A). As a result, TAL effectors have attracted great interest as DNA targeting tools. Like ZFNs, they can be fused to the FokI nuclease domain for genome editing, and since FokI cleaves as a dimer, the TAL effectors function in pairs, binding opposing targets across a spacer over which the FokI domains combine to create the DSB.

1.2 The CRISPR/Cas9 System

The CRISPR/Cas9-mediated genome editing system employs Watson-Crick complementary rules to recognize and cleave the target DNA sequence via a short RNA molecule and the Cas9

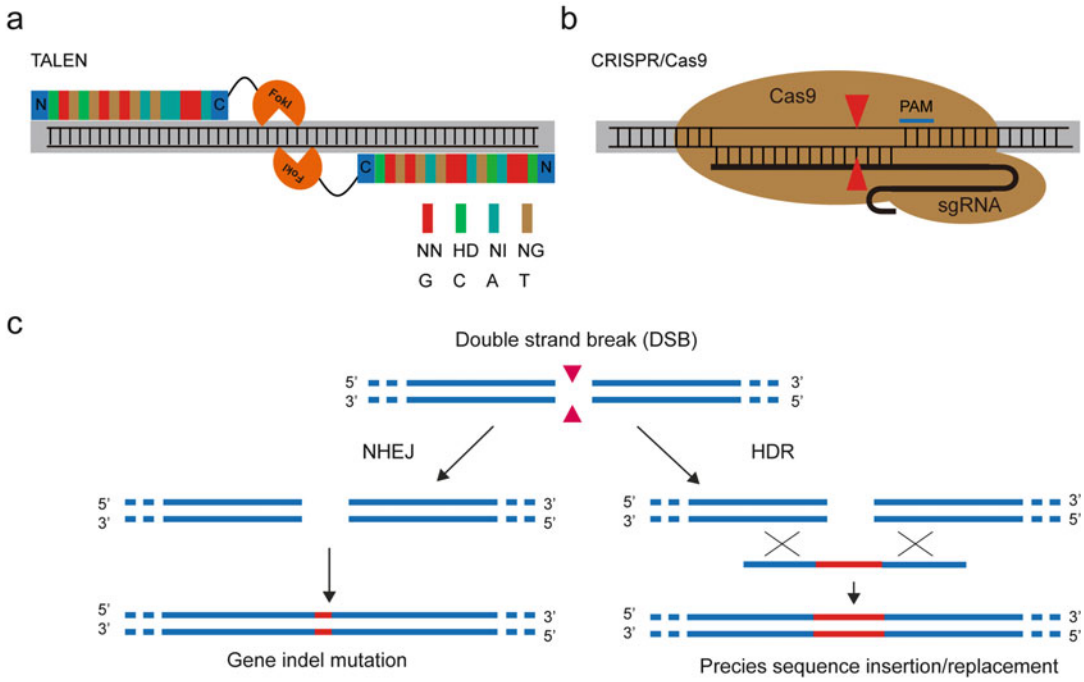

Fig. 1 Targeted genome engineering by the TALEN and CRISPR/Cas9 systems. (**a**) Schematic of the use of TALENs for genome editing. The *colored boxes* denote the TAL effector repeats. Each *color* represents a different repeat variable diresidue (RVD). FokI endonuclease (*orange*) is fused to the C-terminal domain. NI, HD, NN, and NG recognize A, C, G, and T, respectively. (**b**) Schematic of the use of CRISPR/Cas9 for genome editing. sgRNA-mediated recruitment of Cas9 to a target site. The 20 bp of the sgRNA hybridize to the DNA target by Watson-Crick base pairing. In *blue* is the NGG protospacer adjacent motif (PAM) just downstream of the hybridizing base pairs. Cas9 has two nuclease domains (shown by *red arrowheads*) that each cleave one strand of double-stranded DNA. (**c**) Targeted genome engineering by nonhomologous end-joining or homologous recombination using sequence-specific nucleases. NHEJ-mediated repair can result in small deletions or insertions that can disrupt gene function (*left*). HR-mediated repair, involving a homologous DNA template, leads to gene replacement or gene insertion (*right*)

endonuclease (Fig. 1b). The CRISPR/Cas system functions as an RNA-based adaptive immune system in bacteria and archaea [9]. The type II system from *Streptococcus pyogenes* is the best characterized, and it is this system that has been adapted for gene targeting. In the type II CRISPR system, the Cas9 protein is guided by a crRNA-tracrRNA heteroduplex to target and degrade foreign nucleic acids [9]. The two strands of the crRNA-tracrRNA heteroduplex can be fused to generate a chimeric single-guide RNA (sgRNA) that together with Cas9 produces sequence-specific DSBs (Fig. 1b). The target sequence must be complementary to the sgRNA sequence, and must be immediately followed by the correct protospacer adjacent motif (PAM), a 3-bp NGG motif (Fig. 1b). Cas9 has two conserved nuclease domains: the HNH nuclease domain cleaves the strand complementary to the sgRNA, while the RuvC-like nuclease domain cleaves the opposite strand (Fig. 1b).

In this chapter, we provide protocols for TALEN and CRISPR/Cas9-mediated targeted genome mutagenesis in bread wheat, including target site design, construction of TALENs and CRISPR/Cas9, validation of SSN activity in protoplasts, transformation of SSNs in plants and the identification of mutations in transgenic plants. These systems demonstrate the potential of SSNs as tools for wheat genome research and molecular breeding.

2 Materials

2.1 TALEN Assembly and sgRNA Cloning

2.1.1 Reagents

1. Golden Gate TALEN and TAL Effector Kit 2.0 (Addgene).
2. T4 DNA ligase, restriction endonucleases (NEB or Thermo).
3. Plasmid-Safe™ ATP-Dependent DNase (Epicentre).
4. Plasmid Miniprep kit (Axygen).
5. Chemically competent cells of *Escherichia coli* DH5α (TransGen Biotech).
6. Gateway LR Clonase Enzyme mix (Life Tech).
7. pEASY-Blunt cloning vector (TransGen Biotech).
8. BbsI (Fermentas/Thermo Scientific).
9. FastPfu DNA polymerase (TransGen Biotech).
10. High Pure dNTPs, 2.5 mM each (TransGen Biotech).
11. DNA gel extraction kit (Axygen).
12. Wizard Plus midipreps (Promega).
13. Annealing buffer, 10× (OriGene).

2.1.2 Equipment and Consumables

1. Polymerase chain reaction (PCR) thermocycler (Bio-Rad).
2. Environmentally controlled incubators (Eppendorf).
3. Heating water bath (Changfeng).
4. NanoDrop spectrophotometer (Thermo).
5. Standard equipment and reagents for agarose gel electrophoresis (Bio-Rad).

2.2 Protoplast Isolation and Transformation

2.2.1 Reagents

1. 2-(N-morpholino) ethanesulfonic acid (MES), 0.2 M, pH 5.7 (Sigma-Aldrich).
2. Mannitol, 0.8 M (Sigma-Aldrich).
3. $CaCl_2$, 1 M (Sigma-Aldrich).
4. KCl, 2 M (Sigma-Aldrich).
5. $MgCl_2$, 2 M (Sigma-Aldrich, cat. no. M9272).
6. BSA (Sigma-Aldrich).
7. Cellulase R10 (Yakult Pharmaceutical Industry).
8. Macerozyme R10 (Yakult Pharmaceutical Industry).
9. PEG4000 (Sigma-Aldrich).

2.2.2 Solution Buffer

1. Cell wall dissolving enzyme solution: 20 mM MES (pH 5.7), 1.5% (wt/vol) cellulase R10, 0.75% (wt/vol) macerozyme R10, 0.6 M mannitol. Warm the solution at 55 °C for 10 min to inactivate DNases and proteases and to enhance enzyme solubility. Cool the solution to room temperature (RT; 22–25 °C) and add 10 mM $CaCl_2$ and 0.1% (wt/vol) BSA. Finally, filter-sterilize. The enzyme solution should be freshly prepared.
2. W5 solution: 2 mM MES (pH 5.7), 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl. The solution can be stored at RT for up to 2 months.
3. MMG solution: 4 mM MES (pH 5.7), 0.4 M mannitol and 15 mM $MgCl_2$. The solution can be stored at RT for up to 2 months.
4. PEG solution: 40% (wt/vol) PEG4000 in ddH_2O, 0.2 M mannitol and 100 mM $CaCl_2$. The solution should be freshly prepared.

2.2.3 Equipment and Consumables

1. 6-well flat-bottom plates (Nunclon).
2. Single edge razor blades (Feiying).
3. 50 ml round-bottom centrifuge tubes (Haimeng).
4. Environmentally controlled incubator (24 °C) (Yiheng).
5. Microscope, fluorescence microscope (Olympus).
6. 40 μm nylon meshes (BD Falcon).
7. Syringe sterilization filter, 0.45 μm (Sartorius Stedim Biotech, cat. no. 21423103).

2.3 PCR/RE and T7E1 Assay

1. DNA quick plant system (Tiangen Biotech).
2. T7E1 (ViewSolid Biotech).
3. T7E1 buffer, 10× (ViewSolid Biotech).
4. T4 DNA ligase, restriction endonucleases (NEB or Thermo).
5. 10 mM mixed deoxynucleotides (Biodee).
6. Primer pairs (10 μM) (Beijing Genomics Institute).
7. pEASY-blunt cloning vector (TransGen Biotech).

2.4 Wheat Biolistic Transformation

1. Basal callus induction medium MS2.0: MS salts with vitamins, 30 g/l sucrose, 2 mg/l 2,4-D, 500 mg/l casein hydrolysate, and 0.6 g/l $CuSO_4 \cdot 5H_2O$.
2. Basal regeneration medium M1G: MS salts plus vitamins, 30 g/l sucrose and 0.2 mg/l kinetin.
3. Basal rooting medium: Half MS salts and vitamins, supplemented with 30 g/l sucrose.
4. All the media are solidified with 3.0 g/l Phytagel and the pH is adjusted to 5.8 with KOH.

3 Methods

3.1 Wheat Targeted Mutagenesis Strategy

Wheat is a hexaploid species with most genes represented by homologous copies in genomes A, B, and D. Normally, the homologous genes are functionally redundant, and knockout at one locus usually fails to cause a substantial phenotypic change. However, some homologous genes have different functions in each genome, and they need to be individually knocked out to analyze their functions. Thus, the challenge in wheat is to target all the copies of certain genes simultaneously and to target the individual copies of other genes. The SSNs are more flexible and specific in terms of target recognition than traditional techniques, such as EMS induction and T-DNA insertion. Here we summarize some key principles of SSNs design and mutation identification in wheat.

- Analyzing gene copy number and polymorphism. Determine the copy number of target genes using the IWGSC wheat sequence database (http://www.wheatgenome.org/), and then see if the sequences of these copies are polymorphic. It is usually necessary to clone and sequence target genes to acquire genomic and transcriptomic information (*see* **Note 1**).
- Designing SSNs for targeted wheat genes. There are two approaches to designing SSNs that target all the copies of a gene. One approach uses sequence alignment, with the SSN designed to target a conserved region of all the copies (Fig. 3a); the other involves designing different SSNs for each copy in the polymorphic region (specific region) (Fig. 3b). For specific targeting of one copy of a gene, the SSN is also designed for a specific region (Fig. 3b).
- To disrupt gene function, the target site should be located in an exon in the first 1/3–1/2 of the coding sequence. Cutting the gene should then lead to total loss of gene function. Possibly owing to the state of the chromatin of the target loci or the complex genomic background, certain SSNs are inefficient or can even fail to work. To obtain efficient SSNs and avoid repeating experiments, at least five SSNs per target locus should be constructed, and it is worth first testing the efficiencies of the SSNs in protoplasts.

3.2 TALEN Assembly

Because of the repetitive nature of TALEs, construction of the DNA binding monomers by traditional cloning techniques is difficult [16]. There are many methods for constructing TALEs, such as REAL [17] and REAL Fast Assembly [18], but they are not widely used in the laboratory. To generate a small number of TALE proteins in a typical academic laboratory, the Golden Gate Assembly platforms are particularly straightforward and user-friendly.

Golden Gate cloning is a method of assembling multiple DNA fragments in a single reaction. It employs type IIS restriction enzymes to create multiple sticky ends; cloning involves digestion and ligation in the same reaction mixture, and researchers can easily ligate up 2–10 TAL repeats in one reaction. The plasmid kit, 'Golden Gate TALEN 2.0', developed by the Voytas group is specifically designed for cloning TALE nucleases anywhere between 12 and 31 repeats in length [16]. The plasmid kit (kit # 1000000024) and a detailed protocol for assembly can be obtained from Addgene (http://www.addgene.org/). In this protocol, we use the Golden Gate cloning method to assembly TALENs.

3.2.1 Design of TALEN Target Sites

Conserved or specific gene sequences are submitted to TAL Effector–Nucleotide Targeter (TALE-NT) 2.0 soft (https://talent.cac.cornell.edu/) to select TALEN target sites and design arrays of TALEs. When designing a TALEN, the following key points should be considered:

- Since the wheat genome has a high GC content and contains many repeat sequences, target sites containing at least five consecutive identical nucleotides and a high GC content should be avoided; the four nucleotides should be distributed evenly over the TALEN binding sequence.
- The TALEN binding sequence should start with a T, but the last nucleotide is not restricted to a T.
- Each target site can be designated 5′-TN$^{15–18}$ N$^{15–18}$ N$^{15–18}$A-3′; the left TALEN targets the 5′-TN$^{15–18}$-3′ on the plus strand, and the right TALEN targets the 5′-N$^{15–18}$A-3′ on the minus strand (N = A, G, T or C).
- When targeting more homoeoalleles simultaneously, SNPs should be avoided in the conserved TALEN binding region, but can be present in the spacer.

  When targeting one specific copy of an allele, the TALEN binding sequence should have more than 50% polymorphism difference compared with the nontargeted copies.
- If the PCR/RE assay is used to detect TALEN construct activity and the mutations, a restriction enzyme site that is present in the spacer of all the gene copies needs to be selected. If using the T7E1 assay, there is no need to select the restriction.

3.2.2 TALEN Construction

Assembly of a custom TALEN or TAL effector construct is accomplished in 5 days (Fig. 2c) and involves two steps (Fig. 2a, b). The first step consists of ligating arrays of the first ten repeats and arrays of the rest of the repeats separately in array vectors, and the ligations then need to be tested by sequencing (Fig. 2a); the second step consists of ligating the two assembled repeat arrays and the last half

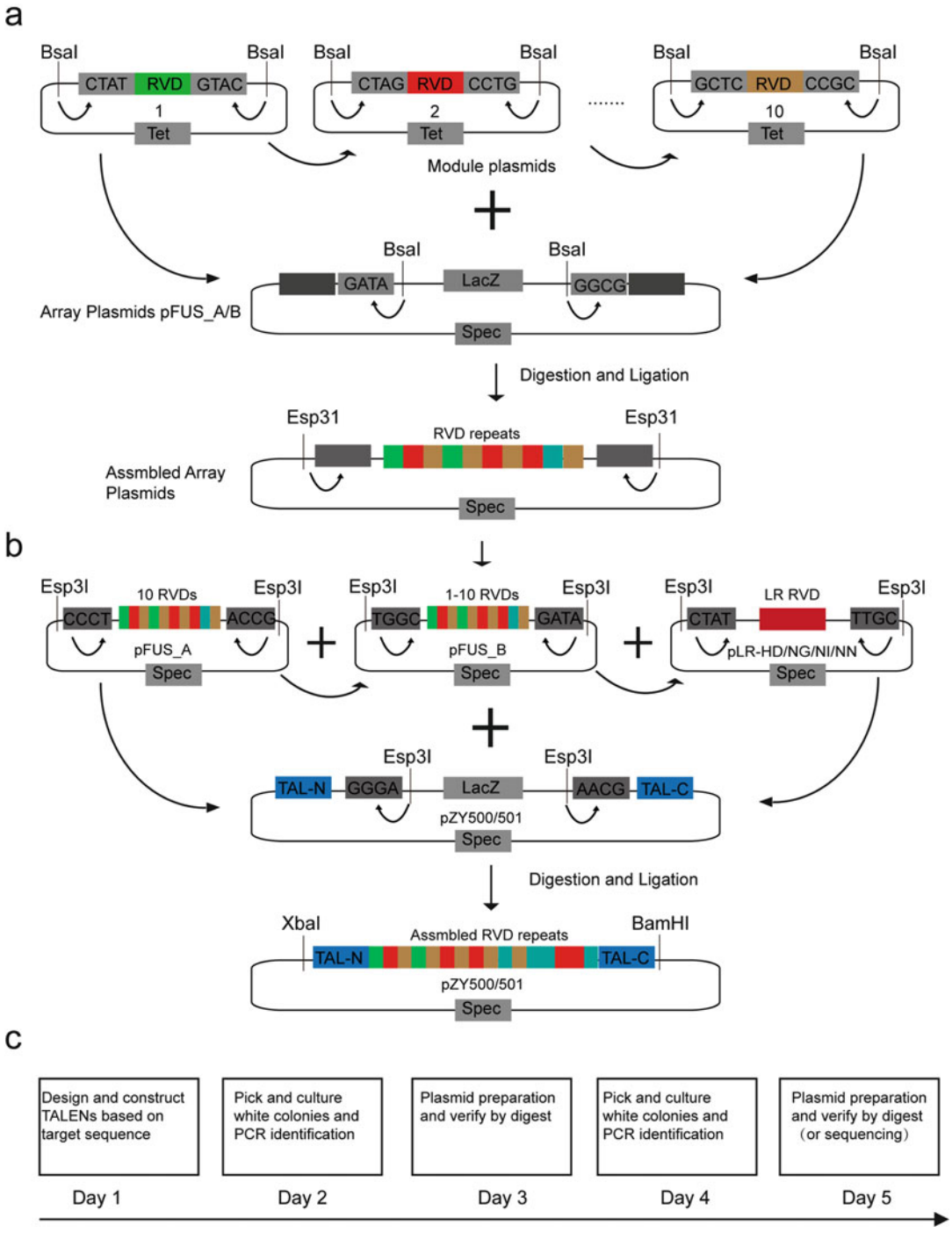

Fig. 2 Making TAL effector and TALEN constructs using the Golden Gate assembly method. (**a**) By using the type IIS restriction endonuclease *BsaI*, 4-bp complementary overhangs can be produced in the RVD plasmid and these should be ligated together once in the order 1–10. The ten modular plasmids recognizing the first ten target DNA base are cloned into the middle array vector pFUS-A. The modular plasmids recognizing the 11th to 20th target DNA base are cloned into the corresponding array vector pFUS-B. (**b**) By using the type IIS restriction endonucleases *Esp3I*, 4-bp complementary overhangs can be produced in the assembled array vector Pfus-A, pFUS-B and the LR RVD plasmid and these can be ligated together, and then ligated into a backbone plasmid (pZY500/501), which includes the truncation of the N-terminus and C-terminus. (**c**) Timeline of the assembly of TALEN and TAL effector constructs

repeat into the backbone vector that includes the truncated TAL-N and TAL-C (pZHY500 for the left TALE and pZHY501 for the right) [19] (Fig. 2b). The assembly of the TALEN requires a high quality TALEN plasmid library of RVDs. The detailed TAL effector arrays are constructed following the protocols in: http: //www.addgene.org/static/cms/files/Golden_TALEN_assembly_v6.pdf. We use as an example here the construction of a *TaMLO* TALEN monomer (T-MLO-L) with a 17 RVD array [20] (Fig. 4a).

- Day 1: Consider the RVD array HD NN HD NG NN HD NG NN HD NG HD NN HD HD NN NG, targeting the sequence 5′-CGCTGCTGCTCGCCGT-3′. Select from the module plasmids those that encode RVDs 1–10 in one array, and 11–16 in another array. The two ligation products are transformed into competent cells to construct pFUS-A [1–10] and pFUS-B [11–15] (Fig. 2a).
- Day 2: check for positive colonies of pFUS-A [1–10] and pFUS-B [11–15] by colony PCR. Start overnight cultures with confirmed clones (Fig. 2a).
- Day 3: Miniprep the plasmids of pFUS-A [1–10] and pFUS-B [11–15], and mix these two plasmids with the last repeat plasmid (pLR-NG) for ligation into pZY500/501 by the Golden Gate cloning method. Transform the ligation product into competent cells to construct pZY500-T-MLO-L (Fig. 2b).
- Day 4: check for positive colonies of pZY500-TMLO-L by colony PCR. Start overnight cultures with confirmed clones.
- Day 5: Miniprep pZY500-T-MLO-L to complete the TALEN assembly; the assembled TALEN can be used to construct the expression vectors for genome editing (Fig. 2b).

In this protocol, in order to transfer TALEN pairs into expression vectors in a straightforward way, we build the TALEN arrays into an intermediate Gateway entry vector pZHY013, which contains two obligate FokI heterodimer domains separated by a T2A translational skipping sequence (Fig. 3c). Figure 3 shows that the assembled TALE arrays are released from pZHY500 and pZHY501 by *XbaI/BamHI*, and subcloned into pZHY013 at the *XbaI/BamHI* site (for the left TALE) and at the compatible *NheI/BglII* site (for the right TALE) [19]. A Gateway LR reaction is performed to move the TALEN pair into the vector pYP010 to construct the TALEN expression vector, which can be used to transform protoplasts and immature embryos (Fig. 3c, e, g).

3.3 Design and Construction of sgRNAs

sgRNA target site selection should follow the procedures outlined above in Subheading 1 “Wheat targeted mutagenesis strategy”. Once a target site is selected, forward and reverse oligos can be

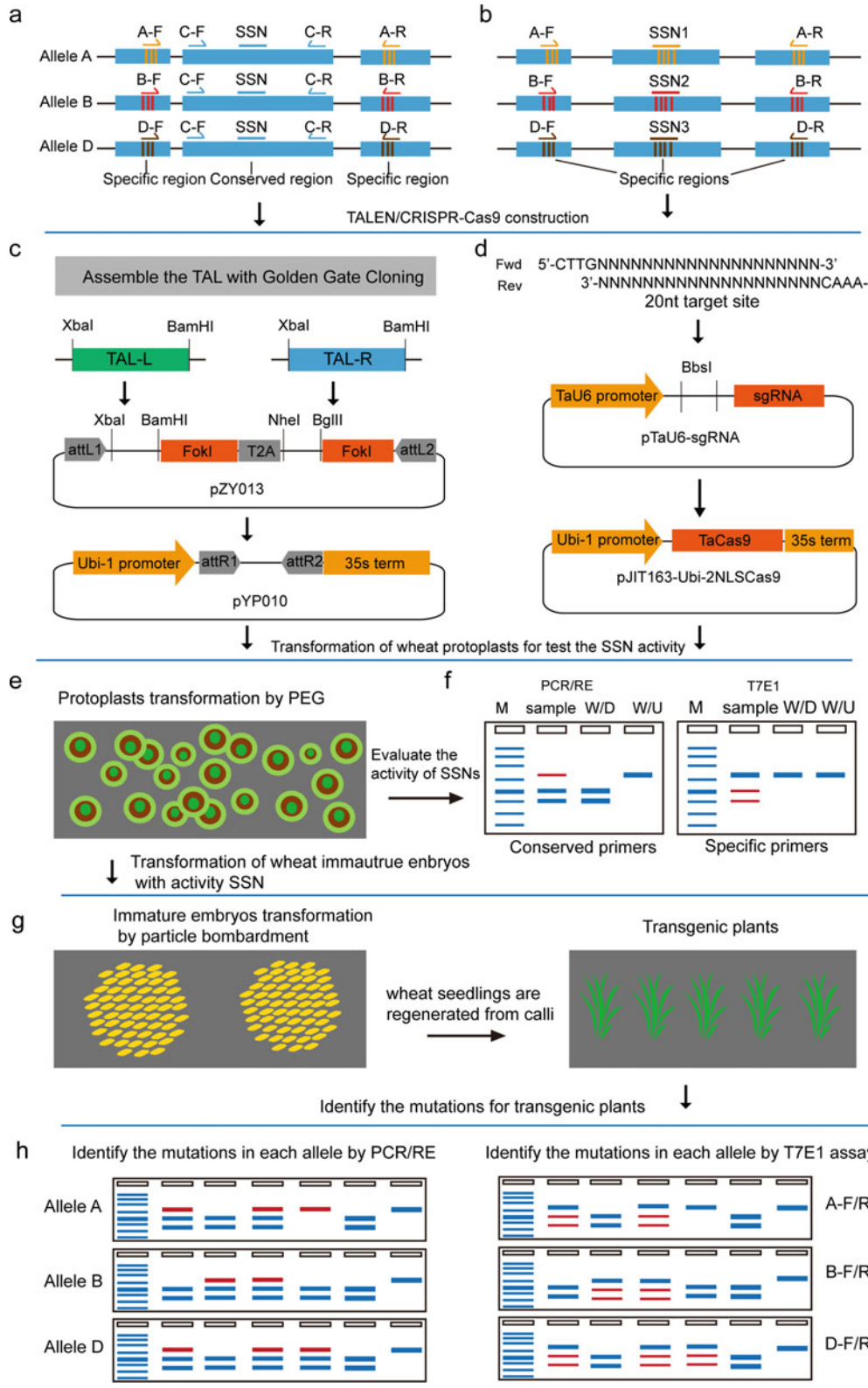

Fig. 3 Overview of wheat genome editing with TALENs and CRISPR/Cas9. (**a**) SSN design for multiple copy editing. (**b**) SSN design for editing individual alleles. (**c**) Schematic for constructing TAL arrays in intermediate vector pZY013 and expression vector pYP010. (**d**) Schematic for cloning wheat guide sequence oligos into a

designed and synthesized for insertion into the sgRNA scaffold vector (Fig. 3d). The oligos should consist of the 20-nt target site with 5′ and 3′ overhangs complementary to the digested scaffold vector (Fig. 3d). In this protocol, these should be *BbsI*-complementary overhangs, which can consist of any of the four bases. The TaU6 RNA polymerase III promoter used to express RNAs from the pTaU6-sgRNA vector is most effective with transcripts that start with an adenine (G) nucleotide, so an G is included immediately 5′ of the target sequence (the fourth of the four additional bases) (Fig. 3d). Primers should be designed and ordered as described in Fig. 3d. The oligo pairs for wheat should be: wheat-Fwd: 5′-CTTGN [17–20]-3′; wheat-Rev.: 5′-AAACN [17–20]-3′. When selecting target sites, the following key points should be taken into account:

- The 20-bp target sequence should immediately precede the 5′-NGG/NAG PAM, which is essential for Cas9 to bind to the target DNA. The 5′NGG should be considered first.
- Target sequences can be designed on both strands. The rule is: 5′-N [17–20]-NGG-3′ for template strand targeting; 5′-CCN-N [17–20]-3′ for complementary strand targeting.
- In order to reduce off-target effects, target sites should be chosen after searching the wheat genome sequence using a BLAST search (http://www.wheatgenome.org/) for the relevant 22-nt sequence—the 20-nt sgRNA-binding sequence plus the GG in the NGG PAM—to make sure that the target sequence is unique (*see* **Note 1**).
- If sgRNA activity is to be detected by the PCR/RE assay, restriction enzyme sites within the target sequences at the Cas9 endonuclease cutting site (3-bp upstream 5′-NGG) will facilitate detection.

Fig. 3 (continued) plasmid containing the sgRNA scaffold. Cotransformation of sgRNA plasmid pTaU6-sgRNA with Cas9 expression plasmid pJIT163-Ubi-2NLS-Cas9 is used for transient transformation of protoplasts and immature embryos. (**e**) Schematic for transformation of transient SSN plasmids into protoplasts. (**f**) Schematic of the PCR/RE and T7E1 assays used to detect indel mutations. For the PCR/RE assay, genomic DNA from SSN-transformed protoplasts or transgenic plants is amplified by PCR with conserved primers. The amplicons are then digested with restriction enzymes that recognize the wild-type target sequences. Mutations introduced by NHEJ are resistant to restriction enzyme digestion because of the loss of the restriction sites, and they result in uncleaved bands (*red band*) in agarose gels. For T7E1, genomic DNA from SSN-transformed protoplasts or transgenic plants is amplified by PCR with specific primers. The amplicons are then denatured and annealed in a thermocycler to generate heteroduplexes. The heteroduplexes can be cleaved by T7EI (*red band*), whereas homoduplexes remain intact. W/D, wild-type digestion. W/U, wild-type no digestion. (**g**) Schematic for stable transformation of SSN plasmids into immature embryos. Transgenic plants obtained from resistant calluses. (**h**) Schematic of the PCR/RE and T7E1 assays used to detect mutations in transgenic plants. Primers specific for each allele are used to amplify the target site region and identify mutations and genotypes

The Cas9 sequence is constructed in another vector, pJIT163 (Fig. 3d). It is driven by the Ubi-1 promoter, and the terminator is 35SCaMV. So two constructs, pJIT163-Ubi-2NLS-Cas9 and pTaU6-sgRNA, are used for transforming wheat protoplasts and wheat immature embryos [20] (Fig. 3d, e, g).

3.4 Evaluation of SSN Activity in Wheat Protoplasts

To validate and measure the activity of SSNs rapidly, the TALEN construct (pYP010-TALEN) can be transformed directly into protoplasts (Fig. 3c), and pTaU6-sgRNA can be cotransformed with pJIT163-Ubi-2NLS-Cas9 into protoplasts for transient expression [20] (Fig. 3d).

3.4.1 Plant Preparation

- Wheat cultivar: all spring wheat and winter wheat cultivars.
- Plant growth conditions: grow the seeds at 25 °C with a photoperiod of 16 h of light and 8 h of dark for ~7–14 days.
- Protoplast isolation.

Usually 1×10^7 protoplasts can be isolated from 20–30 seedlings, and they can be used in 20 separate transformations with 5×10^5 cells per transformation.

Bundle together the stems and sheaths of ~30 wheat seedlings. Cut them into latitudinal strips (by transecting) of ~0.5 mm width using very sharp razor blades. Transfer the strips into petri dishes with 0.6 M mannitol, and incubate them for 10 min in the dark for quick plasmolysis. Filter through nylon meshes, transfer the strips into a 150-ml conical flask containing 50 ml of filter-sterilized enzyme solution and wrap the flask in aluminum foil. Vacuum-infiltrate the strips with cell wall–dissolving enzymes by applying a vacuum (~380–508 mmHg) for 30 min in the dark. Next, incubate the strips in the dark for 5–6 h with gentle shaking (60–80 rpm) at room temperature (RT). After enzymatic digestion, add 50 ml of W5 solution to the conical flask, and shake it gently by hand for 10 s to release the protoplasts.

Collect the protoplasts into three or four 50 ml round-bottomed centrifuge tubes after filtering the mixture through 40-μm nylon mesh. Centrifuge at $100 \times g$ for 3 min at RT in a swinging bucket rotor and remove the supernatant by pipetting. Resuspend the protoplasts in 10 ml of W5 solution and put it on ice for 30 min to let the protoplasts settle. Remove the supernatant by pipetting and resuspend the protoplasts in 4 ml of MMG solution at a final concentration of 2.5×10^6 cells per ml. Optional: Determine the protoplast concentration under a microscope (×100) with a hemocytometer.

3.4.2 PEG-Mediated Transformation of Protoplasts

Prepare the transformed plasmids in a 2-ml microcentrifuge tube. For TALEN transformation, add 20 μg pYP010-TALEN plasmids. For CRISPR/Cas9 transformation, add 20 μg of pTaU6-sgRNA and 20 μg of pJIT163-2NLS-Cas9.

Add 200 μl of protoplasts (5×10^5 cells) and mix gently by tapping the tube. Add 250 μl of freshly prepared PEG solution and mix thoroughly by gently tapping the tube. Incubate the mixture for 20 min in the dark. Add 800 μl of W5 solution to the tube and mix well by inverting the tube to stop the transformation process. Centrifuge at 100 × *g* for 3 min at RT. Remove the supernatant. Protoplasts should be resuspended in 2 ml of W5 solution. Transfer the protoplasts to six-well plates (12-well or 24-well plates can also be used for larger-scale experiments). Wrap the plates in aluminum foil and incubate them at 23 °C for at least 48 h.

In every transformation, a pJIT163-Ubi-GFP positive control is used to evaluate the transformation efficiency. After 48 h incubation, the protoplasts should be collected into 2 ml tubes for extraction of genomic DNA, which is used to detect mutations in the target site.

3.5 Identifying SSN-Induced Mutations

SSN-induced mutagenesis usually consists of the introduction of small insertions or deletions (indels) into the target sequences. Below we briefly describe PCR/RE and T7E1 assays strategies for identifying the mutations in wheat [21] (Fig. 3f, h).

- For the PCR/RE assay, a prerequisite is that the target locus includes a restriction enzyme site; if there is an SSN-induced mutation in the target site, the restriction enzyme site will be destroyed and the mutant amplicon will be resistant to restriction enzyme digestion and produce uncleaved bands in agarose gel electrophoresis (Fig. 3f). Such mutant bands can be further characterized by subcloning and sequencing. When targeting several genes, conserved primers can be used for the PCR amplification and specific primers can be used in the PCR/RE assay (Fig. 3f). This assay can be used to detect mutations in wheat protoplasts and identify mutations in transgenic plants (Fig. 3f, hprerequisite is that the target). Examples of detecting *MLO* mutations with conserved primers and specific primers are shown in Fig. 4a, b, respectively.
- An alternative way to identify mutations is using T7E1, which recognizes and cleaves double-stranded DNA at mismatched sites; for this approach, specific primers need to be designed to amplify the target locus (Fig. 3f, h). The primers are designed to amplify 300–1000 bp surrounding the target site. The PCR products (a mixture of wild type allele and mutant allele) are then denatured and renatured, forming heteroduplexes. The reaction products are digested with T7E1 nuclease, and then analyzed by 2.0% (wt/vol) agarose gel electrophoresis. For example, designing a specific sgRNA to target *TaMLO-A1*, a specific primer is used for PCR amplification, and the T7E1 digestion result shows that the mutation only occurred in the genome A locus (Fig. 4d–f).

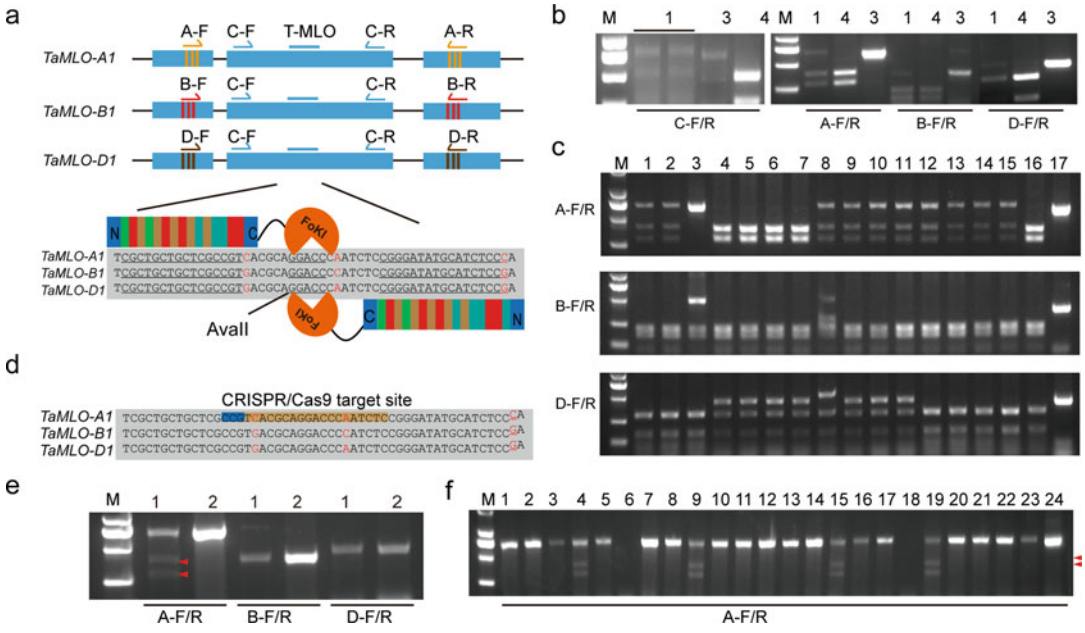

Fig. 4 Example of modification of the *MLO* gene using TALEN and CRISPR/Cas9 technologies. (**a**) Schematic of TALEN target site design to edit three copies of wheat *TaMLO* homoeologs, and primers design. The TALEN-targeted sequences in *MLO-A1*, *MLO-B1*, and *MLO-D1* are underlined, and the *AvaII* restriction site in the spacer for mutation detection is indicated in *blue*. (**b**) Detection of TALEN activity in protoplasts by the PCR/RE method. C-F/R, conserved primers for amplifying the *MLO* target site; A-F/R, B-F/R, and D-F/R, specific primers for amplifying the individual *MLO* target sites in the three subgenomes. (**c**) Outcome of the PCR/RE assay to detect TALEN-induced mutations in 15 representative T0 transgenic wheat plants. (**d**) Site within *MLO-A1* targeted by CRISPR/Cas9. The three SNPs are highlighted in *red*. (**e**) T7E1 assay to detect sgMLO-A1-induced mutations in protoplasts and transgenic plants. A mutation was only detected in genome A in protoplasts (*left figure*) and transgenic plants (*right figure*)

3.6 Transformation of Immature Embryos

Varieties of wheat plant are grown in the field under normal field management conditions. Young caryopses are threshed from spikes 10–14 days post anthesis. They are first surface-sterilized by soaking for 1 min in 70% (v/v) ethanol followed by 2% sodium hypochlorite for 15 min at 28 °C, and then shaken and rinsed in sterile water. Immature embryos (IEs) are isolated under aseptic conditions after removal of the axis.

- Embryos are transformed with the SSNs by the biolistic method. For stable wheat transformation, pYP010-TALEN and a bar selection marker plasmid pAHC20 are cotransformed into immature embryos by the biolistic method. pTaU6-sgRNA, pJIT163-Ubi-2NLS-Cas9, and pAHC20 are mixed at 1:1:1 and cotransformed into immature embryos (Fig. 3g). The protocol for biolistic transformation is based on previous work [22]. We use a PDS1000/He particle bombardment system (Bio-Rad) with a target distance of 6.0 cm from the stopping plate at helium pressure 1100 psi, as previously described.

- Culture media and explant tissue conditions: After bombardment, embryos are transferred to callus induction medium. In the third or fourth week, all calli are transferred to selective regeneration medium containing 5 mg/l phosphinothricin (PPT). PPT is present in all subsequent tissue culture procedures including 2 rounds of regeneration (4 weeks) and two rounds of rooting (4 weeks). After 10–12 weeks, T0 transgenic plants are obtained, transferred into soil, and grown in a greenhouse.

3.7 Screening and Identifying SSN-Modified Wheat Plants in T0 Generation

After obtaining the transgenic plants, use the leaf tissues of the primary transgenic (T0) plants to extract genomic DNA by the DNA quick extract plant system or other methods. PCR amplify the relevant region centered on the SSN target site with conserved primers or specific primers and the genomic DNA as template (Fig. 3h).

- If the target sites include a restriction enzyme site, the sites for each copy are first amplified from the genomic DNA of T0 plants using conserved primers and analyzed by the PCR/RE assay to detect potential mutations. Next, in order to identify which gene copies had been mutated, specific primers needed to amplify each individual copy are used and mutations detected using PCR/RE (Fig. 3h). Confirmation of the genotype at each target site is then obtained by sequencing. For example, an *AvaII* restriction site is present in the middle of the spacer region of *TaMLO* [20], and the corresponding restriction enzyme *AvaII* is useful for initially screening T0 plants for mutations (Fig. 4a).
- If the target site does not contain a restriction enzyme site, mutations are detected by the T7E1 method. First, each target copy is amplified with specific primers. Next, the PCR products are digested with T7E1, and mutated PCR products are cloned into the TA-vector for confirmation by sequencing. As an example, we show the T7E1 assay used to identify mutations induced by CRISPR/Cas9 targeting of *TaMLO-A1* in transgenic wheat plants (Fig. 4f).

3.8 Identifying the Homologous Mutations in Progeny

Harvest seeds from the T0 plants and break their dormancy by incubation at 37 °C for 7 days. Grow the T1 progeny and determine the genotypes of individual plants by PCR/RE with specific primers or T7E1 digestion and sequencing.

- In the PCR/RE assay, the amplicons of homozygous mutants should not be cleaved by the restriction enzyme, and the amplicons of heterozygous mutants should form three bands (the mutated band, the other two digested wild type bands) (Fig. 3f). Purify the uncleaved band for cloning and sequencing.
- For the T7E1 assay, the process can be divided into two steps. First, to screening all the mutant plants in progeny, the PCR

product with a mixture of wild type allele and mutant allele should be tested. When the mutation occurs, the sample can be partially digested with three bands. Next, to identifying whether the heterozygous or homozygous mutants happening, the PCR product with only mutant allele should be used. A totally undigested sample (only one band) indicates the presence of a homozygous mutation; partial digestion (three bands) implies the presence of a heterozygous mutation (Fig. 3f). The mutated PCR products should be cloned for sequencing.

- If no homozygous mutants are obtained in the T1 generation, heterozygotes should be self-pollinated and homozygotes detected among the progeny, using the methods described above (*see* **Notes 2–4**).

4 Notes

1. The wheat sequence database IWGSC (http://www.wheatgenome.org/) does not provide a high-quality hexaploid wheat sequence, but it is constantly updated. So it may not contain the complete sequence of some targeted genes. Hence it is usually necessary to clone and sequence the genes to acquire genomic and transcriptomic information.
2. Some wheat genes may exist in more than three copies. The strategy for knocking out such genes is: (1) Analyze their sequences and classify them into subgroups based on their conserved regions; (2) Design different SSNs for the conserved subgroup regions; (3) Test the activities of these SSNs in protoplasts and then co-transform them into immature embryos by the biolistic method or Agrobacterium-mediated transformation; (4) Identify the mutations using PCR/RE or T7E1 with specific primers for amplification.
3. The three alleles of a given gene may be functionally redundant or have different functions. So, in order to observe the mutant phenotypes, all the homozygous mutants (*aa*, *bb*, *dd*, *aabb*, *aadd*, *bbdd*, and *aabbdd*) should be isolated and examined.
4. To identify mutations in each copy, specific primers are needed, and the genotype of each mutant needs to be confirmed by sequencing.

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Chapter 12

Design and Assembly of CRISPR/Cas9 Reagents for Gene Knockout, Targeted Insertion, and Replacement in Wheat

Tomáš Čermák and Shaun J. Curtin

Abstract

Advances in cereal transformation along with the completion of the wheat genome sequence assembly have increased the demand for tools that perform targeted and specific modifications in this crop plant. This protocol demonstrates the construction of reagents using a comprehensive genome engineering kit to create single and multiple gene "knockouts," site-specific chromosome deletions and gene replacement or "knockins" including the use of geminivirus replicons (GVRs). The reagents allow for both easy construction of simple genome engineering vectors, and "mix and match" swapping of components such as the Cas9, guide RNA and donor template cassettes for gene targeting. In addition, a web-based tool greatly streamlines vector selection, primer design, and vector construction.

Key words Wheat, Genome editing, CRISPR/Cas9, GVRs, Replicons, Gene knockout, Chromosome deletion, Gene insertion, Gene replacement

1 Introduction

Most genome engineering approaches rely on the induction of double-strand breaks (DSBs) to create sequence modifications at targeted genetic loci [1–3]. The DSB is rapidly repaired by one of the host's DNA repair pathways, the nonhomologous end-joining (NHEJ) or the homology-directed repair (HDR). NHEJ repair is an error-prone process that results in imprecise ligation of the DSB leading to nucleotide insertion/deletions (indels) at the target site. Frame-shift mutations often result in disruption of gene function (gene knockout). While indel mutations are readily induced by targeted DSBs, gene inactivation is not always the desired type of modification. The most efficient way to induce custom base substitutions, targeted insertions, or other types of precise modifications requires taking advantage of HDR, which uses a DNA template with homology to the site of the break (a so-called "donor") to copy information and repair the break. If a donor DNA molecule is co-transformed into a plant cell along with the

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DSB-inducing reagent, then modifications incorporated into the donor template can potentially be copied into the target site by HDR in an approach known as gene targeting (GT). However, GT can be challenging due to the fact that the dominant type of DNA repair in plant cells is NHEJ, and HDR is much less frequent. Therefore, several strategies have been developed to increase the rates of GT in plants. One of them, called *in planta* GT [4], is based on stable insertion of the donor template into the plant genome to ensure that at least one copy is present in each cell. The next step is to excise the donor DNA from the transgene by a site-specific nuclease, making it available for the HDR. Another method improves the availability of the donor template by replicating it into a high copy number on a non-integrating geminivirus replicon (GVR) [5] and can be used to create GT plants without the need for stable integration of vector DNA in the plant genome [6]. This approach has recently been shown to efficiently induce precise modifications in wheat cells [7].

The CRISPR/Cas9 platform is the most recent and commonly used reagent for induction of targeted DSBs. It is distinct from the protein-only based zinc finger nuclease (ZFN) [8] and TAL effector nuclease (TALEN) [9–11] platforms in that it consists of the Cas9 protein with DNA cleavage activity and a single-stranded guide RNA (gRNA) sequence conferring target specificity [12, 13]. The 20 bp part of the gRNA sequence on the 5′ end referred to as the gRNA spacer, anneals to the target DNA based on complementarity of bases, adjacent to an NGG motif referred to as the PAM (protospacer adjacent motif). The gRNA sequence is often supplied from a separate transcription unit delivered to the cell along with the Cas9 expression cassette and controlled by an RNA polymerase III promoter, such as the U6 or U3 promoter [14–16]. This is an efficient approach to induce single gene knockouts. To target multiple sites, several gRNAs can be delivered simultaneously. Efficient expression systems have been developed to process transcripts containing arrays of gRNA sequences into individual functional gRNAs by the use of RNA cleaving enzymes such as the Csy4 ribonuclease [17, 18]. CRISPR/Cas9 therefore offers many advantages over ZFNs and TALENs including easy re-targeting to new DNA sequences and multiplex capabilities. To date, CRISPR/Cas9 has been used to edit the genomes of a wide range of organisms including human cells, *Caenorhabditis elegans*, zebrafish, *Drosophila*, mouse, and rat, as well as many plant species, including several crops; *Arabidopsis*, tobacco, soybean, *Medicago*, tomato, rice, wheat, maize, and sorghum [14–16, 19–29].

A recently developed toolkit combines the flexibility and high efficiency of CRISPR/Cas9 with other gene editing tools such as multiplex gRNA expression systems and GVRs for gene editing in plants [27]. There are two main types of reagents in this kit designated “Direct” and “Modular” cloning vectors (Fig. 1). The Direct

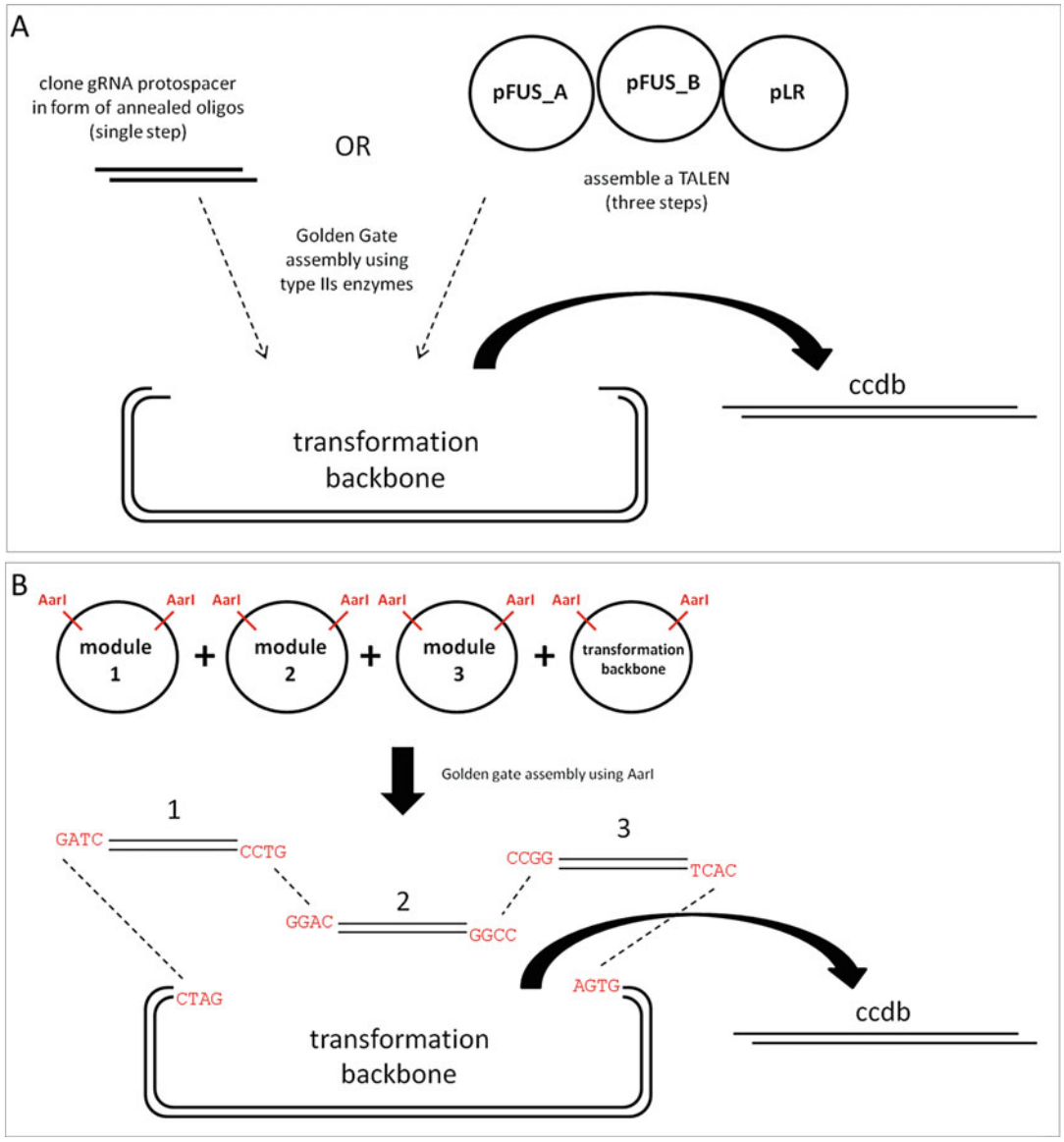

Fig. 1 Two approaches for construction of genome engineering reagents. (**a**) Direct cloning vectors are designed to speed up the cloning process as gRNAs are directly cloned into a transformation backbone (e.g., T-DNA). (**b**) Modular Cloning Vectors enable combination of different functional elements such as gRNAs and donor templates. Custom-selected modules are then assembled together into the transformation backbone

vectors offer the easiest and the quickest way to create CRISPR/Cas9 constructs for gene inactivation or repression. All essential components (selectable marker gene, Cas9, etc.) are already combined in the Direct vector backbones that are ready for transformation after the gRNA spacer sequence(s) are inserted using a single step protocol (Fig. 1a). For all other applications including gene targeting using GVRs, the Modular vectors are used to combine

different components in a two-step protocol (Fig. 1b). Three vector sets designated Module A, Module B and Module C and a set of Transformation Backbones are available for the modular assembly approach. In this approach, some components are available as ready-to-use plasmids, such as the Cas9 expression cassettes in Module A plasmids. gRNA expression cassettes are prepared by inserting the spacer sequence(s) for one or more gRNAs into a Module B plasmid and repair templates for gene targeting can be inserted into a Module C plasmid. In addition, other genes such as GFP are available in specific Module plasmids. The components of Module A, B, and C plasmids are combined in a Transformation Backbone of choice using Golden Gate cloning (Fig. 1b).

This protocol details the design and construction of CRISPR/Cas9 vectors for gene knockout and gene targeting in wheat using our comprehensive genome engineering toolset and should be used in conjunction with the original publication and its associated website (http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/) [27]. While the focus of this protocol is on CRISPR/Cas9 and wheat, a more extensive collection of reagents can be viewed on this website including TALEN-based protocols and tools optimized for other plant species [27].

2 Materials

2.1 General Materials for Use with the Genome Engineering Toolkit

2.1.1 Vectors

1. Vectors from the multipurpose plant genome engineering kit [27]—available from Addgene, Cambridge, MA, USA (http://www.addgene.org/).

2.1.2 Enzymes and Buffers

1. Proofreading DNA polymerase and buffer.
2. Taq DNA polymerase and buffer.
3. Restriction enzyme AarI and AarI oligonucleotide.
4. Restriction enzyme BaeI.
5. Restriction enzyme BanI.
6. Restriction enzyme BsaI.
7. Restriction enzyme Esp3I.
8. Restriction enzyme SapI.
9. T7 DNA ligase and 2× T7 ligase buffer.
10. T4 DNA ligase and 10× T4 DNA ligase buffer.
11. T4 polynucleotide kinase + T4 DNA ligase buffer (contains ATP).
12. 1.33× Gibson assembly master mix: 2 μl T5 exonuclease (1 U/μl), 6.25 μl Phusion DNA polymerase (2 U/μl), 50 μl Taq

DNA ligase (40 U/μl), 100 μl 5× isothermal buffer (25% PEG-8000, 500 mM Tris–HCl pH 7.5, 50 mM MgCl2, 50 mM DTT, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 5 mM NAD), H_2O to 375 μl.

2.1.3 Stock Solutions and Media

1. SOC medium: 5 g/l yeast extract, 20 g/l tryptone, 20 mM dextrose, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride.
2. 50 mg/ml carbenicillin stock. Ampicillin may be used in the place of carbenicillin.
3. 50 mg/ml kanamycin stock.
4. 50 mg/ml spectinomycin stock.
5. LB plates/liquid media with 50 mg/l carbenicillin (ampicillin), 50 mg/l kanamycin or 50 mg/l spectinomycin.
6. 40 mg/ml X-gal dissolved in either dimethylsulfoxide or *N*′,*N*-dimethylformamide.
7. 100 mM IPTG (isopropyl β-d-1-thiogalactopyranoside) dissolved in water and filter-sterilized.

2.1.4 Other Supplies

1. dNTPs.
2. S-Adenosyl methionine (SAM).
3. Gel electrophoresis equipment.
4. QIAprep Spin Miniprep Kit (Qiagen).
5. QIAquick Gel Extraction Kit (Qiagen).
6. Software for DNA analysis and plasmid map viewing.

2.1.5 Cell Strains

1. *E. coli* DH5α chemically competent cells.
2. *E. coli* ccdB resistant strain chemically competent cells (for propagation of certain cloning vectors).

2.2 Materials for Single Gene Knockout Constructs

1. Vectors from the multipurpose plant genome engineering kit [27]—available from Addgene, Cambridge, MA, USA (http://www.addgene.org/).
2. Two complementary oligos containing the gRNA target sequence.
3. Restriction enzyme Esp3I (Module vectors) and/or AarI + AarI oligonucleotide (Direct vectors and assembly of Modules A + B + C).
4. T4 polynucleotide kinase + T4 DNA ligase buffer (contains ATP).
5. T4 DNA ligase and 10× T4 DNA ligase buffer.
6. Gel electrophoresis equipment.

7. DH5α chemically competent cells.
8. SOC medium.
9. 50 mg/ml carbenicillin and/or kanamycin or spectinomycin stock (depending on type of the vector).
10. 40 mg/ml X-gal dissolved in either dimethylsulfoxide or *N*′,*N*-dimethylformamide (only for Direct vectors).
11. 100 mM IPTG (isopropyl β-d-1-thiogalactopyranoside) dissolved in water and (only for Direct vectors).
12. LB liquid medium with 50 mg/l carbenicillin (ampicillin) and/or kanamycin or spectinomycin (depending on type of vector).
13. QIAprep Spin Miniprep Kit (Qiagen).
14. Primers for sequencing:

    ZY015F: 5′-GGAATAAGGGCGACACGGAAATG (Module vectors).

    NB463: 5′-CGAACGGATAAACCTTTTCACG (Direct vectors).

    TC430: 5′-GTTGGATCTCTTCTGCAGCA (for final constructs created by the modular approach).

2.3 Materials for Chromosome Deletion and Multigene Knockout Constructs

1. Vectors from the multipurpose plant genome engineering kit [27]—available from Addgene, Cambridge, MA, USA (http://www.addgene.org/).
2. Primers designed using the online tools available at http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/.
3. Proofreading DNA polymerase and buffer.
4. dNTPs.
5. Restriction enzyme BanI.
6. Gel electrophoresis equipment.
7. QIAquick Gel Extraction Kit (Qiagen).
8. Restriction enzyme Esp3I or BsaI (Module vectors) and/or AarI + AarI oligonucleotide (Direct vectors and assembly of Modules A + B + C).
9. Restriction enzyme SapI.
10. T7 DNA ligase and 2× T7 DNA ligase buffer.
11. DH5α chemically competent cells.
12. SOC medium.
13. 50 mg/ml carbenicillin and/or kanamycin or spectinomycin stock (depending on type of vector).
14. LB liquid medium with 50 mg/l carbenicillin (ampicillin) and/or kanamycin or spectinomycin (depending on type of vector).
15. Taq DNA polymerase and buffer.

16. QIAprep Spin Miniprep Kit (Qiagen).
17. Primers for colony PCR and sequencing:

    TC306: 5′-AGCACTACCAATGATGACCT (Module vectors with PvUbi1 promoter).

    TC320: 5′-CTAGAAGTAGTCAAGGCGGC (Module vectors with CmYLCV promoter).

    TC089R: 5′-GGAACCCTAATTCCCTTATCTGG.

    M13F: 5′-GTAAAACGACGGCCAGT (Direct vectors).

2.4 Materials for Gene Insertion and Replacement Constructs

1. Vectors from the multipurpose plant genome engineering kit [27]—available from Addgene, Cambridge, MA, USA (http://www.addgene.org/).
2. Module vectors created using the protocols for single or multi-gene knockouts, carrying gRNA(s) targeting the site of interest.
3. Primers for amplification of the DNA template for gene targeting.
4. Restriction enzyme BaeI.
5. S-Adenosyl methionine (SAM).
6. Gel electrophoresis equipment.
7. QIAquick Gel Extraction Kit (Qiagen).
8. Proofreading DNA polymerase and buffer.
9. dNTPs.
10. 1.33× Gibson assembly master mix.
11. DH5α chemically competent cells.
12. SOC medium.
13. 50 mg/ml carbenicillin and/or kanamycin or spectinomycin stock (depending on type of vector).
14. LB liquid medium with 50 mg/l carbenicillin (ampicillin) and/or kanamycin or spectinomycin (depending on type of vector).
15. QIAprep Spin Miniprep Kit (Qiagen).
16. Restriction enzyme AarI and AarI oligonucleotide.
17. T4 DNA ligase and 10× T4 DNA ligase buffer.
18. Primers for colony PCR and sequencing:

    ZY015F: 5′-GGAATAAGGGCGACACGGAAATG.

    M13F: 5′-GTAAAACGACGGCCAGT.

    TC430: 5′-GTTGGATCTCTTCTGCAGCA.

    CS433: 5′-GCTGGCCTTTTGCTCACATGTTCTTTCCTGCG.

3 Methods

3.1 Design and Construction of Reagents for Single Gene Knockouts

This protocol describes cloning of a single gRNA spacer under the control of an RNA Pol III promoter in either Module B or Direct vectors. The approach consists of Golden Gate assembly of annealed oligonucleotides containing the gRNA spacer sequence and does not require a separate plasmid digestion and purification step. The Direct vectors with the cloned gRNA spacer can be used directly for *Agrobacterium*/plant transformation to induce single gene knockouts. The gRNA unit in Module B vectors can be further combined with the Cas9 expression cassettes in Module A and additional elements (such as a gene targeting donor or GFP expression cassettes) in Module C with a Transformation Backbone of choice in another Golden Gate cloning step (**steps 13–17** of this protocol). A timeline for construction of Modular and Direct vectors is shown in Fig. 2.

1. Select appropriate vectors using the "Vector Selection" or "List All Vectors" options on the Voytas Lab Plant Genome Engineering Toolkit website (http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/vector.php). When using the "Vector Selection" tool, start by selecting "Direct Cloning Backbone" from the drop-down menu in **step 1**. In **step 2**, select "T-DNA

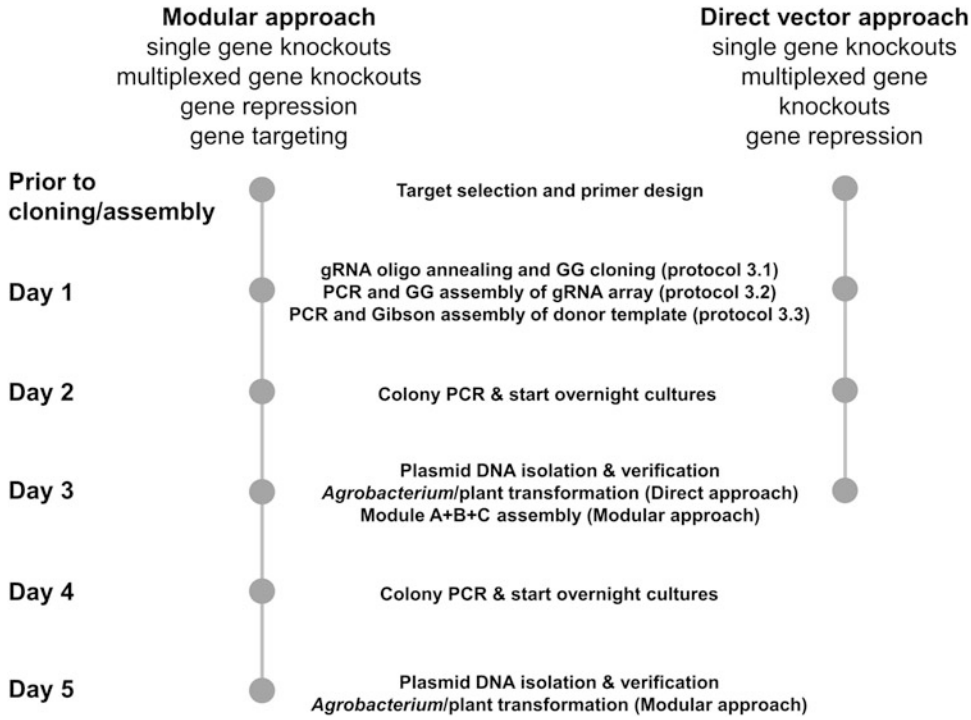

Fig. 2 Timeline for construction of the Direct and Modular vectors. *GG* Golden Gate

(pCAMBIA based, kanamycin for bacterial selection)". In **step 3**, select appropriate Plant Selectable Marker (*see* **Note 1**). In **step 4** select "ZmUbi:TaCas9 + TaU6 gRNA" (*see* **Note 2**) and then submit your selection below. Next page will show the name of the cloning vector along with a link to download the annotated vector sequence. If additional components, such as GFP or Trex2 (*see* **Note 3**) are desired in the final expression vector, the modular cloning approach can be used to accommodate these additional components. In this approach, the gRNA target sequence is inserted into a Module B vector instead of the Direct Cloning Backbone. Use pMOD_B2517 or pMOD_B2518 as the Module B vector (*see* **Note 4**) for this purpose. In addition, Module A carrying the Cas9 gene, Module C with the additional component and a Transformation Backbone will be needed in this approach. For Module A, use pMOD_A1110, Module C and the Transformation Backbone can be selected using the "Vector Selection" tool based on your preferences.

2. Order selected plasmids from Addgene and purify plasmid DNA from 5 ml LB cultures using the QIAprep Spin Miniprep Kit (*see* **Note 5**).
3. Select targets for gene knockout. For a single gRNA, this will be any N_{20}NGG site, although the RNA polymerase III promoters used for gRNA expression in this protocol might pose constraints on the first nucleotide of the target site (*see* **Note 6**). Sequences containing AarI of Esp3I binding sites should be avoided. Online tools such as sgRNA Scorer (https://crispr.med.harvard.edu/) [30] and CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) [31, 32] can be used for target site selection, prediction of on-target activity and identification of potential off-targets (*see* **Note 7**).
4. Design two complementary oligonucleotides as shown in Fig. 3. Replace the X symbols with the first 20 bases of the selected gRNA target site (N_{20}, not including NGG). Replace the Y symbols with the complementary sequence. Make sure

TaU6 sense gRNA oligo
ACTTXXXXXXXXXXXXXXXXXXXX
TaU3 sense gRNA oligo
AAGCXXXXXXXXXXXXXXXXXXXX

antisense gRNA (TaU6 & TaU3)
AAACYYYYYYYYYYYYYYYYYYYY

Fig. 3 Complementary oligonucleotides for insertion of a single gRNA spacer into TaU6 and TaU3 gRNA units in Direct and Module vectors. Promoter-specific sequence overhangs highlighted in red are added to each oligonucleotide. X and Y symbols are replaced with the first 20 bases of the selected gRNA target site and a complementary sequence, respectively

the specific 4 bp overhangs match the selected promoter as in Fig. 3.

5. Phosphorylate the oligonucleotides. Prepare the following reaction:
   - 3 μl 10× T4 DNA ligase buffer (*see* **Note 8**).
   - 3 μl 100 μM sense gRNA oligonucleotide.
   - 3 μl 100 μM antisense gRNA oligonucleotide.
   - 2 μl T4 polynucleotide kinase.
   - 19 μl H_2O.

   Incubate at 37 °C/1 h.
6. Anneal the phosphorylated oligonucleotides. Place the reaction from **step 5** in a PCR machine and run the following program: 95 °C/5 min + ramping down to 85 °C at −2 °C/second + ramping down to 25 °C at −0.1 °C/second + 4 °C hold (*see* **Note 9**).
7. Dilute the reaction 25 times (1 μl phosphorylated oligo mixture + 24 μl H_2O).
8. Insert the annealed oligonucleotides into the selected Module B or Direct Cloning Backbone via Golden Gate cloning. The Golden Gate Reaction will include:
   - 2 μl 10× T4 DNA ligase buffer.
   - 50 ng of selected Module B or Direct Cloning Backbone plasmid.
   - 1 μl of 25× diluted phosphorylated and annealed oligo mixture.
   - 0.5 μl Esp3I if using a Module B **OR** 0.5 μl AarI plus 0.4 μl AarI oligonucleotide if using a Direct Cloning Backbone.
   - 1 μl T4 DNA ligase.
   - H_2O up to 20 μl.
9. Place the Golden Gate reaction in a PCR machine and run the following program: 37 °C/5 min + 16 °C/10 min + 37 °C/15 min + 80 °C/5 min.
10. Transform 5 μl of the Golden Gate reaction into *E. coli*. If using a Module B, DH5α or similar *E. coli* strain sensitive to the presence of ccdB gene should be used and the reaction plated on LB + 50 mg/L ampicillin/carbenicillin. If using a Direct Cloning Backbone, plate on LB + 50 mg/L kanamycin supplemented with X-gal and IPTG. Incubate at 37 °C overnight.
11. On the following day, inoculate 2–3 white colonies (*see* **Note 10**) into 5 ml of LB supplemented with 50 mg/l ampicillin/carbenicillin if using a Module B **OR** LB + 50 mg/L

kanamycin if using a Direct Cloning Backbone. Incubate and shake overnight at 220 rpm, 37 °C.

12. On the following day, purify plasmids from the LB cultures and verify by sequencing using the primer ZY015F for a Module B or NB463 for a Direct Cloning Backbone. If a Direct Cloning Backbone was used, the protocol ends here and the vector is ready for *Agrobacterium*/plant transformation. For a Module B, follow **steps 13–17**.
13. The Module B vector created in **steps 1–12** can be used in the assembly of vectors for gene targeting (*see* the protocol in Subheading 3.3) or assembled into a Transformation Backbone of choice (*see* **Note 11**) along with the selected Module A (for Cas9, use pMOD_A1110) and Module C plasmids (*see* **Note 12**). For Golden Gate assembly of Modules A + B + C into the selected Transformation Backbone, set up the following Golden Gate reaction:
    - 2 μl 10× T4 DNA ligase buffer.
    - 75 ng of the selected Transformation Backbone.
    - 150 ng of the selected Module A plasmid.
    - 150 ng of the Module B plasmid from **step 12**.
    - 150 ng of the selected Module C plasmid.
    - 0.4 μl AarI oligonucleotide (comes with the AarI enzyme).
    - 0.5 μl AarI.
    - 1 μl T4 DNA ligase.
    - H_2O up to 20 μl.
14. Place the Golden Gate reaction in a PCR machine and run the following cycle: 10× (37 °C/5 min + 16 °C/10 min) + 37 °C/15 min + 80 °C/5 min + 4 °C hold.
15. Transform 5 μl of the Golden Gate reaction into *E. coli* (DH5α or similar, but sensitive to the presence of ccdB gene) and plate on LB + 50 mg/L spectinomycin if using a non-T-DNA transformation backbone **OR** LB + 50 mg/L kanamycin if using a T-DNA transformation backbone. Incubate at 37 °C overnight.
16. On the following day, inoculate 1–2 colonies (*see* **Note 13**) into 5 ml of LB supplemented with 50 mg/l spectinomycin if using a non-T-DNA transformation backbone **OR** LB + 50 mg/l kanamycin if using a T-DNA transformation backbone.
17. On the following day, purify plasmids from the LB cultures. The final vector can be verified by colony PCR or restriction digest analysis and used for *Agrobacterium*/wheat transformation.

3.2 Design and Construction of Reagents for Multigene Knockouts and Chromosome Deletions

This protocol describes cloning of multiple gRNA spacers into arrays controlled by an RNA Pol II promoter in either Module B or Direct vectors. The approach is based on Golden Gate assembly of PCR products containing parts of the gRNA sequence and Csy4 binding sites. The Module B/Direct vectors serve both as the cloning vector and as the template for PCR amplification of individual gRNA parts. $n + 1$ (n = number of gRNA spacers) parts (PCR products) are assembled into the vector by Golden Gate assembly using two distinct type IIs restriction enzymes, including SapI and one of three other enzymes—Esp3I, BsaI or AarI, selected by the user. While SapI opens the vector backbone, the second type IIs enzyme creates complementary overhangs on each of the PCR products to allow for ligation in a specified order. Primers are designed using a dedicated online tool. When the gRNA array is expressed along with the Csy4 ribonuclease (provided in the Direct vector backbone or in Module A as a P2A fusion to Cas9), it will be processed into individual functional gRNA units. The Direct vectors with the cloned gRNA array can be used directly for *Agrobacterium*/plant transformation to induce multiplexed gene knockouts. gRNA arrays in Module B vectors can be further combined with the Csy4-P2A-Cas9 expression cassette in Module A and additional elements in Module C with a Transformation Backbone of choice in another Golden Gate cloning step (**steps 13–17** of protocol in Subheading 3.1). A timeline for construction of Modular and Direct vectors is shown in Fig. 2.

1. As for single gene knockouts, appropriate vectors can be selected by opening the link to the Webtools for the Voytas Lab Plant Genome Engineering Toolkit (http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/vector.php) and clicking on the "Vector Selection" or "List All Vectors" link. To select a "Direct Cloning Backbone," follow the first step in Subheading 3.1, but select "ZmUbi:Csy4-P2A-TaCas9 + PvUbi1:gRNAs with Csy4 spacers" in **step 4** of the selection process (*see* **Note 14**). To find a "Module B," select one of the multi gRNA cloning cassette options in **step 2** (*see* **Note 15**) and the PvUbi1 or CmYLCV promoter in **step 3** (*see* **Note 16**).
2. Order selected plasmids from Addgene and purify plasmid DNA from 5 ml LB cultures using the QIAprep Spin Miniprep Kit (*see* **Note 5**).
3. Select targets for the gene(s) of interest (GOI) (*see* **Note 17**) or two targets flanking the gene's genomic locus to be deleted (*see* **Note 18**). Any N_{20}NGG site can be used as a target without any constraints on the first nucleotide, although gRNA spacers that contain AarI sites or start with GTG (which creates AarI recognition site when placed downstream of a Csy4 site)

should be avoided to ensure accurate assembly when using Module B plasmids. Online tools such as sgRNA Scorer (https://crispr.med.harvard.edu/) [30] and CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) [31, 32] can be used for target site selection, prediction of on-target activity and identification of potential off-targets (*see* **Note 7**).

4. Generate a list of all target DNA sequences in fasta format as in the example below and save as a ".txt" file. Only use the first 20 nucleotides (N_{20}) of each target without the NGG PAM sequence. The two gRNAs below target the wheat phytoene desaturase gene [29].

   >GOI_gRNA1

   TTTGCCATGCCAAACAAACC

   >GOI_gRNA2

   GGCGCCCTTAAATGGAGTGT

5. Open the "Primer Design and Map Construction" tool at (http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/assembly.php). Use the "Browse..." button to select the text file with target DNA sequences in fasta format, select the target vector identified in **step 1**, promoter (*see* **Note 16**), type IIs restriction enzyme (*see* **Note 19**), splicing system (*see* **Note 15**), and click "Submit". An example of the output generated by the program is shown in Fig. 4.

6. Order primers from the output and download the vector map for your reference.

7. Generate PCR template for amplification of the first gRNA unit containing the promoter by digesting the target vector with the restriction enzyme BanI (*see* **Note 20**). Mix about ~2 μg of the plasmid DNA with 2 μl of CutSmart™ buffer and 1 μl of BanI enzyme and add ddH_2O to 20 μl. Incubate the reaction at 37 °C for 1 h and run on an agarose gel.

8. Extract the promoter and gRNA scaffold-containing fragment from the gel using the QIAquick Gel Extraction Kit (*see* **Note 21**). This is usually the longest fragment. For exact size refer to the map of the selected target vector.

9. Amplify the gRNA units by PCR using a proofreading DNA polymerase.

   (a) Set up a PCR reaction for each primer pair designed by the Primer Design and Map Construction tool. The number of reactions will be n + 1 (n = number of gRNA targets). Use the BanI fragment isolated in **step 8** as the template in the PCR reaction 1 and the undigested vector in all other PCR reactions:

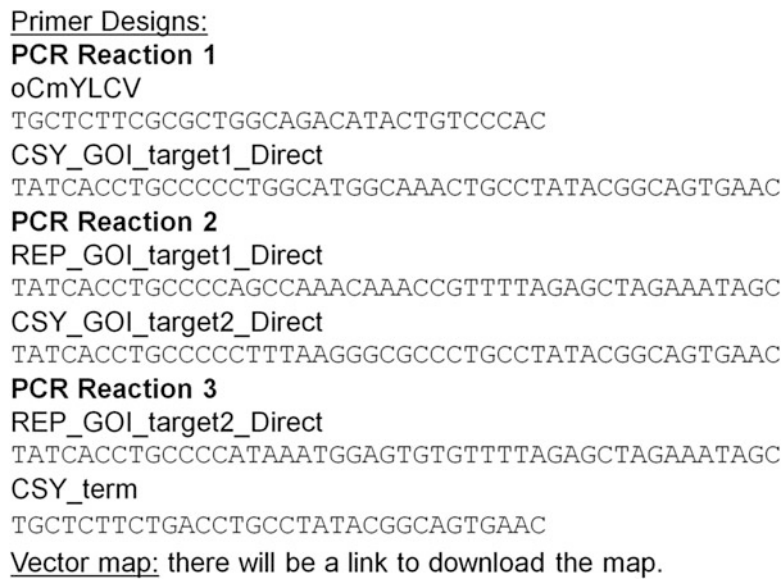

Fig. 4 Output from the Primer Design and Map Construction tool showing sequences of primers used in each PCR reaction. In addition, a vector map is automatically generated and can be downloaded and viewed using commonly used software tools for DNA analysis

- 1× reaction buffer.
- 1 μl 10 mM dNTPs.
- 2.5 μl 10 mM forward primer.
- 2.5 μl 10 mM reverse primer.
- 5–20 ng template.
- 1 U heat-stable, proofreading DNA polymerase (*see* **Note 22**).
- H_2O up to 50 μl.

(b) Place the PCR reaction in a thermocycler and run the following program:
98 °C/1 min + 30× (98 °C/10 s + 60 °C/15 s + 72 °C/15 s) + 72 °C/2 min + 4 °C hold

10. Confirm successful amplification by running 5 μl of each PCR product on a gel. The product of PCR reaction 1 will be longer, ~2000 bp for the PvUbi1 promoter or ~500 bp for the CmYLCV promoter. Products of all other reactions will be ~150 bp long.
11. Dilute each PCR product ten times with H_2O (*see* **Note 23**). Do not purify the PCR products (*see* **Note 24**).
12. Assemble the PCR amplified gRNA units into the selected vector.

(a) Prepare a Golden Gate reaction:
- 10 μl 2× T7 DNA ligase buffer.
- 50 ng of the selected vector.
- 0.5 μl of each 10× diluted PCR product.
- 0.5 μl SapI (*see* **Note 25**).
- 0.5 μl of the type IIS restriction enzyme selected in **step 5** (*see* **Note 26**).
- 1 μl T7 DNA ligase.
- H_2O up to 20 μl.

(b) Mix the reaction by pipetting up and down several times.

(c) Place the Golden Gate reaction in a thermocycler and run the following program:
10× (37 °C/5 min + 25 °C/10 min) + 4 °C hold (*see* **Note 27**).

13. Transform 5 μl of the Golden Gate reaction into *E. coli* (DH5α or similar, but sensitive to the presence of ccdB gene) and plate on LB + 50 mg/L of appropriate antibiotic (refer to the vector map created by the Primer Design and Map Construction tool for the type of antibiotic). Incubate at 37 °C overnight.
14. On the following day, screen 8–10 colonies (*see* **Note 28**) for the presence of the correctly assembled gRNA array by colony PCR. Use TC306 (PvUbi1) or TC320 (CmYLCV) as the forward primer depending on the type of promoter used, and TC089R (B and C modules, non-T-DNA Direct vectors) or M13F (T-DNA Direct vectors) as the reverse primer depending on the type of vector used. Upon completion, run the PCR reactions on an agarose gel. The products from correct clones will form a ladder of bands, where each band represents one gRNA unit. The full length product of expected size will be the most abundant band. The size of this band will depend on the number of gRNA units assembled and the type of promoter and splicing system. Refer to the vector map created by the Primer Design and Map Construction tool to determine the size of the PCR product. Inoculate 1–2 correct clones into 5 ml of LB supplemented with 50 mg/l of appropriate antibiotic and incubate and shake overnight at 220 rpm, 37 °C.
15. On the following day, purify plasmids from the LB cultures. The final vector can be verified by sequencing using the same primers that were used for colony PCR in **step 14**. If a Direct Cloning Backbone was used, the protocol ends here and the vector is ready for plant transformation. If the gRNA array was assembled into a Module B, this plasmid can be further used in assembly of vectors for gene targeting (*see* the protocol in Subheading 3.3) or assembled into a Transformation

Backbone (*see* **Note 11**) along with the selected Module A (for Csy4-P2A-Cas9, use pMOD_A1510) and Module C plasmids (*see* **Note 12**). Follow **steps 13–17** of protocol in Subheading 3.1 for assembly of Modules A, B and C into transformation backbones.

3.3 Design and Construction of Reagents for Gene Insertion and Replacement

This protocol describes the construction of vectors for both the *in planta* method and the GVR-mediated approach for gene insertion or replacement. Each gene targeting (GT) construct is prepared using the two-step modular cloning approach based on the assembly of the intermediate modules A, B and C into a transformation backbone. Module A carries the Cas9 nuclease expression cassette, Module B contains the gRNA expression cassette and Module C is used to add the DNA donor template for gene targeting. While Module A is a ready-to-use plasmid, Modules B and C must be prepared by the user by inserting the gRNA spacer and donor template sequences, respectively. Protocols for insertion of gRNA spacers into Module B vectors have been described in Subheadings 3.1 and 3.2. The cloning approach for the assembly of the donor template into Module C is described below. All three modules are then assembled into a transformation backbone of choice. A timeline for construction of gene targeting vectors is shown in Fig. 2.

1. Select appropriate Module vectors and the Transformation Backbone. For Module A, use pMOD_A1110 (ZmUbi:Cas9, use with a single gRNA assembled as described in Subheading 3.1) or pMOD_A1510 (ZmUbi:Csy4-P2A-Cas9, use with two or more gRNAs assembled as described in Subheading 3.2). Select Module B as in protocol of Subheadings 3.1 or 3.2 depending on the number of gRNAs used (*see* **Note 29**). To clone the donor template, an empty Module C (pMOD_C0000) can be used. Alternatively, a Module C vector containing a GFP expression cassette can be selected using the "Vector Selection" tool at http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/vector.php, or from the list of all vectors. The choice of the Transformation Backbone will depend on the transformation method and the gene targeting technique used. Open the vector selection tool and select "Transformation Backbone (Accepts Modules A + B + C)" in **step 1**. If the gene targeting vector will be used for protoplast or biolistic transformation, select non-T-DNA in **step 2**, for *Agrobacterium*-mediated transformation, select T-DNA. In **step 3**, select appropriate selectable marker (*see* **Note 1** and **30**). In **step 4**, select "none" for the *in planta* gene targeting approach, or "WDV" for the GVR approach (*see* **Note 31**).
2. Order selected plasmids from Addgene and purify plasmid DNA from 5 ml LB cultures using the QIAprep Spin Miniprep Kit (*see* **Note 5**).

3. Select gRNA target site(s) in the locus of interest (*see* **Note 32**) and proceed to construct the Module B with one or two gRNAs following protocol Subheading 3.1 (**steps 3–12**) or Subheading 3.2 (**steps 3–14**).
4. In parallel, insert the donor template for gene targeting into the selected Module C vector. Design the following two or three (in case of targeted insertion) (*see* **Notes 33** and **34**) pairs of primers for Gibson assembly of the donor sequence. Primer pair #1 (1F + 1R, Fig. 5a) to amplify ~1 kb (*see* **Note 35**) of sequence upstream of the cleavage site where the modification is being introduced. Primer pair #3 (3F + 3R, Fig. 5a) to amplify ~1 kb (*see* **Note 35**) of sequence downstream of the

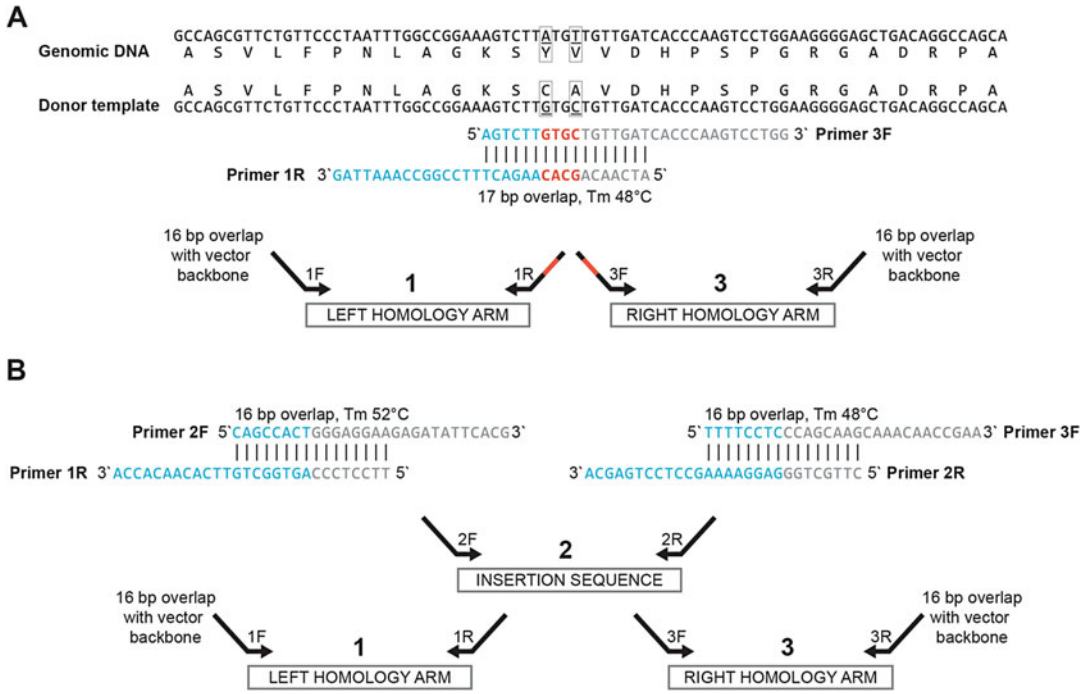

Fig. 5 Donor template design for two different gene targeting approaches. (**a**) Donor design for targeted substitution of two DNA bases resulting in two amino-acid substitutions. Only two fragments (*left* and *right* homology arms) amplified using two primer pairs (1F + 1R and 3F + 3R) will be assembled. The base substitutions are positioned in the primer overhangs within the region where the two primers overlap. The DNA binding regions (specific to the gene of interest) of primers 1R and 3F are shown in *cyan* and *gray*, respectively. The modified region is highlighted in *red*. The overlap between the primers 1R and 3F is depicted with short vertical lines representing base pairing. Overhangs on primers 1F and 3R are complementary with the *BaeI* linearized Module C backbone. (**b**) Donor design for targeted insertion. Three fragments (*left* and *right* homology arms plus the insertion sequence) amplified using three primer pairs (1F + 1R, 2F + 2R, and 3F + 3R) will be assembled. The DNA binding regions (specific to the gene of interest) of primers 1R and 2R are shown in *cyan* and the binding regions of primers 2F and 3F in *gray*. The overlaps between primers 1R and 2F, and 2R and 3F are depicted with short vertical lines representing base pairing. Overhangs on primers 1F and 3R are complementary with the *BaeI* linearized Module C backbone

cleavage site. Add the following overhang to the forward primer #1 (1F):

5′-CGCGTAGTCCTCGGTA(23 bp gRNA binding site)-3′—for *in planta* GT vectors (*see* **Note 36**)

OR

5′-CGCGTAGTCCTCGGTA-3′—for GVR vectors

Add the following overhang to the reverse primer #3 (3R):

5′-TGACTTGAAGTACACTC(23 bp gRNA binding site)-3′—for *in planta* GT vectors

OR

5′-TGACTTGAAGTACACTC-3′—for GVR vectors

For targeted insertion, design primer pair #2 to amplify the sequence being inserted. Design overhangs on reverse primer 1 (1R), forward primer 2 (2F), reverse primer 2 (2R), and forward primer 3 (3F) such that primer 1R with 2F and 2R with 3F overlap by 16 bp, as in Fig. 5b. Calculate the melting temperature (Tm) of each sequence overlap by adding two °C for each AT pair and four °C for each GC pair. For the overlap of example primers 1R and 2F in Fig. 5b this will be 4 (CG) + 2 (AT) + 4 (GC) + 4 (CG) + 4(CG) + 2 (AT) + 4 (CG) + 2 (TA) + 4 (GC) + 4 (GC) + 4 (GC) + 2 (AT) + 4 (GC) + 4 (GC) + 2 (AT) + 2 (AT) = 52 °C. If the Tm is below 48 °C, extend the overlap until the Tm is at least 48 °C.

5. PCR amplify the DNA fragments for Gibson assembly of the donor template (*see* **Note 37**).
   (a) Set up a 50 µl PCR reaction for each homology arm, and, optionally, for the insertion sequence:
      - 10 µl 5× Q5 reaction buffer (*see* **Note 38**).
      - 1 µl dNTPs.
      - 2.5 µl 10 mM forward primer.
      - 2.5 µl 10 mM reverse primer.
      - 10–100 ng template DNA.
      - 0.5 µl Q5 DNA polymerase (*see* **Note 38**).
      - H_2O up to 50 µl.
   (b) Place the PCR reaction in a thermocycler and run the following cycle:

      98 °C/1 min + 40× (98 °C/10 s + XX°C/20 s + 72 °C/XXs) + 72 °C/2 min + 4 °C hold (*see* **Note 39**).
6. Run full volumes of all reactions on an agarose gel, excise the bands of correct sizes and purify using the QIAquick Gel Extraction Kit. Measure DNA concentration using a spectrophotometer.

7. Prepare the Module C vector backbone for cloning of the donor template by digestion with BaeI (*see* **Notes 37** and **40**). Set up the digestion reaction by mixing 2–3 μg of the vector with 2 μl CutSmart buffer, 1.25 μl 320 μM SAM, 1 μl BaeI, and water to 20 μl. Incubate for 1 h at 25 °C.
8. Run the full volume of the reaction on a 1% agarose gel, excise the linearized vector fragment and purify using the QIAquick Gel Extraction Kit (*see* **Notes 21** and **41**). Measure DNA concentration using a spectrophotometer.
9. Insert the donor template into the Module C vector by Gibson assembly.
   (a) Set up a Gibson assembly reaction:
      - 15 μl of the 1.33× Gibson assembly master mix.
      - 50 ng BaeI linearized, purified Module C vector backbone.
      - Each purified PCR fragment (insert) in threefold molar excess over vector.
      - H_2O up to 50 μl.
   (b) Incubate for 1 h at 50 °C.
10. Transform 5 μl of the reaction into *E. coli*, plate on LB + 50 mg/L carbenicillin/ampicillin and incubate plates overnight at 37 °C.
11. Identify correct clones by colony PCR using primer ZY015F along with a primer specific to the donor sequence (*see* **Note 42**). Inoculate 1–2 correct clones into 5 ml of LB supplemented with 50 mg/l of carbenicillin/ampicillin and incubate and shake overnight at 220 rpm, 37 °C.
12. On the following day, purify plasmid DNA from the LB cultures. The vector can be verified by restriction digest and sequencing using primers ZY015F, CS433 and internal primers specific to the donor sequence.
13. Assemble the selected Module A, Module B with inserted gRNA spacers from **step 3** and Module C with inserted donor sequence from **step 12** into the selected Transformation Backbone by Golden Gate cloning following protocol Subheading 3.1, **steps 13–17**.

4 Notes

1. Hygromycin (PvUbi2:hptII) or phosphinothricin (PvUbi2:bar) resistance genes expressed from the switchgrass ubiquitin promoters are recommended for wheat.

2. Alternatively, "ZmUbi:TaCas9_dead + TaU6 gRNA" can be selected for transcriptional downregulation instead of gene knockout. This vector contains catalytically dead Cas9 lacking endonuclease activity, which is known to interfere with transcriptional elongation and inhibit gene expression when targeted to a gene locus [33].
3. Trex2 is an exonuclease that drives resection of the DNA ends at the newly formed DSB and increases the mutagenicity of the DSB by increasing the length and frequency (about 2–2.5 fold) of deletions [27].
4. The only difference between pMOD_B2517 and pMOD_B2518 is the type of promoter for gRNA expression. pMOD_B2517 contains the wheat U3 promoter (TaU3) and pMOD_B2518 contains the wheat U6 promoter (TaU6). Both promoters should be similarly active.
5. Prepping the plasmids with kits from other suppliers may reduce efficiency of the reaction.
6. Polymerase III promoters usually require a specific base in the first position of the transcribed sequence for efficient transcription. For the TaU3 promoter it is adenine (A) and for the TaU6 promoter it is guanine (G). For this reason, we recommend targeting sites that start with an A or a G, such that the target sequence is $AN_{19}NGG$ or $GN_{19}NGG$, respectively. However, the first nucleotide did not have any effect on gene editing frequencies in rice when the OsU3 and OsU6 promoters were used for gRNA expression [34]. Therefore, if a $AN_{19}NGG$ or $GN_{19}NGG$ site cannot be identified in the gene of interest, sites starting with other nucleotides may be tested as an alternative.
7. The wheat genome is not currently available on the CRISPR-P website but is expected to be available soon.
8. 10× T4 DNA ligase buffer is the preferred buffer since it contains ATP at sufficient concentration that would otherwise need to be added to the T4 PNK buffer.
9. Alternatively, the oligonucleotides can also be annealed by boiling the reaction in a water bath for 2 min and lettting it cool down gradually.
10. Correct clones can be identified by colony PCR using the sense gRNA oligonucleotide as the forward primer and ZY015F (Module B) or NB463 (Direct Cloning Backbone) as the reverse primer. However, colony PCR is usually not necessary thanks to the high efficiency of the Golden Gate reaction.
11. Three types of transformation backbones are available in the Voytas lab Multipurpose Plant Genome Engineering kit. These include T-DNA backbones for *Agrobacterium*-mediated

transformation, and non-T-DNA backbones with or without selectable marker genes for particle bombardment or protoplast transformation, respectively. Appropriate Transformation Backbones can be identified using the "Vector Selection" tool at http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/vector.php by selecting "Transformation Backbone (Accepts Modules A + B + C)" in **step 1**, or from the list of all vectors also available on the website.

12. Module A plasmids contain the Cas9 expression cassettes and Module C plasmids contain additional genes (such as GFP or Trex2) or can be empty. Appropriate Module A and Module C plasmids can be identified using the "Vector Selection" tool at http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/vector.php.
13. Correct clones can be identified by colony PCR using a forward primer specific for the sequence between the AarI sites in the A, B or C used in the assembly reaction (such as TC430 specific for the HSP terminator in Module A) and primer M13F (for T-DNA vectors) or pCR8R1 (for non-T-DNA vectors) as the reverse primer. However, colony PCR is usually not necessary due to the high efficiency of the Golden Gate reaction.
14. Alternatively, "ZmUbi:Csy4-P2A-TaCas9_dead + PvUbi1:gRNAs with Csy4 spacers" can be selected for transcriptional downregulation instead of gene knockout.
15. The preferred multi-gRNA expression system uses the Csy4 ribonuclease to process the transcript into individual gRNA units. Although this system offers the highest efficiency [27], two other systems are available, using tRNA [35] or ribozyme-based [36] processing. The advantage of these systems is that they do not require additional proteins (such as the Csy4 ribonuclease for Csy4 array processing). However, the ribozyme system has been shown to induce mutations with suboptimal efficiency using this toolkit. For the most efficient Csy4 system, two types of Module B plasmids are available for cloning up to six or more than six gRNA spacers. If more than six sites are targeted, the option for seven and more gRNAs should be selected. This Module B plasmid is designed to accept six gRNA spacers, but can be combined with another array of six gRNA spacers cloned into module pMOD_C2200 using the same procedure.
16. The PvUbi1 promoter provides optimal expression in wheat, but decreases the cloning efficiency due to its size (~2 kb). Although not directly tested with this protocol in wheat, the viral CmYLCV promoter provides sufficient expression of the gRNA array in tomato, tobacco, *Medicago truncatula*, and barley [27], is highly active in a wide variety of both dicot

and monocot species [37] and provides optimal cloning efficiency thanks to its smaller size (~500 bp).

17. We have previously shown that the majority of the blunt DNA breaks created by CRISPR/Cas9 are repaired precisely [27]. To prevent precise restoration of the original sequence, we recommend targeting each gene for deletion with two (or more) gRNAs which not only increases the efficeincy of mutagenesis but also simplifies mutant identification since simple PCR can be used to screen for the longer deletions.
18. Note that the deletion frequency decreases with increasing deletion size.
19. This protocol was optimized using the Esp3I enzyme and therefore we recommend using Esp3I, where possible. However, Esp3I cannot be used with all combinations of promoters and cloning vectors and in some cases BsaI or AarI must be used instead. The "Restriction enzyme" drop-down menu only shows the suitable enzymes based on the selected target vector and promoter.
20. Due to the presence of two Csy4 repeats in each template vector, two products can be amplified when amplifying the first gRNA unit since the reverse primer binds to the repeated sequence. This is prevented by using BanI digested plasmid as the template. BanI cleaves the gRNA repeat sequence and separates the two Csy4 repeats.
21. The purified fragment can be stored at −20 °C and used for multiple reactions.
22. The protocol was optimized using Phusion DNA polymerase and therefore we recommend using Phusion or another type of proofreading DNA polymerase. We have not achieved good results in the subsequent cloning steps when Taq polymerase was used for amplification and we recommend avoiding the use of Taq polymerases in this step.
23. This is an essential step as using non-diluted products decreases cloning efficiency by several times.
24. Purification is not necessary and we have achieved better results with non-purified PCR products.
25. SapI tends to settle in the tube. To ensure sufficient activity, the enzyme solution should be mixed by pipetting up and down several times before adding to the reaction.
26. If AarI is used, also add 0.4 μl of the AarI oligonucleotide supplied with the enzyme to ensure optimal activity.
27. It is essential to NOT heat inactivate the reaction. Heat inactivation decreases transformation efficiency due to the presence of PEG in the reaction buffer. Heat inactivating the reaction may result in very few to no colonies.

28. The cloning efficiency decreases with the number of gRNA spacers cloned and may be as low as 50–60% with six gRNAs. Therefore we recommend screening 8–10 colonies. The cloning efficiency is much higher when cloning 2–3 gRNA spacers, and therefore screening 2–4 colonies is sufficient.
29. Using one gRNA is sufficient to induce gene targeting; however, if longer stretches of DNA are being replaced, then two gRNAs to delete the intervening part are recommended.
30. The *in planta* approach requires selection to identify plants with stably integrated Cas9 and donor template transgene; however, selection is not essential when using GVRs, since we have shown that gene targeting can occur without the integration of the genome engineering reagent into the genome [6]. Therefore selection for such integrations will work against plants with successful gene targeting but no integrated selectable marker. Optionally, the selectable marker can be included in the donor template instead when using GVRs, to be inserted at the targeted site and allow for selection of all gene targeting events. In such case, vectors with no selectable marker should be chosen and selection gene integrated in the donor template by the Gibson assembly approach described in the protocol.
31. WDV (wheat dwarf virus)-based GVRs have been shown to work efficiently in wheat cells [7], although it should be noted that whole wheat plants have not yet been recovered using this approach.
32. The target site should be as close to the site of modification/insertion as possible. If a longer DNA sequence is being replaced, it is recommended to remove the region using two gRNAs targeting sites flanking the intended deletion. Note that if the targeted modification/insertion does not disrupt the gRNA binding site(s), the modified target may potentially be recleaved again resulting in imprecise repair. This can be prevented by introducing additional silent mutations in the donor template that will disrupt the gRNA target site, ideally within the PAM sequence which is essential for cleavage by Cas9, or as close as possible to the PAM.
33. More than three fragments can be assembled if necessary, for example if additional mutations are being introduced at different positions of the donor template. We recommend not exceeding 5–6 fragments for optimal cloning efficiency.
34. Sequences used as homology arms or for insertions should not contain AarI recognition sites. The presence of an AarI site would interfere with the Golden Gate assembly of Modules A, B and C. If necessary, AarI sites can be removed by introducing silent mutations in the donor template sequence using the same procedure as for the introduction of sequence modifications shown in Fig. 5a.

35. The length of each homology arm is important. Generally, the longer the homology is, the more efficient the HDR will be. We recommend keeping the length of each homology arm within the range of 700–1000 bp. The opposite is true for the length of the insertion sequence. The longer it is, the lower the efficiency of precise incorporation of such sequence will be. We have successfully targeted insertion of up to 2 kb long sequence into the plant genome.
36. The sequences of gRNA binding sites may or may not be included in the primers 1F and 3R to allow for the release of the donor template from the chromosome. This should be the binding sequence for the same gRNA that is used to induce the DSB in the gene of interest. Alternatively, a different gRNA may be used, but has to be included in the gRNA expression cassette in Module B. When building GVR vectors, gRNA binding sites are not necessary, as the donor availability is improved by replication of the donor template into high copy number, rather than by release from the chromosome.
37. **Steps 5–6** can be done simultaneously with **steps 7–8**.
38. In this example we are using the Q5 polymerase, but any proofreading polymerase can be used instead.
39. The optimal annealing temperature for the primers can be determined using the NEB Tm calculator (http://tmcalculator.neb.com/#!/). Use 30 s for each 1 kb of amplified sequence in the extension step.
40. BaeI will open and linearize the vector backbone and create ends compatible with Gibson overhangs on primers 1F and 3R. BaeI excises its binding site from the vector by making two staggered cuts on each side of the recognition sequence. The resulting DNA ends will have 5 bp overhangs that are incompatible with each other and thus prevent religation of the vector without the donor template.
41. We recommend using Qiagen MinElute columns and elute the DNA in 10 μl of water to get high concentration.
42. We recommend selecting a primer in the first assembled fragment of the donor sequence, such that when this primer is used with ZY015F which binds to the plasmid backbone, the PCR product will span all assembled fragments. Although the product will be relatively long (2–4 kb depending on the length and number of the assembled donor fragments), we routinely perform colony PCR resulting in products of similar size using the standard Taq polymerase from NEB.

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Chapter 13

Doubled Haploid Transgenic Wheat Lines by Microspore Transformation

Sachin Rustgi, Nii O. Ankrah, Rhoda A.T. Brew-Appiah, Yue Sun, Weiguo Liu, and Diter von Wettstein

Abstract

Microspores are preferred explant choice for genetic transformation, as their use shortens the duration of obtaining homozygous transformants. All established gene-delivery methods of particle bombardment, electroporation, and cocultivation with *Agrobacterium tumefaciens* were optimized on androgenic microspores or derived tissues. In the biolistic gene delivery method 35–40 days old haploid microspore embryoids were used for genetic transformation, whereas freshly isolated androgenic microspores were used for genetic transformation in the electroporation and *Agrobacterium* cocultivation-based methods. The genetic transformation methods of biolistic gene-delivery and electroporation gave rise to the chimeric plants, whereas the method involving cocultivation with *Agrobacterium* yielded homozygous transformants. These methods were tested on a large number of cultivars belonging to different market classes of wheat, and found to be fairly independent of the explant genotype. Other benefits of using microspores or derived tissues for transformation are: (1) a few explant donors are required to obtain desired transformants and (2) the time required for obtaining homozygous transformants is about 8 months in case of spring wheat genotypes and about a year in case of winter wheat genotypes.

Key words Genetic transformation, Particle bombardment, Microspore embryogenesis, Electroporation, *Agrobacterium tumefaciens*

1 Introduction

Wheat is one of the most widely cultivated and traded cereals with a hectarage of 222.3 million in 2014/15 (http://www.statista.com). Wheat yield is increasing sturdily, but not in synergy with an increase in global population. Factors such as changing edaphoclimatic conditions, and urbanization of arable land impose additional pressure on agriculture, in particular on wheat production [1]. Wheat is a national staple in many countries, and accounts for about 20% of the total food calories consumed worldwide. It also provides approximately half of the global demand for dietary proteins [2]. Wheat not only satisfies human needs for calories and

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protein but also serves as a primary source of a wide range of essential nutrients such as vitamins B, E, and A, antioxidative compounds like phytosterols, phenolic acids, alkylresorcinols, lignans, choline, and betaine, a number of mineral elements including Ca, Mg, Fe, Zn, and Cu, and dietary fibers such as β-glucans and arabinoxylans [3–6]. In addition of being a source of these health promoting nutrients wheat and derived products are elicitors of a number of frequent diet-induced health issues, in particular gluten intolerance, sensitivity and allergy, collectively referred to as the "gluten syndrome" [7, 8]. These disorders cumulatively affect more than 7.5% of the US population [9, 10]. In particular, the gluten intolerance dubbed celiac disease, alone affects more than 71 million individuals around the globe (about 1% of the world population), which makes it one of the most devastating disorders of the gastrointestinal tract [11]. In addition of being major elicitors of the dietary disorders wheat gluten proteins are unusually rich in amino acids proline (15%) and glutamine (35%) and deficient in essential amino acids lysine, threonine, methionine, and histidine [12]. Collectively, it suggests that to meet the future demands for healthy and surplus food the two priority breeding targets are: (1) increasing wheat productivity under marginal environments, and (2) enriching wheat grains for health promoting nutrients on the expense of undesirable elements. Genetic transformation is one of the ways to achieve wheat genotypes with desirable characteristics in the reasonable amount of time. In this direction, two stable genetic transformation systems were developed in wheat. Both of these systems were designed to use scutellar calli as explants with particle bombardment or cocultivation with *A. tumefaciens* as the transgene delivery method [13–15]. These procedures, though widely used, have inherent problems: A major drawback is the lack of a reliable regeneration system after transformation of the morphogenic tissue. These methods often require preparation of large number of calli derived from many explant donors to achieve the desired transfromants. Another disadvantage is time and labor required to obtain homozygous transgenic plants in the desired genetic background [16]. Collectively these procedures take multiple years to get the genetically true-breeding lines of selected transformants suitable for commercial application. Additionally, only a limited number of genotypes can be transformed with a reasonably high efficiency [17].

An alternative to avoid these problems is the use of microspores as explants for genetic transformation. Microspores are specialized haploid cells with embryogenic potential. Being a synchronized mass of cells with morphogenic potential, the microspores offer enormous opportunities for genetic manipulation of desired genotypes at a single-cell level. In view of the potential benefits of using microspores and derived tissues as explants substantial efforts have been made in the past to develop genetic transformation methods.

Based on the developmental stage targeted for transformation these procedures can be broadly classified into two groups: gametophytic and sporophitic [18]. The gametophytic route includes: (1) mature pollen-based transformations where foreign DNA is delivered into the pollens before pollination or applied to stigma before/after pollination and (2) microspore maturation based transformation where foreign DNA was delivered into microspores, cultured in vitro into mature pollens and used for pollination to obtain transformants. The sporophytic route includes microspore embryogenesis based transformation procedures where microspores were induced/reprogrammed toward sporophytic pathway to produce gametic-embryos. All of the above mentioned procedures have their own advantages and disadvantages, where the latter approaches have an obvious advantage as they allow production of homozygous doubled haploid transgenic plant in one generation after diploidization or by spontaneous doubling.

Although wheat doubled haploids were successfully produced in early 1990s the initial efforts to transform wheat microspores using microprojectile bombardment resulted in only transient expression of the marker genes [19, 20]. Later on transformation of mature wheat pollens with pDPG165 (the *Bar* gene expressing vector) by electroporation followed by vacuum drying and pollination of receptive stigma resulted in stable wheat transformants [21]. Additionally, in a recent effort, cocultivation of anther culture derived haploid wheat embryos with *A. tumefaciens* resulted in stable doubled-haploid wheat transformants expressing the barley *HVA1* gene [22]. More recently, an alternative technique for direct delivery of the protein molecules into microspores has been proposed that utilizes the natural ability of cell penetrating peptides to internalize protein, DNA or nanoparticles into the living cells [23]. In this study, the Tat2 peptide was shown to efficiently transduce GUS enzyme (272 kDa) in its functional state in the wheat microspores [23]. The real potential of this technique in agriculture is yet to be fully realized.

In view of the importance of stable doubled haploid transformants in agriculture, the major quest of the present communication is to deliver the reliable procedures of transforming androgenic microspores or derived tissues to the wheat community. The basis of obtaining doubled haploid transgenic wheat plants is rooted in an efficient genotype independent doubled-haploid production method established in the senior author's laboratory [24]. The procedure relies on chemical inducer formulations to trigger microspore embryogenesis that also ensures delivery of high frequencies of green plants and reduces the formation of albino regenerants [25]. Use of nursing ovaries to increase the frequency of microspore embryogenesis was also recommended in this procedure [26].

2 Materials

2.1 Plants

1. For microspore electroporation based genetic transformation method seven wheat cultivars Express, Chris, Perigee, Hollis, WestBred 926, Farnum, and Louise were used. These cultivars belong to three market classes of common wheat in the USA (for selection criteria, *see* **Note 1**).
2. For microspore transformation by cocultivation with *A. tumefaciens* the following spring wheat cultivars: Chris, WED 202-16-2, and NPBCT were used as microspore donors.
3. For genetic transformation of microspore embryoids by particle bombardment WestBred 926 was used as explant donor.

2.2 Bacterial Strains and Binary Vectors

1. *A. tumefaciens* strain AGL-1 was used for genetic transformation of embryogenic microspores. AGL-1 carries a disarmed Ti plasmid, which is derived from the hypervirulent, attenuated tumor-inducing plasmid pTiBo542 by precise excision of the T-region [27]. The Ti plasmid of AGL-1 also carries an insertion in the *recA* gene that stabilizes the recombinant plasmid and renders the strain resistant to carbenicillin.
2. Double cassette binary vector RS 128/Xyl was used for the genetic transformation of androgenic microspores by cocultivation with *A. tumefaciens*. One of the cassettes in RS 128/Xyl consists of the bialaphos resistance (*bar*) gene cloned in frame with the maize *ubiquitin* promoter with its first intron and nopaline synthase (*nos*) terminator between one set of the T-DNA left and right borders. The second cassette in RS 128/Xyl consists of a codon-optimized version for 1,4-β-xylanase gene cloned in frame with barley D-hordein gene promoter and its signal peptide and *nos* terminator between a second set of the T-DNA borders. Plasmid RS 128/Xyl was derived from another plasmid pAM470 with kanamycin resistance [28]. Significance of using a binary vector and an intron sequence between the promoter and the gene of interest are respectively described in **Notes 2** and **3**. Three single cassette vector pRB107, pRB113, and pUbi.GFP were used in the microspore electroporation based genetic transformation procedure. Vector pRB107 consists of the β-glucuronidase (*GUS*) gene cloned in frame with the cauliflower mosaic virus (CaMV) *35S* promoter and *nos* terminator. Similarly, vector pRB113 consists of the codon-optimized version of the *Trichoderma harzianum* endochitinase (*ThEn42*) gene cloned in frame with the CaMV *35S* promoter and *nos* terminator. In the single cassette vector, pUbi.GFP the *GFP* (green fluorescent protein) gene is cloned with the maize *ubiquitin* promoter and its first intron and *nos* terminator. Significance of using codon optimization in

expressing prokaryotic genes in the plant cells is described in **Note 4**. All of the above mentioned single cassette vectors were developed in pUC19 backbone with ampicillin resistance. The single cassette vector pDPG165 that was used for biolistic transformation of microspore embryoids consists of *Bar* gene cloned in frame with CaMV *35S* promoter and *Tr7* (T-DNA transcript number 7) terminator. Vector pDPG165 carries the *ampR* gene for ampicillin resistance.

2.3 Stock Solutions

1. The preparation of the induction medium Northwest Plant Breeding-99 (NPB-99) and regeneration medium 190-2 is described in Table 1.
2. The preparation of the Modified Murashige and Skoog 5 (MMS5) regeneration/rooting media is described in Table 2.
3. Luria–Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 mL of deionized H_2O. Adjust the pH to 8.0 with 5 N NaOH. Adjust the volume of the solution to 1 L with deionized H_2O. For solid medium add bacto agar to 15 g/L before autoclaving, sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.
4. Kanamycin stock solution (50 mg/mL): 500 mg kanamycin sulfate powder in 10 mL deionized H_2O. Filter-sterilize antibiotic solution using a 0.22 μm syringe filter. Aliquot (200 μL) and store at −20 °C.
5. Acetocarmine solution: 0.5 g carmine in 100 mL of 45% boiling acetic acid. Heat for another few minutes in a fume hood while stirring. Cool down the solution to room temperature, add one to two drops of a saturated ferric acetate solution and filter. Store acetocarmine solution at room temperature.
6. Colchicine stock solution (0.1%): 1 g colchicine in a liter of sterilized deionized water. Store the solution in refrigerator (take necessary precautions while handling colchicine). For working solution add five drops of dimethyl sulfoxide (DMSO) to 10 mL of the colchicine stock solution. Store at 4 °C.
7. $CuSO_4$ solution (2 mM): 500 mg $CuSO_4 \cdot 5H_2O$ in 1 L 0.4 M mannitol solution. Store at 4 °C.
8. Mannitol solution (0.4 M): 72.85 g mannitol in 1 L of sterilized deionized H_2O. Store at 4 °C.
9. Maltose solution (21%): 21 g maltose in 100 mL of sterilized deionized water. Store at 4 °C.
10. Pretreatment solution: Maltose (90 g/L), 2-hydroxynicotinic acid (0.1 g/L), 6-benzylaminopurine (2 mg/L), 2,4-D (10 mg/L), and gibberellic acid (3 mg/L). Adjust the pH to 7.0 and filter-sterilize.

Table 1
Composition of induction medium NPB-99[a] and regeneration medium 190-2[b]

Component		NPB-99 (mg/L)	190-2 (mg/L)
Macro	$(NH_4)_2SO_4$	232	200
	KNO_3	1415	1000
	$CaCl_2 \cdot 2H_2O$	83	–
	$Ca(NO_3)_2 \cdot 4H_2O$	–	100
	KH_2PO_4	200	300
	$Mg(SO_4) \cdot 7H_2O$	93	200
	KCl	–	40
Micro	H_3BO_3	5	3
	$CoCl_2 \cdot 6H_2O$	0.0125	–
	$CuSO_4 \cdot 5H_2O$	0.0125	–
	KI	0.40	0.50
	$MnSO_4 \cdot 4H_2O$	5	8
	$Na_2MoO_4 \cdot 2H_2O$	0.0125	–
	$ZnSO_4 \cdot 7H_2O$	5	3
Iron	Na_2 EDTA	37.3	37.3
	$FeSO_4 \cdot 7H_2O$	27.8	27.8
Amino acids	Glycine	–	2
Vitamins	Pyridoxine–HCl	0.5	0.5
	Nicotinic acid	0.5	0.5
	Thiamine–HCl	5	–
	Glutamine	500	–
Sugars	Myo-inositol	50	100
	Sucrose	–	30,000
	Maltose	90,000	–
Hormone	Kinetin	0.2	–
	2,4-D	0.2	–
	PAA	1	–
pH		7.0	6.5
Gelling	Phytagel	–	4000

Medium 190-2 is autoclaved at 240 °F for 30 min.
[a]NPB-99 = *N*orthwest *P*lant *B*reeding-99 [Zheng MY, Liu W, Weng Y, Polle E, Konzak CF (2003) Production of doubled haploids in wheat (*Triticum aestivum* L.) through microspore embryogenesis triggered by inducer chemicals. In: Maluszynski M, Kasha KJ, Froster BP, Szarejko I (eds.) Doubled haploid production in crop plants. Kluwer Academic Publishers, pp 83–94]
[b]Wang XZ, Hu H (1984) The effect of potato II medium for triticale anther culture. Plant Sci Lett 36: 237–239

Table 2
Composition of *M*odified *M*urashige and *S*koog 5 (MMS5[a]) regeneration/ rooting media (pH 5.8)

Component	mg/L
Macro salts	
1. KNO_3	1400
2. NH_4NO_3	300
3. KH_2PO_4	170
4. $MgSO_4 \cdot 7H_2O$	370
5. $CaCl_2 \cdot 2H_2O$	440
Iron source	
$FeSO_4 \cdot 7H_2O$	27.8
Na_2 EDTA	37.5
Micro salts	
$MnSO_4 \cdot H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4 \cdot 7H_2O$	8.6
$CoCl \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	2.5
$NaMoO_4 \cdot 2H_2O$	0.25
KI	0.83
Other components	
Glutamine	975
Myo-inositol	300
Thiamine–HCl	0.4
Nicotinic acid	0.5
Pyroxidine–HCl	0.5
Maltose monohydrate	30,000
PAA	0.2
Kinetin	0.5
Phytagel	3000
GA_3	0.5
Amino acids, U2.5	355

[a]Modified from the original MS media [cf. Kasha KJ, Simion E, Miner M, Letarte J, Hu TC (2003) Haploid wheat isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Froster BP, Szarejko I (eds.), Doubled haploid production in crop plants. Kluwer Academic Publishers, pp 77–81]

11. Electroporation buffer: 98.1 mg acetosyringone in a liter of 0.4 M mannitol. Supplement the electroporation buffer with plasmid DNA at a final concentration of 10 ng/mL.
12. Timentin: 200 mg timentin in a liter of deionized H_2O.
13. $CaCl_2$ (2.5 M): 11 g $CaCl_2 \cdot 6H_2O$ in 20 mL of sterilized deionized H_2O. Sterilize the solution by passing it through a 0.22 μm filter.
14. Spermidine (0.1 M): 255 mg spermidine in 10 mL of sterilized deionized H_2O. Store at −20 °C.

2.4 Specific Laboratory Equipment and Supplies

1. Refrigerated centrifuge.
2. Glass bead sterilizers (BS-1000).
3. Waring blender and vessel.
4. Gene gun (PDS-1000/He™ and Hepta™ Systems).
5. Inverted microscope with camera.
6. Stereomicroscope.
7. Table top horizontal airflow workstation.
8. Electroporator (Gene Pulser Xcell™ Total System).
9. pH meter.
10. Incubator shaker and tissue culture chamber.
11. Magnetic stirrer.
12. Vortex mixer.
13. Spectrophotometer.
14. Filter paper disks.
15. Parafilm.
16. Nylon mesh.
17. Magenta boxes.
18. Sterile petri dishes with lids.
19. Sterile filter-stopped tips.
20. Standard pipettes.
21. Sterile screw-cap polypropylene centrifuge tubes (15 mL).

3 Methods

3.1 Growth Conditions and Selection of Spikes

1. Plants of selected genotypes are cultivated in 20 × 25 cm pots using Sunshine #1 potting mixture (SunGro Horticulture, Bellevue, WA, USA) in a glasshouse maintained at the day temperature of 20–23 °C and night temperature of 14–16 °C and a photoperiod of 18 h.
2. Fertilizer is applied once a week with nutrient water containing 200–250 ppm of water-soluble fertilizer [nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O)].
3. Primary tillers at the Feeke's stage 10–10.1, showing a 3–5 cm slit in the boot are excised below the second node and placed in a clean container with distilled water (Fig. 1a, b). These boots are expected to have microspores at the mid to late-uninucleate stage of development (specifically in the florets located in the central part of the immature spike; *see* **Notes 5** and **6**).
4. Stage of microspore development is determined by microscopic examination of crushed anthers mounted with acetocarmine or

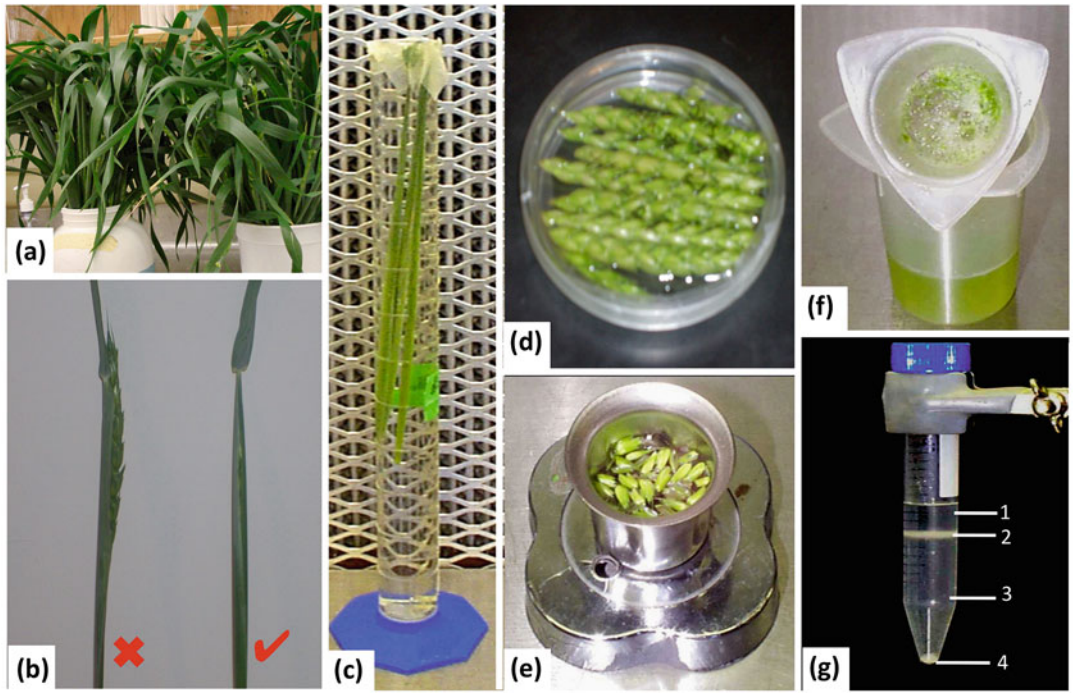

Fig. 1 Isolation of wheat microspores and use as explant for genetic transformation. (**a**) Wheat boots collected from greenhouse at the Feeke's stage 10–10.1. (**b**) Spikes at different developmental stages are indicative of microspore developmental stages: 25% of spike exposed out of the flag leaf indicative of binucleate microspores with a generative and a vegetative nucleus (too old), hence markerd with a "×", and spikes with 3–5 cm slit in boot indicative of microspores with a single haploid nucleus (optimal), hence markerd with a "√". (**c**) Spikes under pretreatment. (**d**) Spikes being sterilized with 10% commercial bleach solution, before microspore isolation. (**e**) Blender cup with florets. (**f**) 100-μm mesh with debris and a beaker containing filtrate with viable microspores. (**g**) Density centrifugation showing embryogenic microspores at the interphase of mannitol and maltose; *1* = 0.4 M mannitol solution, *2* = premitotic viable microspores, *3* = 21% maltose solution, and *4* = dead microspores

water (use phase contrast when water is used for mounting). At uninucleate stage, the microspores are round in shape with a nucleus located opposite the germination pore.

5. Tillers can be stored by dipping them up to the second node (from the top) in water at 4 °C for a week without a significant loss of microspore viability.

3.2 Pretreatment

1. The boots are pretreated with 2 mM $CuSO_4$ solution prepared in 0.4 M mannitol for 10–14 days at 4 °C. This pretreatment increases the possibility of obtaining green seedlings (Fig. 1c) (*see* **Note 7**).
2. For boots derived from Chris, WED 202-16-2, and NPBCT the following pretreatment conditions are used: tillers with mid to late uninuclear microspores were placed below the second node from the top in a flask containing pretreatment solution

(*see* Subheading 2.3 for details), and incubated at 33 °C for 48–72 h.

3. Either of the pretreatments described in **steps 1** and **2** work equally well for Chris.
4. After pretreatment the spikes are sterilized for 10 min with 10% commercial bleach solution (active ingredient 6.15% sodium hypochlorite) (Fig. 1d).

3.3 Isolation of Microspores

1. Transfer sterilized spikes into an autoclaved MC2 Waring blender cup containing 50 mL of 0.4 M mannitol solution (Fig. 1e). For this step use a chilled blender cup (after autoclaving store the blender cup in a refrigerator).
2. Blend spikes for 5 s at 2200 rpm in a MC2 Waring blender, pass the resulting slurry through an autoclaved 100-μm mesh to reduce microspore debris.
3. Rinse microspore debris from mesh into blender cup with 50 mL of 0.4 M mannitol and blend for another 5 s at 2200 rpm in a MC2 Waring blender (extended period of blending may cause damage to the microspores). Filter with 100-μm mesh as in **step 2**. Rinse the mesh cup three times with 5 mL of 0.4 M mannitol solution each time (Fig. 1f).
4. Pour filtrate onto an autoclaved 50-μm mesh filter to trap viable microspores. Rinse the mash with 10 mL of 0.4 M mannitol solution.
5. Invert the mesh cup and rinse off microspores that were trapped on the mesh with 15 mL of 0.4 M mannitol into a beaker.
6. Transfer the resulting microspore suspension to a 15 mL tube and centrifuge at 900 rpm (149 × *g*) for 5 min.
7. Pour off supernatant and add 10 mL of 0.4 M mannitol and centrifuge at 900 rpm for 5 min.
8. Pour off supernatant and resuspend the microspores in 2 mL of 0.4 M mannitol solution and layer it over 10 mL of 21% maltose solution.
9. Density gradient centrifugation at 800 rpm (118 × *g*) for 3 min separates the nonembryogenic microspores from the embryogenic microspores, the latter accumulating at the interphase between the maltose and the mannitol solutions (Fig. 1g).
10. Transfer 2–3 mL of the upper part of the band to a new 15 mL tube and add 10 mL of mannitol. Centrifuge at 1000 rpm (184 × *g*) for 5 min, pour off supernatant and repeat this process two more times. Finally suspend the pellet in 2–5 mL of the NBP-99 medium.
11. At this time point the pretreated microspores are handled differently in the three genetic transformation methods.

3.4 Microspore Electroporation-Based Transformation Procedure

1. Centrifuge microspore suspension at 900 rpm (149 × *g*) for 5 min. Pour off supernatant and add 0.5 mL of electroporation buffer (cf. Subheading 2.3), and supplement it with plasmid DNA at a final concentration of 10 ng/mL (*see* **Note 8**).
2. Incubate the suspended microspores and plasmid DNA at room temperature for 5 min, and pipette the microspore suspension into a chilled 2 mm cuvette for electroporation.
3. Preset a protocol with the following parameters on the Bio-Rad Gene Pulser Xcell unit (Fig. 2a) to give the electrical pulse: voltage = 375 V, capacitance (μF) = 150, resistance (W) = 200, cuvette = 2 mm, and duration = exponential decay pulse. Put the cuvette containing suspended microspores and plasmid DNA into the shock chamber of the GenePulsar and press the pulse button to deliver the voltage (*see* **Note 9**).
4. After electroporation, pour the microspore suspension from the cuvette into a 60 × 15 mm or 35 × 10 mm petri dish. Gently wash cuvette with 3 mL of induction medium (NPB-99) into the petri dish (Fig. 2b). Microspores should be plated within a density range of 1×10^4–1×10^7 microspores per milliliter NPB-99 solution (cell density determined using hemocytometer).
5. Microspores are cocultured with live ovaries of same or a different wheat variety (mature ovaries work best). Three ovaries per milliliter of electroporated microspore suspension is used. Dishes are covered with aluminum foil, sealed with Parafilm and incubated at 28 °C in dark without shaking for embryoid development.
6. At 20 or 21 day of incubation, transfer dishes containing microspore embryoids to a incubator maintained at 28 ± 1 °C and equipped with a shaker (shake ~50 rpm to ensure even distribution of nutrients) (Fig. 2c–e).
7. Follow microspore development during the incubation period under inverted microscope. Cultures following developmental pathway II or III should be discarded at day 20 (*see* **Note 10**).

3.5 Microspore Transformation by Cocultivation with *A. tumefaciens*

3.5.1 Preparation of AGL-1 Competent Cells

1. Streak AGL-1 cells from the glycerol stock onto the LB agar plates, and incubate it at room temperature for 1–2 days or until single colonies appear.
2. Inoculate 5 mL of LB medium with a single colony on the plate, and culture it overnight shaking at a speed of 250 rpm at the room temperature.
3. Use 1 mL of this over night culture to inoculate a liter of LB medium, and culture it overnight shaking at a speed of 250 rpm at room temperature or until an optical density (OD) of 1.5–2.0 at A_{600} is achieved.

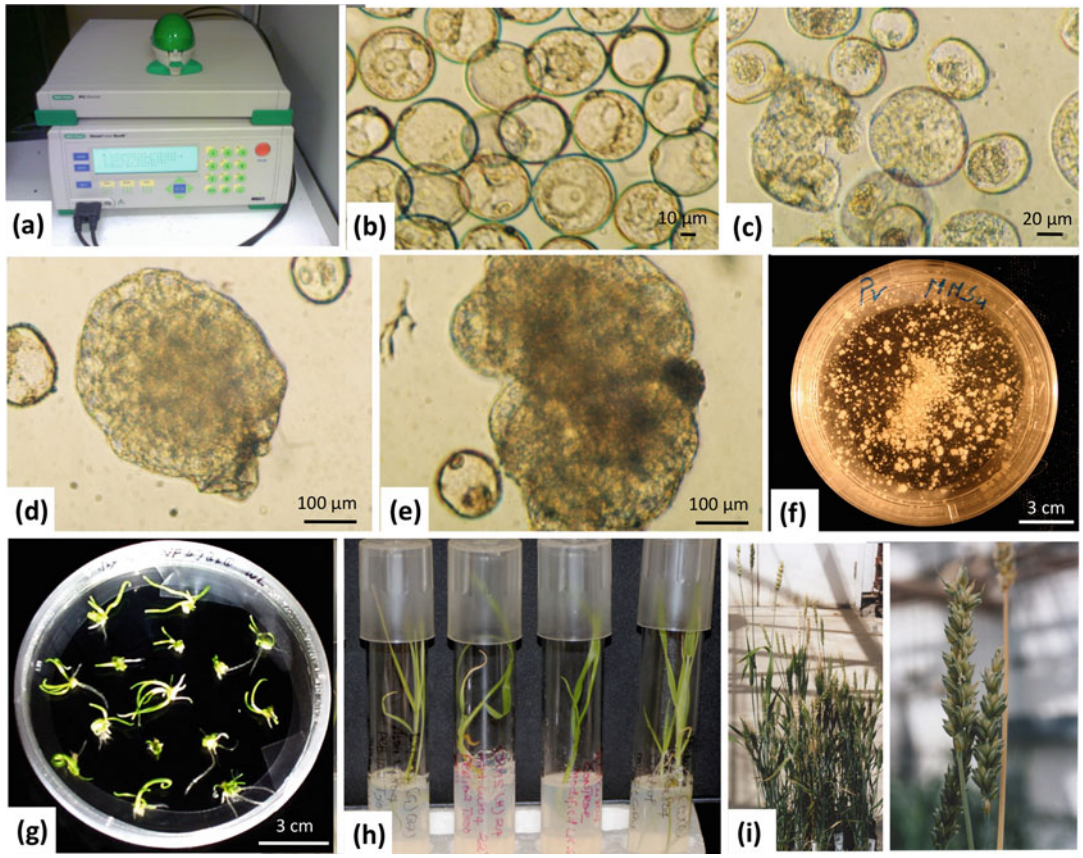

Fig. 2 Chronological display of events in bread wheat microspore cultures from microspore isolation to plant regeneration: (**a**) Bio-Rad Gene Pulser Xcell unit, used for microspore electroporation. (**b**) Embryogenic microspores after 7 days in induction media. (**c**) Embryogenic microspores after 14 days in induction media. (**d**) Embryogenic microspores after 21 days in induction media. (**e**) Embryogenic microspores after 28 days in induction media. (**f**) Embryoids growing on regeneration media, (**g**) Plantlets growing on regeneration media, (**h**) Plantlets transferred to test tube. (**i**) Transgenic plants growing in greenhouse

4. At this stage harvest the cells by centrifugation at 4500 × *g* for 5 min, and resuspend the pellet in 15 mL of cold sterile deionized H_2O. Again harvest the cells by centrifugation at 4500 × *g* for 5 min, and repeat this step three times.
5. After final centrifugation resuspend the clean cells in 5 mL of cold sterile deionized H_2O with 10% glycerol, prepare 150 µL aliquots of the competent cells in 1.5 mL Eppendorf tubes and store them at −80 °C after snap freezing in liquid nitrogen.

3.5.2 Transformation of Competent AGL-1 Cells by Electroporation

1. Thaw competent cells on ice, and dispense 40 µL of it into a prechilled cuvette with 0.2 cm slit. Add 1 µL of the vector DNA, dissolved in H_2O and preadjusted to a final concentration of 100 ng/µL to the cuvette, and mix the ingredients by

tapping. Allow the ingredients to settle down at the bottom of the cuvette (avoid air bubbles).

2. Place the cold and dry cuvette in the holder of the Bio-Rad Gene Pulser II, and electroporate at the following setting: 1.25 kV/cm, 25 μF, and 200 Ω. Immediately add 1 mL of the LB medium to the cuvette after electroporation.
3. Transferred the cell suspension to an Eppendorf tube and incubate it at room temperature in a shaker maintained at the speed of 200 rpm for 1 h. Plated 100 μL of the cells on a LB agar plate supplemented with kanamycin at a concentration of 50 μg/mL. Usually the colonies appear within 2–3 days, which indicates successful transformation of AGL-1 cells.
4. Isolate plasmid DNA using a plasmid miniprep kit or home made chemicals, and use it in PCR to confirm presence of the vector DNA in the AGL-1 cells.
5. Strip a positive single colony on a new LB agar plate supplemented with kanamycin (50 μg/mL), and incubate it at room temperature. Strip new LB agar plates every 2 weeks for a period of 2 months.
6. Use fresh AGL-1 cells from the latest plate for cocultivation with microspores. Alternatively, use these cells to make glycerol stock.

3.5.3 Preparation of AGL-1 Cells for Cocultivation with Androgenic Microspores

1. Culture freshly prepared AGL-1 cells containing the binary vector in 5 mL of the LB medium supplemented with kanamycin (50 μg/mL) on a shaker maintained at 200 rpm and 20 °C for 48 h or until OD of 1–1.5 at A_{600} is achieved.
2. Centrifuge the AGL-1 cells at 2500 rpm (Beckman F0630 Rotor) for 2 min, discard supernatant and resuspend the pellet in 2 mL of NPB-99 medium.

3.5.4 Microspore Preparation and Transformation

1. The method for obtaining microspores is as described in Subheading 3.3.
2. Add AGL-1 cells to 5 mL of microspores suspension in NPB-99 medium contained in 60 × 15 mm plate (*see* Subheading 3.3). After addition of AGL-1 cells seal plate with parafilm and incubate it for 45 min at 25 °C.
3. Carry out cocultivation of embryogenic microspores with 20% of *A. tumefaciens* cells in the culture medium at days 0 of the embryoid induction culture.

3.5.5 Methods for Elimination of A. tumefaciens post Cocultivation

1. To eliminate AGL-1 cells from the cocultivation medium pass it through a 38 μm filter it will retain the microspores on the filter, as microspores are larger (40–50 μm) than *Agrobacterium* cells (<5 μm). Subsequently, rinse the filter with NPB-99 medium to wash out the remaining AGL-1 cells.

2. Collect and reculture microspores in NPB-99 medium for embryoid production. Add timentin to the culture medium at a concentration of 200–400 mg/L to kill the escaped *Agrobacterium* cell.
3. Add 3–5 fresh ovaries to the plate and incubate it in dark at 25 °C for embryoid production.
4. At 20 or 21 days of incubation, transfer dishes containing embryoids to a incubator maintained at 28 ± 1 °C and equipped with a shaker (shake at ~50 rpm to ensure even distribution of nutrients).
5. Follow microspore development during the incubation period under inverted microscope. Cultures following developmental pathway II or III should be discarded at day 20 (*see* **Note 10**).

3.6 Biolistic Transformation of Microspore-Derived Calli

3.6.1 Preparation of Gold Particle Stock Solution

1. Weigh 60 mg of 0.6 μm gold particles in 1.5 mL Eppendorf tube. Add 1 mL of 100% ethanol to it and vortex the ingredients for 3 min. Centrifuge the mixture at 16,873 × *g* for 2–5 min using a bench top microcentrifuge and discard the supernatant.
2. Add 1 mL of 70% ethanol to the pellet and mix ingredients by vortexing for 2 min. Incubate tube for 15 min at room temperature. Mix contents at least three times during this period. After incubation centrifuge tube at 16,873 × *g* for 3 min, and discard the supernatant.
3. Add 1 mL of filter-sterilized deionized H_2O to the tube, vortex the ingredients for 10 s and let it stand for 1 min. Centrifuge tube at 16,873 × *g* for 2 min, and discard the supernatant.
4. Repeat **steps 3** for two times.
5. Add 1 mL 50% glycerol to the Eppendorf tube and store it at −20 °C for later use (*see* **Note 11**).

3.6.2 Preparation of Gold Particles for Bombardment

1. Vortex the previously stored gold particle solution until fully suspended. Pipette out 50 μL of the gold suspension into a 1.5 mL microcentrifuge tube.
2. Add following ingredient to the gold suspension in the stepwise fashion, and vortex for 5 s after each addition: 18 μL of plasmid DNA (pDPG165) adjusted at a concentration of 1 μg/μL, 50 μL of freshly prepared filter-sterilized 2.5 M $CaCl_2$, and 20 μL of 0.1 M spermidine free-base. After addition of all ingredients vortex the mixture for 30–40 min at 4 °C. Subsequently, add 200 μL of absolute ethanol to the mixture, vortex the suspension for 5 s and centrifuge it at 3000 rpm for 30 s. Remove supernatant, and repeat this step four times.
3. After final centrifugation step, resuspend the pellet in 30 μL of 100% ethanol, keep the mixture on ice until used.

3.6.3 Coating the Microcarrier

1. Submerge microcarriers in 100% ethanol in a petri dish. Using a forceps remove microcarriers from ethanol and dry them on Kimwipes.
2. Load microcarriers into the disks.
3. Vortex the gold suspension and quickly transfer 10 μL of it to center of the microcarrier and let it dry on the microcarrier.

3.6.4 Preparation of Plant Material for Microprojectile Bombardment

1. The method for obtaining microspores is as described in Subheading 3.3.
2. Transfer microspores to a 60 × 15 mm petri dish at a minimum density of 1×10^4/mL and a maximum density of 1×10^7/mL, as estimated with the hemocytometer (Fig. 3a).
3. Add mature ovaries of same or different wheat variety to the culture medium. Use 6–9 ovaries per dish (i.e., 3 ovaries per milliliter of culture medium). Cover dishes and seal with parafilm.
4. Incubate petri dish at 28 °C in the dark for embryoid development. After 20–21 days of incubation, transfer petri dish to an incubator maintained at 28 °C and equipped with a shaker.
5. Transfer 35–40 days old microspore embryoids to the regeneration media in 60 × 15 mm petri dish and arrange them in the centre of the petri plate prior to the bombardment (Fig. 3b–d).

3.6.5 Microprojectile Bombardment

1. For microprojectile bombardment the following steps are carried out in a serial order.
2. Turn on the vacuum pump.
3. Turn on the Helium cylinder valve slowly to 14,000 kpas, and set the regulator to 1500 psi.
4. Turn on power to the chamber.
5. Place rupture disc into centre of screw-on cylinder, and tighten the screw cylinder with torque tool.
6. Place stooping screen and microcarrier into the holder and screw lid.
7. Place tissue on shelf (second from the bottom) and close the door.
8. Turn on vacuum switch. When the vacuum gauge reads 27, it is ready to fire.
9. Turn vacuum switch to “hold”, and press “fire” switch.
10. After firing turn the vacuum switch to vent.
11. Bombarded embryoids plated on a regeneration media (190-2) are kept in the dark for 3 days at 28 °C and then exposed to 16 h light for shoot growth (Fig. 3e–g).

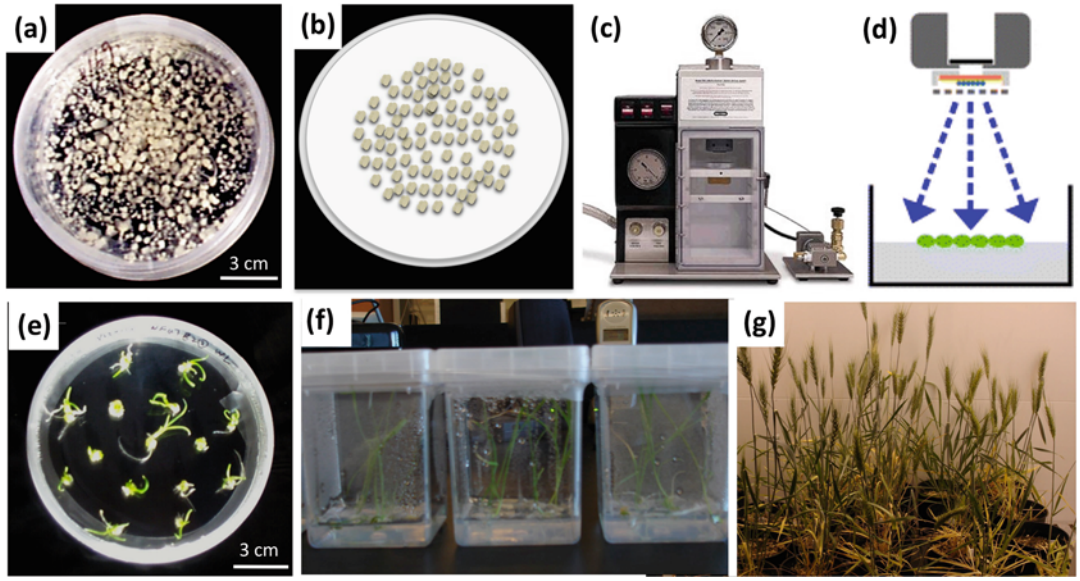

Fig. 3 Biolistic transformation of microspore embryoids. (**a**) 20–21-day-old microspore embryoids in induction medium. (**b**) Diagrammatic representation of 35–40-day-old microspore embryoids transferred to the regeneration media in 60 × 15 mm petri dish and arranged in the centre of plate prior to bombardment. (**c**) Gene gun. (**d**) Diagrammatic representation of the microcarrier launch assembly and the bombardment process. (**e**) Plantlets growing on regeneration media. (**f**) Plantlets transferred to Magenta box. (**g**) Transgenic plants growing in greenhouse

3.7 Regeneration

1. When embryos are approximately 2 mm (45 days old, do not keep them longer in induction media) in size transfer them to the regeneration medium (190-2) (about 20 embryos per 100 mm petri dish) (Figs. 2g and 3e).
2. In a laminar hood, transfer green germinated plantlets to test tube or magenta box containing the rooting medium (MMS5). Allow the plantlets to grow until the apex reaches the lid of the test tube or magenta box (Figs. 2h and 3f). Transfer the plants to pots containing the Cornell mix and place the root trainer in a growth cabinet or glasshouse (Figs. 2i and 3g).

3.8 Colchicine Treatment

1. Plants at tillering stage are appropriate for colchicine treatment. Removed plant from the pot and thoroughly wash to eliminate soil particles. Trim roots down to 2–3 in. and remove one third of the shoot to expose the lateral bud (Fig. 4).
2. Subsequently submerge the roots and crown in the colchicine working solution for 7 h (bubble solution with air during treatment). After colchicine treatment thoroughly wash the plant under running water and replant in soil.

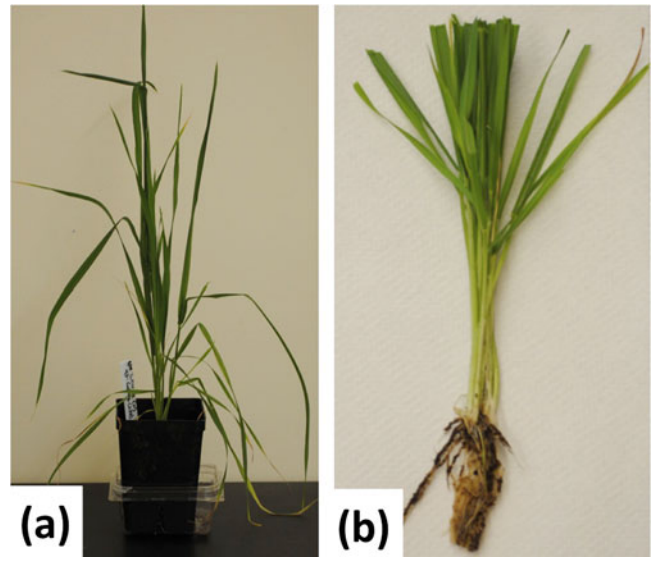

Fig. 4 Colchicine treatment. (**a**) Developmental stage of the regenerated plant used for colchicine treatment. (**b**) Plant after trimming off the old roots and shoots

3.9 Determination of Ploidy by Chromosome Analysis

1. Germinate seeds of the colchicine treated plants at room temperature in germination boxes. Move germination boxes to 4 °C for a day (to synchronize growth). Excise root tips when ~2–3 in. in size. Transfer root tips to 1.5 mL Eppendorf tubes containing chilled water followed by fixation at 0 °C in an ice bucket containing ice water slurry. Pretreat root tips for 20–22 h. After pretreatment replace water with 3:1 solution of absolute ethanol and acetic acid. Keep the tubes for 1 week at room temperature and then store at 4 °C until used.
2. Stain root tips in acetocarmine solution for 1 h, boil for a minute and then squash in 45% acetic acid. Put cover slip and gently tap the slide and heat gently on the flame of a spirit lamp. Press the coverslip evenly with thumb and observe under 10×, 40×, and 63×/100× (use immersion oil) optical lenses. Count 42 chromosomes to confirm a euhexaploid wheat cell.

4 Notes

1. Seven wheat cultivars for microspore electroporation based transformation method were selected based on their yield potential, performance under tissue culture and high end-use quality in their respective market classes. Five hard red spring (HRS) wheat cultivars: Chris, Express, Hollis, WestBred 926 and an early maturity super dwarf Perigee were selected in this market class. All of these cultivars have high grain protein

content required for production of leavened or flat breads. One hard red winter (HRW) wheat cultivar Farnum with high gain yield and protein content, required for production of high quality leavened and flat breads, was selected. One soft white spring (SWS) wheat cultivar Louise with low grain protein content, required for baking cookies and sponge cakes was selected. Similarly, spring wheat cultivars Chris, WED 202-16-2 (hard white spring), and NPBCT were selected based on their high culture-response [24] or transformability by particle bombardment [17].

2. The major advantage of using double cassette binary vector is in the development of "marker-free" transgenic plants, which has been independently demonstrated by studies conducted in tobacco, rice, and barley. These studies suggested that both T-DNA segments of a double cassettes vector cotransfer with a frequency of ~50%, and frequently insert into different chromosomal locations or to different chromosomes. It allows the two cassettes one with the selectable marker gene and the other with the gene of interest to segregate independently in the T_2 generation [28].
3. Integration of an intron in the selectable marker gene cassette in a binary vector prevents *A. tumefaciens* from expressing this gene, and the plant selective agent accelerates the removal of *A. tumefaciens* after cocultivation. The other advantage of introducing an intron between the promoter and the gene of interest is during the molecular characterization of transformants, where origin of the transcript can be tracked back to the transgene (integrated in plant genome) by the intron-splicing assay [29].
4. High expression of the enzyme and its activity in the endosperm of the mature cereal grain requires codon optimization of the encoding gene sequence to a C + G content of >60%, and synthesis as a precursor with a signal peptide for transport through the endoplasmic reticulum and targeting into the storage vacuoles [28]. Keeping the codon usage bias in account further increases the transgene expression [30].
5. Spike collection at the appropriate developmental stage is of paramount importance, as it maximizes the possibility of getting microspores at uninucleate stage and avoid the possibility of getting chimeric plants.
6. We do not expect chimerism in microspore-derived transformants; however to eliminate this possibility, 1–14 individual spikes from each green regenerant were tagged and checked for transgene integration(s). For this purpose, a part of flag leaf was collected and used for DNA extraction. To our surprise, in case of microspore electroporation and biolistic transformation

approaches, DNA samples collected from the same plant showed chimerisms, where only 1–4 leaf samples collected from individual tillers of a plant exhibited transgene integration. In wheat the harvested androgenic microspores represent a pool of uninucleate and binucleate microspores with generative and vegetative nuclei, which could lead to chimerism [29, 31]. Since we have not applied any selection during microspore propagation, it is possible that the nascent embryonic cells formed after a few cell divisions, might have dropped the foreign DNA and given rise to a chimeric callus. In view of the above mentioned possibilities, use of double cassette vector or cotransformation with vector carrying selectable marker gene is highly recommended in both microspore electroporation and particle bombardment based genetic transformation methods. The regeneration of chimeric plants after electroporation of microspores can be also explained by the integration of transgene in wheat genome after initial mitotic division(s). Transgenerational inheritance of transgene is an indicative of the transgene integration at some time point in the plant development, but it is difficult to determine the exact timing of the event. According to Kumlehn [31] "from a practical point of view, chimerism among primary transgenics does not constitute a serious problem, as this character is not sexually transmitted." Moreover, formation of a zygote from fusion of transgenic and nontransgenic gametes is an unlikely event.

7. A pretreatment of immature spikes with $CuSO_4$ solution (500 mg/L) at 4 °C for 10 days and incubation of microspores after transfection sequentially at 28 °C in NPB-99 medium and at 26 °C in MMS5 medium (Tables 1 and 2) result in recovery of large numbers green regenerants. Application of copper sulphate during pretreatment has shown to decrease the number of albinos in wheat as previously shown in barley, but the exact mechanism underlying the beneficial effect of copper is not understood [32].
8. To avoid electrical arcing during the electroporation process we improvised and used a minimal electroporation buffer, which is largely devoid of salts (in this minimal buffer acetosyringone serves as a preservative).
9. A lower voltage is used to cause minimal damage to the androgenic microspores; however, it may decrease the rate of transfection. The selection of the electroporation voltage largely depends on the wheat genotype and a voltage ranging from 250 to 500 V can be effectively used to transfect different wheat genotypes [29].
10. It is important to determine if the cultures are following correct developmental pathway through time-lapse tracking of

microspore cultures [29, 33]. The immature microspore with the single haploid nucleus can undergo three different developmental pathways: (1) The microspore retains a thin layer of cytoplasm with few organelles and membranes and does not develop the intine wall further. At a week the nucleus moves into the center and starts to divide after chromosome doubling. At day 19–21 the exine wall ruptures and the proembryos start to emerge (Fig. 2d). (2) This pathway is characterized by the early display of star-like morphology (a hallmark of embryogenic potential) in microspores. But in this case the multicellular structure forming under the exine wall was unable to break it after the 20 days of growth. (3) The third pathway does not allow the microspores to develop beyond day 7. Microspore cultures that develop according to pathways (2) and (3) should be discarded at day 20.

11. Gold particles stock solution can be prepared 1 day in advance and can be stored at −20 °C for at least 4–6 months.

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Chapter 14

Doubled Haploid Laboratory Protocol for Wheat Using Wheat–Maize Wide Hybridization

Meenakshi Santra, Hong Wang, Scott Seifert, and Scott Haley

Abstract

In traditional wheat breeding, the uniformity of lines derived from a breeding population is obtained by repeated selfing from the F1 which takes several generations to reach homozygosity in loci controlling traits of interest. Using doubled haploid technology, however, it is possible to attain 100% homozygosity at all loci in a single generation and completely homogeneous breeding lines can be obtained in 1–2 years. Thus, doubled haploid technology may significantly reduce cultivar development time. Two major methods for producing wheat doubled haploids are androgenesis (anther culture and microspore culture) and embryo culture using wheat–maize wide hybridization, the latter being the most effective and widely used method. The method of wide hybridization between wheat and maize is laborious but is widely successful for rapidly obtaining homozygous lines. This technique includes six major steps: emasculation of the wheat flower; pollination of the emasculated flower with maize pollen; hormone treatment; embryo rescue; haploid plant regeneration in tissue culture medium; and chromosome doubling. It has been observed that the efficiency of doubled haploid production depends on both maize and wheat genotypes, good plant health and proper greenhouse conditions (without disease, insects, or drought stress), and proper conduct of all procedures. Therefore, the procedures may need minor modification in order to produce higher numbers of embryos, haploid green plants, and doubled haploid plants.

Key words Wheat, Doubled haploid, Homozygous, Wide hybridization, Embryo rescue

1 Introduction

The use of doubled haploid (DH) plants has revolutionized modern plant breeding and genetic mapping studies in many important crops, specially maize, oilseed rape, sunflower, wheat, barley, rice, potato, citrus, and apple [1]. Rapid development of homozygosity shortens the breeding cycle time in wheat, leading to more rapid genetic gain and responsiveness to production and market challenges in commercial markets. The use of DHs may cut in half the time needed to produce a commercial cultivar in crops such as winter wheat [2]. Besides reducing time, DH technology fixes rare alleles and may play an important role in evaluation of genetic diversity. Thus, doubled haploid technology is useful in plant

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improvement for gene transfer and production of breeding lines, and in general biology for genome mapping and studies of chromosome behavior and phylogenetic relationships.

Doubled haploids are homozygous plants developed by androgenesis (microspore and anther culture), gynogenesis (ovary and ovule culture), and wide hybridization. In wheat, the wide hybridization method is the most effective for producing doubled haploids [3]. The method of crossing between species of the same or different genera is called wide hybridization. In the manipulation, exploitation, and study of alien genomes through wide hybridization, the first step required is to make a hybrid. The alien genome can then be studied and utilized to various degrees from the whole genome to single chromosomes to specific gene transfer by manipulating the hybrid genome through either natural recombination or specialized techniques, depending on the genomic relationship of the two species. However, crossability barriers often make hybridization difficult. These barriers, caused by gene action, genome incompatibility, different ploidy levels of species, and the environment, lead to no or poor seed set, reduced hybrid seed development, or sterility of the hybrid plant. In nature, hybridization is suppressed owing to less favorable conditions for fertilization, hybrid seed development, and survival of F1 sterile plants. Under artificial conditions, however, genotypic variation for crossability, multiple pollinations, application of growth regulators, and in vitro techniques can be exploited to make wide hybridization possible. In making the hybrid, seed abortion is the major barrier, largely caused by the failure of proper endosperm development and its inability to nourish the embryo. The technique of rescuing the embryo from an aborting seed into a plant in vitro is called embryo rescue or embryo culture. Thus, in embryo rescue following wide hybridization, the synthetic medium provides the life support for the hybrid embryo. The technique has been used routinely for several decades, but procedures and modifications are often incompletely described in publications. Wide hybridization has been becoming popular in wheat since late 1970s which has been described in several literatures [4–8]. In this chapter, we report an improved method for wheat doubled haploid production using wide crossing with maize.

2 Materials

General Lab Supplies and Equipment: The following general supplies, reagents, and equipment are required for establishment of the DH laboratory.

1. Wheat seeds and sweet maize varieties. Wheat F1 seeds are most commonly used though some labs may be DH production from the F2 or other generations.

2. Glassine bags, surgical scissors, forceps, scalpels, and stapler.
3. Laboratory glassware such as conical flasks, beakers of different volumes, pipets, magnetic stirrers, culture tubes, and baby jar bottles.
4. Refrigerator, laminar flow cabinet, distilled water unit, autoclave, pH meter, digital balance with four-digit precision, stirrer with hot plate, and aluminum foil.
5. Hydrochloric acid (1 N HCL) and sodium hydroxide (1 and 10 N NaOH) solutions to adjust the pH of the media.
6. Sodium hypochlorite (bleach) and cheesecloth to sterilize the hybrid seeds produced by wide hybridization.
7. Alcohol burner or germinator™ 500 (also called germ terminator).
8. Wheat varieties: Desired wheat parents are crossed to get F1 seeds prior to this technique. Winter wheat seedlings are routinely vernalized at 2 °C for 8 weeks before planting. Spring wheat seeds can be planted directly without any vernalization.
9. Maize varieties: Early maturing varieties are preferred in order to reduce the time required to get them to flower and maintain them. Seeds of a Canadian sweet maize variety were obtained from Washington State University's wheat doubled haploid laboratory. Three early maturing varieties (all are sweet maize hybrid) were obtained from Burpee Seed Company. These three varieties were SE 1 (early & often hybrid), SU (early sunglow hybrid), and SE 2 (Peaches & Cream hybrid).
10. 2,4-D solution for spray: Dissolve 213 mg/L 2,4-D (Sigma) in 10–15 mL of 70% ethanol or 95% if necessary and then the volume is made up to 1 L and the pH is adjusted to 10.36.
11. Media preparation: Multiple media are used to culture the haploid embryos [3]. Half strength MS media can be prepared in laboratory using each component of micro, macro, and iron source. Alternatively, commercially available full strength MS media can be used by diluting to half strength. We use M519 basal media diluting into half strength (2.22 g/L M519 from Phytotechnology Labs, 30 g/L sucrose, 3 g/L phytagel, 0.5 mL PPM, 200 mg/L ascorbic acid and pH 5.75) in culture tubes and baby jar bottles. PPM is plant preservative mixture. The medium is sterilized by autoclaving at 121 °C for 15 min. The media can be used immediately. For small embryos, half strength MS media is prepared with phenyl acetic acid (PAA) and kinetin (auxin and cytokinin) with a concentration of 0.5 mg/L. Filter-sterilized hormones are added to warm sterilized media and then media are distributed into sterile culture tubes.

12. The colchicine stock solution (5%) is prepared by dissolving 5 g colchicine in 50 mL dimethylsulphoxide (DMSO) solution directly into a glass bottle. A working solution (0.05%) is prepared using 5 mL of the colchicine stock solution, 15 mL DMSO, 10 drops of Tween 20, 100 μL of GA_3 (1 mg/mL stock), brought to 1 L volume with double distilled water in an amber-colored bottle.

3 Methods

3.1 Maize Planting and Management

Maize was planted 3 weeks before transplanting vernalized wheat seedlings and is then planted every week to ensure a continuous supply of pollen. At every planting time, seven to ten pots (3 gallon) were planted with three seeds in each pot. We mix Pro mix mycorrhizae soil and green grade (clay soil) with osmocote, a slow-releasing fertilizer. Pots were watered as needed and were fertilized weekly with a fertilizer solution (general purpose fertilizer). The maize plants are grown in a separate greenhouse from the wheat as it favors a warmer environment than wheat, especially at night. Its day and night temperature requirement is 30/20 °C with a 16 h day–8 h night photoperiod. Maize plants should be healthy in order to get viable pollen and should be monitored from time to time for diseases and pests (*see* **Note 1** and Fig. 10).

3.2 Wheat Vernalization, Planting, and Management

F1 plants obtained from crossing between desired parents are used as female parents for the wide hybridization. The plants should be healthy without incidence of diseases or pests (*see* **Note 1**). Winter wheat needs 8 weeks of vernalization whereas spring wheat seeds may be planted directly in pots. Before vernalization winter wheat seeds are germinated on blotter paper and transferred to 4 °C for imbibing. Sometimes dormancy in recently harvested winter wheat seeds may be reduced by using gibberellic acid (GA_3) treatment (0.01 g/L) for germination. As soon as vernalization is over, wheat seedlings are transplanted two seedlings per one gallon pot. Wheat favors day and night greenhouse temperatures of 25/15 °C with a 16 h day–8 h night photoperiod. Pro mix mycorrhizae soil with osmocote fertilizer is used and once the wheat starts flowering, a general purpose fertilizer is applied. Watering is done on alternate days or if the soil appears dry. Staggered planting of wheat seedlings by 7–10 days may be an option to spread the workload and reduce the need for large amounts of maize pollen on any single day.

3.3 Emasculation

Selection of the correct developmental stage of the wheat floret is very important. A floret approximately 2/3 of the way up the spike should be checked to determine if the spike is ready for emasculation. Each wheat floret contains three anthers along with a pistil and

the process of removing three anthers form each floret is called emasculation. If the anthers are starting to change from a dark green color to a light green color and the stigma is fluffy, the spike can be emasculated. The stigma is the hairy tip of the pistil. Starting at the bottom of the spike, emasculation is done by removal of the central florets of each spikelet leaving only the two lateral florets. The lemma, palea and glumes of each floret are trimmed down to the shoulder with a small sharp scissor without damaging the stigma. Anthers are then removed using a forceps from the lateral florets in a systematic pattern from the bottom to the top of the spike. The top or bottom spikelets may be completely removed if they are too small to emasculate. If the spikes have awns, they should be cut fairly close to the glumes before starting to emasculate. Once emasculation is complete, the spike is bagged with a glassine bag that is properly labeled with the name of the cross and the day of emasculation (*see* **Note 2** and Figs. 1, 2, 3, 4, 5, and 6).

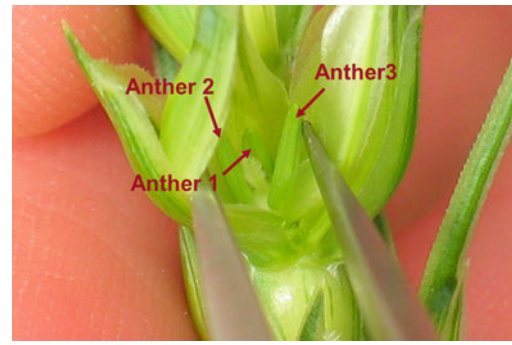

Fig. 1 Male reproductive parts

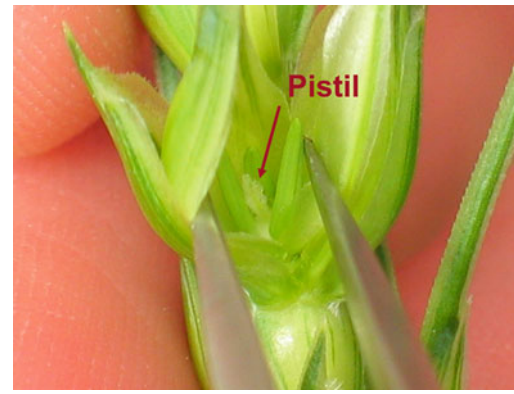

Fig. 2 Female reproductive part

Fig. 3 A wheat spike ready to be emasculated

Fig. 4 Clipping of spikelets prior to anther removal during the emasculation process

3.4 Pollination

Anthers are collected from maize plants when the anthers are starting to emerge, being very careful not to disturb the other anthers as the pollen will be lost. The end of the anther should look like it is opening up. Anthers from multiple maize plants are collected at the same time and placed in a petri dish. The glassine bags are removed from the emasculated wheat spikes 1 day after emasculation, or when the stigma has a feathery appearance. The color of the pollen should be light or pale yellow for optimum viability and it should be used within 15–20 min, prior to turning an orange color (Fig. 7). The pollen is then brushed onto each stigma on the emasculated spikes using a small paint brush; the pollen should flow easily from

Fig. 5 Process of emasculation of wheat flowers

Fig. 6 An emasculated wheat flower

Fig. 7 Fresh pollen collected in a petri dish

the anther to the emasculated florets. Once the entire spike is pollinated, the date of pollination is recorded on the glassine bag and the bag is replaced over the spike (*see* **Notes 3–5**).

3.5 Hormone Spray

The day after pollination the spikes should be sprayed with 2,4-D (Sigma) solution (213 mg/L with pH 10.36). The hormone treatment is applied by spraying the solution on the spike using a small aerosol sprayer bottle. The hormone treatment should be done within 24–48 h after pollination. After hormone treatment, the glassine bags are replaced and secured with a stapler (*see* **Note 6** and Figs. 8 and 9).

3.6 Excision of Embryo or Embryo Rescue

From 16 to 19 days after pollination, spikes are cut off leaving approximately 20 cm of stem with the spike; spikes with stems are placed in a container of double distilled water (Fig. 11). The caryopses (hybrid seeds) are removed carefully with forceps

Fig. 8 Hormone treatment

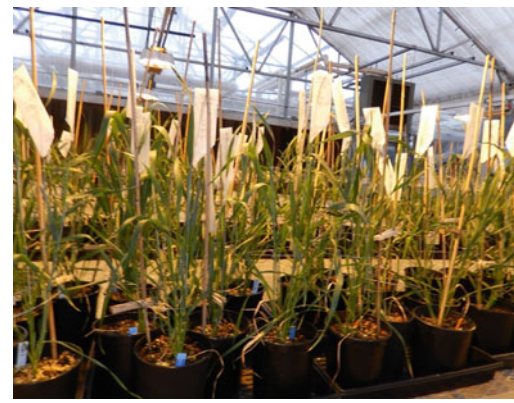

Fig. 9 A part of green house with pollinated and hormone treated wheat heads

Fig. 10 Growing maize plants

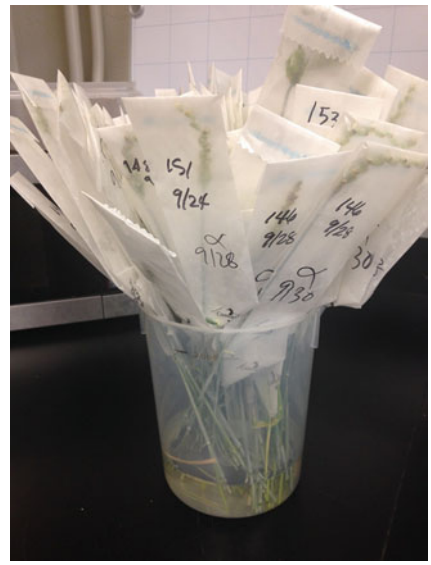

Fig. 11 Harvesting pollinated and hormone treated wheat heads after 16 days

(Fig. 12). The culture of the embryos should be done under sterile conditions in a laminar hood. Embryos should be rescued within 7 days after collecting the spikes. The spikes are kept at 4 °C until embryos are rescued. The caryopses are surface sterilized with 70% alcohol for 1 min. The ethanol is drained and the seeds are covered with a 60% sodium hypochlorite (bleach) solution for 10 min with 2 or 3 drops of Tween 20. It is better to keep the bleach solution on

Fig. 12 Removal of hybrid seeds from wheat–maize hybrid heads

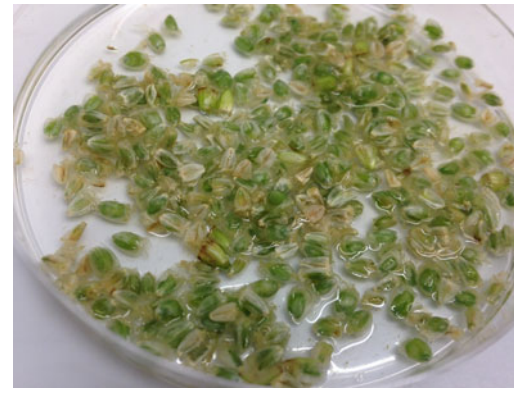

Fig. 13 Sterilization of wheat–maize hybrid seeds

a gentle shaker. The bleach solution is poured off and seeds are rinsed five times with sterilized double distilled water. Seeds are transferred into sterile petri dishes and then dissected carefully; all tools and work surfaces are sterilized multiple times during a work session (Figs. 13 and 14). For dissection, a dissecting microscope and dissecting kit, comprised of sharp forceps and scalpels, is required. Dissection can be achieved in one of the following two ways.

From the embryo end, hold the caryopsis with the forceps, and cut the brush end of the caryopsis using a scalpel. Tilt the open end of the caryopsis toward the microscope with the forceps. The embryo is a small white or translucent structure within the caryopsis. Since there is no solid endosperm present, it should be fairly easy to see if there is an embryo present. Another way to open the caryopsis is to steady the caryopsis with forceps or scalpel and use another set of forceps to grab the seed coat 2/3 of the way from the embryo end and pull back exposing the embryo. Sometimes if there are too many harvested seeds to complete embryo rescue in a single

Fig. 14 Embryo rescue under stereo microscope

Fig. 15 Regeneration of rescued embryos in dark incubator

day the sterilized seeds may be kept in sterile petri dishes at 4 °C for 2–3 days. The haploid embryos can be recognized easily because the caryopsis will be filled with fluid instead of endosperm and the embryo will be loose. A solid endosperm means it was pollinated with wheat pollen and is not haploid. The embryo is placed on the petri dish with the nose like structure up and scutellum side down on the media. Make sure that the embryos are placed on the media in the correct orientation to encourage embryo germination instead of callus production (*see* **Notes 7** and **8**).

3.7 Regeneration of Embryos

After excision, the embryos are placed in ½ strength Murashige–S-koog (MS) basal medium [9]. The embryos are placed in a dark incubator at 23 °C for 1–2 weeks (Fig. 15). Once good roots and shoots form, the small plantlets are transferred to a lighted incubator at 23 °C (Fig. 16). The lighted incubator is maintained using a 16 h day–8 h night photoperiod. Sometimes very small embryos are

Fig. 16 Regenerated plants with well-developed roots and shoots in a lighted incubator

rescued and these embryos do not grow in normal hormone-free media. Those small embryos are placed in hormone media fortified with kinetin and phenyl acetic acid (PAA).

3.8 Vernalization of Haploid Plantlets for Winter Wheat

Once the regenerated plants show good root and shoot development in the tubes or vials, we place them in a refrigerated chamber at 4 °C for 8 weeks. Regenerated haploid plants are vernalized prior to colchicine treatment. Treatment with colchicine after vernalization requires more time but leads to greater survival of the haploid plants. We transfer haploid plants to potting soil in flat trays after 8 weeks and treat them with colchicine solution when plants become healthy. Vernalized plants are planted into a soil medium with good nutrition in a flat having 48–96 cells. Once plants reach the 5–6 leaf stage, we remove them from the soil and wash the roots to remove the soil. The roots are then trimmed to about 1 in length prior to dipping into the colchicine solution.

Spring wheat does not need vernalization and spring haploid plants with well-developed roots and shoots are planted directly to flats and then they are treated with colchicine after about 2 weeks (or when they reach the 5–6 leaf stage).

3.9 Colchicine Treatment

In our laboratory, we treat the plants with colchicine after vernalization for winter wheat. Colchicine is an alkaloid obtained from *Colchicum* species. It is very toxic and must be handled with care and appropriate protection (gloves, safety glasses, and lab coats). Colchicine interferes with microtubule organization and inhibits normal chromatid separation during mitosis, resulting in a cell with double the chromosome number. Treatment is done at the 5–6 leaf stage. Colchicine treatment is done under a fume hood and plants are given overnight treatment (14–15 h) under dark.

Following the overnight treatment, the colchicine solution is poured into a waste bottle and roots are washed in tap water for several minutes. Roots of rinsed plants are kept in tap water for

2–3 h before planting them into pots or soil beds (Fig. 17). For the first few days following transplantation, plants are frequently sprayed with a water mist in order to prevent desiccation until they are well established in the soil. With proper nutrition and moisture the plants will reach maturity in approximately 4 months (Fig. 18).

Fig. 17 Planting chromosome-doubled plants in a soil bed in the greenhouse

Fig. 18 A wheat spike with seeds in chromosome doubled plant

Using our protocol we are able to get 50–90% success in chromosome doubling. The efficiency of chromosome doubling shows a wide variation depending on the wheat genotype.

4 Notes

1. F1 wheat plants and maize plants should be healthy without any diseases, insects or drought stress. It is challenging to maintain complete disease, pest and drought free condition of the plant materials throughout the DH production procedure. But continuous observation on each plant for the mentioned factors improves the efficiency of the procedure. For example if we start seeing the onset of any disease or pest then we immediately spray preventive chemicals to encourage the plants to grow healthy tillers.
2. Emasculation should be done carefully so that the stigmas are not damaged. The flag leaf should not be removed or damaged since it provides nutrition to the embryos.
3. A pollen mixture from several maize varieties improves seed set compared to using pollen from only one maize variety. It is advisable to do pollination twice over 2 days to increase seed set.
4. Information about the cross, date of emasculation and date of pollination are recorded on the glassine bag. While some laboratories use different colored tags to track the procedures done (pollination, hormone spraying, etc.) in our laboratory we write all the information on the glassine bag to simplify the procedure.
5. Good maize pollen production is critical and can be challenging to maintain over the course of the wheat flowering period. The numbers of pots and planting dates of maize seeds may need to be adjusted for individual environments to ensure proper nicking of the maize and wheat.
6. Hormone treatment should be done within 24–48 h after pollination. Proper care should be taken while spraying 2,4-D, since it is a toxic chemical.
7. The seed is composed of pericarp, endosperm, and embryo. The pericarp covers the embryo and the endosperm and forms a brush at the apex of the seed. The embryo is at the end opposite to the hairy tip. The scutellum forms the largest part of the embryo. It is attached to the embryonic axis (bulging side), and its lower side is flat. Pair of forceps and a scalpel are needed for incision; an opening can be made with the forceps used to peel off the pericarp. The embryo is uncovered only partly and a little more pericarp can be peeled away. It is a simple question of breaking the seed and getting the embryo out. Any variation in the operation is fine as long as the embryo is not injured.

Occasionally, the naked embryo may not stay on the seed and may come out into the petri dish. Lifting the embryo is easy if the scalpel is moist. A somewhat dehydrated embryo will stick to the surface moisture of the scalpel. Care must be taken to avoid injury to the embryonic axis or push the embryo into the medium if the embryo is too small to handle with the scalpel. The scalpel or a dissecting needle with a tip moistened with the culture medium or sterile water can be used to pick up the embryo. Embryos should be placed in the medium with the nose like structure pointing upward so that shoot formation is not hindered by the medium.

8. Water is sprinkled over recently chromosome doubled plants frequently (3–4 times in a day for 1 week) using water misting nozzles to help them recover from the toxic effect of colchicine and to form new tillers. In our greenhouse, we use soil beds rather than pots for growing chromosome-doubled plants as the soil beds provide greater water holding capacity and more optimal growing conditions. Larger pots (3 gallon pot) are better than 1 gallon pot to grow chromosome doubled plants. The soil should be aerated a few times at the beginning to encourage additional tiller production. The aeration is done by loosening the soil with a small garden shovel.

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Chapter 15

Real-Time PCR for the Detection of Precise Transgene Copy Number in Wheat

Angelica Giancaspro, Agata Gadaleta, and Antonio Blanco

Abstract

Despite the unceasing advances in genetic transformation techniques, the success of common delivery methods still lies on the behavior of the integrated transgenes in the host genome. Stability and expression of the introduced genes are influenced by several factors such as chromosomal location, transgene copy number and interaction with the host genotype. Such factors are traditionally characterized by Southern blot analysis, which can be time-consuming, laborious, and often unable to detect the exact copy number of rearranged transgenes. Recent research in crop field suggests real-time PCR as an effective and reliable tool for the precise quantification and characterization of transgene loci. This technique overcomes most problems linked to phenotypic segregation analysis and can analyze hundreds of samples in a day, making it an efficient method for estimating a gene copy number integrated in a transgenic line. This protocol describes the use of real-time PCR for the detection of transgene copy number in durum wheat transgenic lines by means of two different chemistries (SYBR® Green I dye and TaqMan® probes).

Key words Durum wheat, Genetic transformation, Real-time PCR, Transgene copy number

1 Introduction

The most commonly used transformation technologies, such as particle gun (biolistic) or *Agrobacterium tumefaciens*, usually lead to the integration of delivered DNA (transgenes, intragenes, cisgenes) in multiple copies at the same or different chromosomal locations [1]. Thus, estimation of gene copy number represents a crucial branch of genetically modified (GM) crop research, as it can severely influence the expression level and the genetic stability of both exogenous and endogenous genes. While multiple copies of a transgene may be useful for over-expression experiments, single-copy transformation events are desirable for most applications as they are supposed to ensure a greater stability over generations of subsequent breeding [2]. Thus, a reliable method is required allowing researchers to select in early stages of plant development only primary transformants carrying one or few integrated copies, which

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are more likely to yield high-level expression of the exogenous gene and can be more successfully destined to field cultivation.

Transgene copy number in plants is traditionally determined by Southern blotting. While reliable, this technique is laborious, time consuming, requires large amounts of starting plant tissue and involves the use of hazardous radioisotopes. Moreover, Southern analysis may fail in detecting the exact number of transgene copies if these integrated in the host genome as a rearranged pattern. Recently, a new method has been suggested to estimate transgene copy number in transformed plants based on quantitative Real-Time PCR [3, 4]. This new approach has successfully been applied to several crop species such as bread [5] and durum [6] wheat, maize [7, 8], rice [9], soybean and peanut [10], *Nicotiana attenuata* [11], *Citrus* [12], tomato [13], liverwort [14], and tea [15].

Real-Time PCR allows detection of a target DNA sequence by means of an absolute or relative quantification. In the first case, the exact level of the sequence in a sample is directly inferred from a standard curve prepared from a dilution series of standard templates of known concentration, usually represented by a plasmid containing the cloned gene of interest. Besides, relative quantification allows to determine the upregulation or downregulation of a gene of interest relative to a calibrator, and it is mainly used in gene expression studies. In both cases, DNA level variation during amplification is registered by following a reporter molecule.

Reporter molecules used in real-time reactions can be nonspecific DNA-binding fluorophores (e.g., SYBR® Green I), fluorophore-labeled primers (e.g., LUX®) or sequence-specific probes (e.g., TaqMan®, Scorpions®). While DNA-binding dyes are low cost and easy to use, they show the disadvantage of binding to any double-stranded DNA, thus leading to the detection of both target sequence and nonspecific amplification products. On the contrary, oligonucleotide probes specifically bind only to the target sequence allowing a higher level of detection specificity, and enabling the quantification of several targets in the same sample within a single PCR reaction.

Real-time PCR is the method of choice for the quantification of DNA and RNA abundance. It is used in several applications both in animal and plant field such as: microbiological analyses [16], monitoring of viral load in infected plants [17–21], detection of allergens [22, 23], testing of vaccines [24], detection of genes involved in toxinogenesis [25, 26], testing food adulteration or contamination with transgenic DNA [27]. Recently, this quantitative PCR assay is being applied also in a growing number of molecular analyses including gene expression detection, expression profiling, SNP analyses, and validation of microarray data.

Here we report the protocol for the use of quantitative real-time PCR to determine transgene copy number in transformed durum wheat (*Triticum turgidum* L. var. durum) lines, by using two different chemistries: SYBR® Green I dye and TaqMan® probes.

2 Materials

2.1 Plant Material and DNA Constructs

1. *Transgenic lines*: durum wheat lines transformed with the transgenic constructs.
2. *Control lines*: not transformed lines of the same wheat cultivars.
3. *Plasmid vectors*: the circular constructs used in the genetic delivery, containing the transgenes of interest.

2.2 Controls for Real-Time PCR Experiments

1. Not transformed lines of the wheat cultivars.
2. NTC (No Template Control): reaction mix free of template DNA.
3. NAC (No Amplification Control): reaction mix free of DNA polymerase.

2.2.1 Negative Controls

2.2.2 Positive Controls

The plasmid vectors delivered in the genetic transformation.

2.3 SYBR Green® PCR Master Mix

SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs, dUTP and optimized buffer components.

2.4 Platinum Quantitative PCR SuperMix UDG

AmpliTaq Gold DNA Polymerase, dNTPs, dUTP, uracyl-N-glycosilase UNG.

3 Methods

3.1 Preparation of DNA

1. Isolate genomic DNA from control and transformed wheat lines by using standard procedures. Assess purity and integrity of extracted DNA by reading 260 nm/280 nm absorbance ratio and visualizing on 0.8% (w/v) agarose gel.
2. To obtain suitable quantity to use as standard templates for building calibration curves, transform plasmid vectors into *E. coli* competent cells by *heat-shock* method, select them on LB solid medium containing 50 mg/L ampicillin. Use standard procedures for plasmid preparations. Check each plasmid preparation for the inserted transgene by means of PCR amplification with specific primer pairs, and by endonuclease digestion followed by electrophoretic separation on 1.8% (w/v) agarose gel.
3. Calculate the exact DNA concentration of transformed and nontransformed (control) wheat lines by means of fluorimetric measurement, then convert this value into the number of copies of genome/μL using the following formula: (DNA concentration [g/L]/Molecular weight [g/mol]) × Avogadro no. × 10^{-6} (*see* **Note 1**).

3.2 Calibration Curves

Prepare serial dilutions starting from known concentrations of standard templates (vector plasmids), and run each in triplicate in SYBR® Green reactions (*see* **Note 2**). Obtain calibration curves for each primer-probe system by plotting the log of starting standard DNA amounts versus the corresponding Ct. (*see* **Notes 3** and **4**).

3.3 Real-Time PCR Reactions

3.3.1 Optimization of Primers and Probes Performance

1. For each transgene design suitable primers to amplify short DNA fragments in a 50–150 bp range (*see* **Note 5**). Set a PCR with a gradient of different annealing temperatures for each designed primer pair, in order to check for specificity and determine the best annealing temperature. Analyse amplification products for the expected molecular sizes by electrophoresis in 1.5% (w/v) agarose gel.
2. Optimise primer concentrations for TaqMan® and SYBR® Green I assays by running preliminary Real-Time PCR reactions with nine different combinations of primer concentration (50, 300 and 900 nM for each primer), then choose those giving the highest endpoint fluorescence and the lowest Ct value (*see* **Note 6**).

3.3.2 TaqMan® Reactions

Perform TaqMan® reactions in a 15 μL total reaction volume containing 1× Platinum Quantitative PCR SuperMix UDG (AmpliTaq Gold DNA Polymerase, dNTPs, dUTP, uracyl-N-glycosilase UNG), 500 nM of ROX passive reference, optimized concentration of specific probe and primers, 5 ng of sample DNA (*see* **Note 7**), and water to the final volume. Use the following thermal parameters: 1 cycle at 50 °C for 2 min (activation of UNG), 1 cycle at 95 °C for 10 min (DNA polymerase activation), followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (combined annealing and extension). Amplify positive and negative controls along with samples. Run all samples in triplicate. Repeat each experiment twice.

3.3.3 SYBR Green® I Reactions

Perform SYBR Green® reactions in a 15 μL total reaction volume containing 1× SYBR Green® PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs, dUTP and optimized buffer components), 500 nM of ROX passive reference, optimized primers concentration, 5 ng of sample DNA, and water to the final volume. Run the same cycling thermal parameters as for TaqMan assays, with an additional dissociation step (melting curve) performed as follows: 1 cycle at 95 °C for 15 s, 1 cycle at 60 °C for 30 s, and 1 cycle at 95 °C for 15 s (*see* **Note 8**). Amplify positive and negative controls along with the samples. Run all samples in triplicate. Repeat each experiment twice.

3.4 Calculation of Transgene Copy Number and Statistical Analysis

Determine transgene absolute quantity in each transformed line by comparing the Ct of samples against the calibration curve built from serial dilutions of standard templates (plasmid vectors). Calculate the precise number of integrated transgenic sequences in each wheat line from the ratio between the absolute quantity of each transgene and the genome copy number (*see* **Note 9**).

4 Notes

1. As durum wheat is an allotetraploid species, refer the transgene copy number to each haploid genome.
2. To accurately quantify all the samples, opportunely adjust the least and most diluted points of the calibration curves so that samples Ct values fall within the linear section of the curve. For each gene, run the standard curve on the same plate as the samples in order to ensure good reproducibility of Ct values, as these may vary if run at different times and plates.
3. Optimize the calibration curves by adjusting the threshold and the baseline till you obtain an R square value (R^2 or Pearson Correlation Coefficient) very close to 1 and a slope of about −3.3. The threshold is the fluorescence value slightly above the background fluorescence measured before exponential growth starts. Thresholds is either calculated according to an algorithm of the real-time PCR software or set manually.
4. As real-time absolute quantification is based on the linear correlation between starting amount of the template DNA and fluorescence intensity at the Ct, preliminary SYBR Green experiments are necessary to test the linearity between Ct and log copy number. This allows to evaluate if the amount of the template and its Ct vary linearly during real-time PCR.
5. As shorter amplicons work more efficiently, primer pairs have to be designed that amplify short DNA segments.
6. All the real-time PCR amplifications are run in presence of specific primer pairs for the target genes. TaqMan probe is usually drawn slightly downstream of one of the primers. In case of exogenous genes showing sequence similarity to endogenous wheat genes, primers for the detection of each transgene should be opportunely designed as one complementary to the vector sequence and the other one annealing to the insert sequence. The use of primer pairs encompassing the vector sequence ensures the amplification of only the transgenic insert, avoiding any endogenous homologous sequence.
7. For each wheat line, use a single DNA extract in all the experiments to ensure good reproducibility and allow a correct quantification.

8. While in TaqMan® assays amplification specificity is ensured by the use of a probe annealing to the gene of interest, in SYBR® Green reactions primer specificity is checked by performing a melting curve following each amplification reaction. This dissociation step allows the detection of any nonspecific amplification product, including primer-dimers, as multiple thermal transitions.
9. Estimation of precise transgene copy number by real-time PCR needs an endogenous control gene, but choosing an appropriate internal gene can be problematic. In this protocol we optimized the detection of transgene copy number by using as a control the same vectors containing the cloned transgenes. In this way, a correct concentration of plasmid copies can be made in order to obtain an accurate standard curve that is a key element for the quantitative assay.

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Chapter 16

Endogenous Reference Genes and Their Quantitative Real-Time PCR Assays for Genetically Modified Bread Wheat (*Triticum aestivum* L.) Detection

Litao Yang, Sheng Quan, and Dabing Zhang

Abstract

Endogenous reference genes (ERG) and their derivate analytical methods are standard requirements for analysis of genetically modified organisms (GMOs). Development and validation of suitable ERGs is the primary step for establishing assays that monitoring the genetically modified (GM) contents in food/feed samples. Herein, we give a review of the ERGs currently used for GM wheat analysis, such as *ACC1*, *PKABA1*, *ALMT1*, and *Waxy-D1*, as well as their performances in GM wheat analysis. Also, we discussed one model for developing and validating one ideal RG for one plant species based on our previous research work.

Key words Endogenous reference gene, Genetically modified wheat, GMO detection

1 Introduction

1.1 General Principle and Definition of Endogenous Reference Gene (ERG) in GMO Analysis

Although genetically modified organisms (GMOs) have been developed and commercially planted by many countries and regions during the past two decades, controversies over safety issues have always been hot topics in public discussions. In global area, the debate on whether or not genetically modified staple food crops (wheat and rice) should be allowed is still ongoing. To protect consumers' rights, many countries and regions have issued regulations and legislations to strengthen commercial GMO release and labeling management [1–3]. To effectively implement GMO labeling regulations, a lot of efforts have been made to develop sensitive and accurate assays for identification and quantification of GMOs, such as qualitative PCR and quantitative real-time PCR.

Reliable qualitative PCR and quantitative real-time PCR GMO assays should be developed on the basis of the selection of appropriate endogenous nuclear DNA sequences as endogenous reference genes (ERGs) [4]. For qualitative PCR assays, ERGs are used

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not only to confirm the identity of plant species, but also as control to confirm the quality of extracted DNAs [5, 6]. For real-time quantitative PCR assays, ERGs are used for estimation of the total amount of plant genomic DNA in tested samples, and GM contents are calculated by the copy number ratio of transgenic specific sequences to plant genomic DNAs [5, 6].

An ERG for a specific plant species generally has three characterizations, which are good species specificity, single copy or low copy number, and low heterogeneity among different cultivars [7]. ERGs should be taxon-specific, meaning that their DNA sequences should be unique for their target species and have no or very low homology to other species [7]. Copy number is also important, especially for the quantification of GM contents. One ideal ERG should be single copy in haploid genome [7]. Low heterogeneity among cultivars means that there should be no allelic variation and change of copy number of an ERG among different plant cultivars [7].

ERGs conforming to these characterizations have been developed and validated for common GM plant species, such as rice, tomato, cotton, canola, papaya, sunflower, and potato [8–17]. Qualitative PCR and quantitative real-time PCR assays based on these ERGs have also been established and applied in the routine analysis of GM food/feed samples.

2 Currently Used Reference Genes for GM Wheat Detection

Wheat (*Triticum aestivum* L.) is one of the major staple food crops grown on more than 17% of the global cultivated land [18]. It is also the last major cereal crop to be genetically modified due to its complex genome structure, recalcitrance to tissue culture, and challenges of gene transfer using *Agrobacterium* mediated method [19, 20]. Although no GM wheat has been commercialized so far, several GM wheat events, such as herbicide-tolerant and Fusarium-resistant GM wheat, are in the pipeline [21, 22]. Up to date, there are five ERGs (*Waxy-D1*, *ACC1*, *PKABA1*, *ALMT1*, and *PRP*) that have been reported and used for GM wheat analysis, and the corresponding conventional PCR and real-time PCR assays based on the validated primers and probes (Table 1) were also established as well [16, 23–26].

2.1 Waxy-D1 Gene

In 2005, Lida et al. reported that *Waxy-D1* gene is suitable to be used as ERG for GM wheat analysis [23]. The *Waxy-D1* gene (accession no. AF113844) were selected and validated from several candidates according to the copy number and taxon specificity requirements of ideal ERG genes. These candidates also include *TaSUT* (accession no. AF408845), *CbpII* (accession no. J02817), *Lr1* (accession no. S79983), and the wheat *GSS* region (accession no. AJ440705). The *waxy* genes encode the *waxy* protein (granule-

Table 1
The primers and probes of the five endogenous reference genes for GM common wheat (*Triticum aestivum* L.) detection

Endogenous reference gene	GenBank Acc number	PCR system	Name	Sequence (5′–3′)	Amplicon size (bp)	Reference
Waxy-D1	AF113844	Qualitative PCR	wx012-5′	GTCGCGGGAACAGAGGTGT	102	[23]
			wx012-3′	GGTGTTCCTCCATTGCGAAA		
		Quantitative real-time PCR	wx012-5′	GTCGCGGGAACAGAGGTGT	102	
			wx012-3′	GGTGTTCCTCCATTGCGAAA		
			wx012-T	FAM-CAAGGCGGCCGAAATA AGTTGCC-TAMRA		
ACC1	AF029895	Qualitative PCR	tri1	TGCCCATTGTCGGCCTTA	93	[16]
			tri2	GCATTCCAACCATCTGCCC		
		Quantitative real-time PCR	tri1	TGCCCATTGTCGGCCTTA	93	
			tri2	GCATTCCAACCATCTGCCC		
			tri12 p	FAM-TGCCTCGACAACACC ATCGCTATCC-TAMRA		
PKABA1	M94726	Quantitative real-time PCR	UnivF	CAAGTATGTCATAGAGATTTGAA	87	[24]
			UnivR	GTAACCGAAGTCACAAATCT		
			TAest	FAM-TCGCACCTCGGCT-MGBNFQ		
ALMT1	AB081804	Qualitative PCR	ALMT1-FP2	AATGACTGTGCCGTCTCCAGT	95	[25]
			ALMT1-RP2	ACAGAGCCGTGTTCTCTGCA		
		Quantitative real-time PCR	ALMT1-FP2	AATGACTGTGCCGTCTCCAGT	95	
			ALMT1-RP2	ACAGAGCCGTGTTCTCTGCA		
			ALMT1-probe	VIC-CGTGAAAGCAGCGGAAAG CCTCAGA-TAMRA		
PRP	X52472	Qualitative PCR	PRP8F	GCACCCATGATGAGTAC TACTATTCTGTA	117	[26]
			PRPds6R	TGCAAACGAATAAAAGCATGTG		
		Quantitative real-time PCR	PRP8F	GCACCCATGATGAGTACTACT ATTCTGTA	117	
			PRPds6R	TGCAAACGAATAAAAGCATGTG		
			PRP-Taq5	FAM-CTGTGCACATGACTCA GTTGTTCTTTCGTG-TAMRA		

bound starch synthase, EC 2.4.1.21), which is involved in amylase synthesis in the plastid of plants [23]. In common wheat, the *waxy* gene is organized in a triplicate set of single-copy homeoloci, *Wx-A1*, *Wx-B1*, and *WxD1*, derived from chromosomes 7A, 4A, and 7D, respectively. The *Wx-D1*-specific PCR primer (DRSL) was developed as a marker to classify partial waxy wheat. Based on these published information, a region of the *Wx-D1* gene with the length of 1323 bp was sequenced and selected to design the primer pair (wx012-5′/3′) and TaqMan probe (wx012-T) for further validation of the suitability of *Wx-D1* gene as an ERG (Table 1). In the test of taxon specificity, a 102 bp DNA band with expected size was amplified only from the common wheat in the qualitative PCR assay using wx012-5′/3′ primer set, and there were no amplification products observed from the other tested species including barley, rye, oats, Italian millet, common millet, soybeans, buckwheat, rice, rapeseed, maize, and sesame, other than wheat. The result indicated that the Wx012 system provides a highly specific PCR condition to common wheat in the tested species. Meanwhile, the same result was obtained from the quantitative real-time PCR assay employing the same primers (wx012-5′/3′) and TaqMan probe (wx012-T). The intraspecies variability of the Wx012 region was investigated by qualitative PCR and quantitative real-time PCR among 19 common wheat varieties belonging to five classes of commercial common wheat. The result of qualitative PCR showed that expected PCR products of identical size and equivalent intensity were obtained in all 19 tested varieties, and the results of quantitative PCR exhibited similar amplification plots and comparable Ct values ranging from 27.56 to 28.50 from all varieties. These results suggest that the copy number of the Wx012 region is identical among the varieties tested. For Southern blot analysis, wheat genomic DNA was digested with *Bam*HI, *Eco*RI, and *Fba*I, and the result showed that the Wx012 region is a single copy in haploid genome (two copies in common wheat genomic DNA).

Since the *Wx-D1* gene is confirmed for all three characterizations of an ideal ERG, qualitative and quantitative real-time PCR assays using *Wx-D1* gene as ERG were established. For the qualitative PCR assay, the limit of detection (LOD) was 6.4–7.6 copies; the qualitative PCR assay could detect as low as 0.5% of target DNA in one reaction. In quantitative real-time PCR assay, a linear correlation (coefficient counts from 0.996 to 0.999) with a slope of −3.4 to −3.7 was obtained between the Ct value and the initial DNA quantity. The LOD is about 15.1 genomic copies. Reproducibility was also evaluated and the coefficient of variation (CV%) values are ranged from 1.015 to 2.475, the standard deviation (SD) values are ranged from 0.256 to 0.880. All of these results indicate that PCR assays using wx012 as ERG is suitable to be applied in common wheat DNA analyses.

2.2 Acetyl-CoA Carboxylase Gene (acc1)

In 2005, Hernandez et al. performed in silico investigation of common wheat sequences from conservative low copy number gene families and selected *acetyl-CoA carboxylase* (*acc1*) gene (acc. no. AF029895) as a candidate for common wheat ERG [16]. The taxon specificity of *Acc1* gene was evaluated among 20 different plant species, which are either phylogenetically related to wheat or frequently found in food, and PCR amplification of *Acc1* was only observed in common wheat but not in any of the other tested species. The intraspecies variability of *Acc1* was evaluated by conventional and real-time PCR assays employing 28 wheat (18 durum and 10 bread wheat) cultivars. In conventional PCR analysis, amplicons with expected size and similar intensity were obtained from all tested cultivars. For real-time PCR analysis, no significant differences of Ct values could be detected among either durum wheat cultivars or bread wheat cultivars. However, Ct values of real-time PCR between durum and bread wheat cultivars are statistically different ($p < 0.01$), with mean Ct value of 32.98 (0.64) for durum wheat cultivars and 25.93 (0.40) for bread wheat cultivars. This Ct difference is indicative of sequence variance of *Acc1* gene between durum and bread wheat cultivars [16]. Southern blot experiments reported wheat acc1 polypeptides was encoded by small multigene families. The established real-time PCR assay using *Acc1* as ERG has LOD of 1 genome copy and LOQ of 10 genome copies. When used in the test of nine food products, PCR assays using *Acc1* as ERG can detect wheat DNA in wheat germ, breakfast cereals, whole wheat bran, plaited bread sticks, biscuits, bread, and wholemeal bread. These results suggest that PCR assays using *Acc1* gene as ERG is specific, sensitive, and reliable, and can be used to determine the presence of wheat ingredients in food samples.

2.3 PKABA1 Gene

In 2006, Rønning et al. selected *PKABA1* as a suitable endogenous reference gene for wheat DNA analysis after searching for single-copy wheat genes in public sequence databases EMBL and GenBank [24]. *PKABA1* encodes a wheat serine/threonine protein kinase (EMBL/GenBank accession number M94726). *PKABA1* gene is also present in many other crops with difference DNA sequences including wheat, barley, and rice. PCR primers and probe were designed based on wheat *PKABA1* gene sequence. The taxon specificity and allelic variation of *PKABA1* among different species were evaluated in real-time PCR assay. The results showed that no amplification was observed in any of the other species that are either closely related to wheat or distantly related but frequently found in food. When tested using DNA amounts of approximately 2500 copies haploid genome in ten different wheat cultivars, the observed Ct values varied from 25.4 to 27.1. This low Ct variation in real-time PCR indicates that the copy number of *PKABA1* per haploid genome was constant among the tested

wheat cultivars. The LOD of developed *PAKBA1* real-time PCR assay was about 5 copies haploid genome. The developed *PAKBA1* real-time PCR assay was also applied successfully to detect wheat contents in eight processed food products.

2.4 ALMT1 Gene

In 2007, Vautrin et al. selected the *ALMT1* gene as a common wheat ERG through database search and sequence analyses [25]. The *ALMT1* gene (gb/AB081804) is a single locus gene that codes for an aluminum-activated malate transporter in common wheat. There are two allelic versions of *ALMT1* reported in all wheat cultivars that have been tested: ALMT1-1 and ALMT1-2, differing at only six nucleotides. To detect the *ALMT1* gene, quantitative real-time PCR assay targeting a conserved region of the two allelic sequences was developed. In taxon-specificity test of this assay, fluorescent signal and Ct values were only observed in common wheat samples (Pr22r18 and Talent). Allelic variation assessment of the real-time PCR assay showed that the mean Ct value for 15 common wheat cultivar DNAs was 24.02 ± 0.251 in a range from 23.60 to 24.47, suggesting the copy number of *ALMT1* gene is constant among the 15 common wheat cultivars. The standard curve of *ALMT1* quantitative real-time PCR assay has an acceptable linearity with R^2 of 0.998, the absolute LOD of 2 haploid genome copies, and the limit of quantification of 20 haploid genome copies. These results showed that the real-time PCR assay developed on the *ALMT1* gene is suitable to be used as an endogenous reference gene for PCR-based detection and quantification of *T. aestivum*-derived DNA.

2.5 PRP Gene

In 2012, Imai et al. reported that the *proline-rich protein* (*PRP*, Acc No. X52472) gene can be used as an endogenous reference gene of common wheat (*Triticum aestivum L.*) and durum wheat (*Triticum durum* L.) [26]. To validate that *PRP* conforms with the three criteria of ERG, a specific region of *PRP* gene sequence was selected as target to develop a real-time PCR assay. Barley, rye, oats, Italian millet, common millet, sorghum, buckwheat, rice, rapeseed, corn, common wheat, and durum wheat were used in *PRP* taxon specificity test, and the results showed that the expected amplicon could only be obtained from the common wheat and durum wheat DNA samples. The intraspecific variability of *PRP* was tested by both conventional and real-time PCR, and the results showed that the intensities of the amplification products and Ct values of eight common wheat and durum wheat samples were very similar, with no nonspecific amplification. The Southern blot analysis revealed that the *PRP* gene can be either a single copy or double copies in the common wheat haploid genome. The *PRP* real-time PCR assay demonstrated a highly linear relationship between Ct values and the amount of genome DNA, and the

LOD value was 5 haploid genome copies. All these results indicated that the *PRP* gene was a suitable ERG for GM wheat detection.

3 Evaluation of the Performance of the ERGs in Real-Time PCR Quantification Assays

Although five ERGs and their PCR assays have been developed for GM wheat detection according to the three typical characterizations of a suitable ERG, more work needs to be done to further validate these ERGs before they can be applied in routine analysis globally, such as testing the intraspecific variability among different wheat cultivars planted worldwide, and evaluating the performance of their corresponding real-time PCR assays [5, 6]. SNPs often exist in different cultivars of the same crop, such as in maize and rice [5, 6]. For a reported maize ERG, *adh1* gene, large bias was observed in real-time PCR analysis due to one SNP in the target sequence [27].

In order to fully evaluate the intraspecific variability of some reported common wheat ERGs among different cultivars, Huang et al. performed a comprehensive test employing 43 wheat and its relatives collected from worldwide [28]. For all 43 samples, target DNA sequences of *ACC1*, *PKABA1*, *ALMT1*, and *Waxy-D1* gene were amplified and sequenced, and SNPs were discovered in the real-time PCR target regions of *Acc1* gene, *ALMT1* gene, and *PKABA1* gene among different varieties. For *Acc1* gene, there was a SNP of C to T in durum wheat line Cannizzo, located at the eighth nucleotide from the 5′ end of the forward PCR primer. For *ALMT1* gene, a SNP of A to C was located at the second nucleotide from the 5′ end of the forward primer in common wheat Zhongguochun, and another SNP of G to A was located at the fourth nucleotide from the 5′ end of the probe in common wheat line Zhengmai 9023. For *PKABA1* gene, a SNP of G to A was found at the 11th nucleotide from the 5′ end of the probe region in wheat lines Zhoumai 16 and Zimai 12. No SNP was observed in the amplicon of *Waxy-D1* gene among all tested wheat lines. Furthermore, the effects of these revealed SNPs on real-time PCR assays were evaluated. The PCR efficiencies of *Acc1*, *ALMT1*, and *PKABA1* genes in wheat lines with SNPs were mostly below 0.90, lower than the PCR efficiencies when using lines containing no SNPs. For instance, the PCR efficiency of *ALMT1* gene in Zhongguochun and Zhengmai 9023 were 0.893 and 0.868, respectively. These results indicate direct effects of SNPs on PCR efficiency, which is decreased when SNP mismatches with target sequences are present.

The performance of real-time PCR assays of these ERGs were also evaluated employing 37 hexaploid wheat lines and two

octoploid triticale lines in Huang's work. The results showed that the lowest variation (judged by standard deviation, SD) of Ct values was achieved for *Waxy-D1* gene. The *PKABA1* assay had the largest Ct value variations in all 39 lines. The *ALMT1* and *Waxy-D1* gene assays appeared to be relatively consistent among all tested lines. GeNorm analysis also showed the highest *M* values for *PKABA1* gene while *Waxy-D1* gene had the lowest *M* values. The overall evaluation using calculated *M* values in GeNorm analysis suggest that the *Waxy-D1* real-time PCR assay is more consistent than the other assays, with the best PCR performance among all four genes. Based on the combination of high species specificity, no SNPs in target sequence, and high PCR performance among different wheat lines, *Waxy-D1* gene was selected as the most suitable candidate among the reported wheat endogenous reference genes, and its real-time PCR assay would be very useful in establishing accurate and creditable quantitative PCR assay for GM wheat.

4 The Difficulties of Developing Suitable ERG and Its Detection System for Common Wheat

Although five ERGs have been developed for wheat, it is still difficult in selecting the best one for universal GM wheat analysis. There are complicated genotypes and karyotypes among wheat species (*Triticum* L. genus), such as diploid (genotype BB, DD and AA, including *Aegilops tauschii*, *Aegilops speltoides*, *and Triticum urartu*), tetraploid (*Triticum durum*, genotype BBAA), Hexaploid (genotype BBAADD, such as zhongmai 175), and octoploid triticale (genotype BBAADDRR, such as Xiaoyan 22).

Based on reported qualitative PCR amplification results, amplicons of *Acc1* gene and *PKABA1* gene were not only observed in all hexaploid common wheat lines, but also in *Aegilops speltoides Tausch* (BB), *Triticum urartu* L. (AA), and durum wheat (BBAA), indicating that *Acc1* and *PKABA1* assays have low specificity for common wheat. *ALMT1* and *Waxy-D1* gene amplicons could not be amplified from *Aegilops speltoides Tausch* (BB), *Triticum urartu* L. (AA), and durum wheat (BBAA), indicating these two genes are more suitable for identifying common wheat species. The results also showed that *ACC1* gene is from B genome, *PKABA1* from A genome, and *Waxy-D1* and *ALMT1* genes are from the D genome of wheat. Therefore, it is impossible to specifically identify the Chinese octoploid triticale from common wheat lines by employing any gene from haploid A, B, and D genome. Apparently, the genotype and karyotype situations should be considered carefully when selecting endogenous reference genes for wheat.

The gene allele stability among different cultivars is another important factor in developing endogenous reference gene assays for GM wheat detection. It can be evaluated by target DNA sequencing/alignment and PCR performance analysis. For example, sequence alignment of target DNA from different wheat cultivars revealed SNPs in some lines. SNPs in real-time PCR primer/probe annealing regions could affect PCR efficiency in quantitative analysis of GM contents, virus, and microorganisms [29, 30].

A collaborative ring trial validation of the developed ERG real-time PCR assays might further improve their reliability and applicability when comparing the robustness of the assays are evaluated in different laboratories, using various PCR equipments and different brands of PCR mastermix. Therefore, collaborative ring trial validation should be performed before an ERG and its detection system can be released for GMO routine analysis.

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Chapter 17

Phenotypic Characterization of Transgenic Wheat Lines Against Fungal Pathogens *Puccinia triticina* and *Fusarium graminearum*

Jagdeep Kaur, Dilip Shah, and John Fellers

Abstract

Leaf rust (LR) and Fusarium head blight (FHB) caused by *Puccinia triticina* and *Fusarium graminearum*, respectively, are among the most damaging fungal diseases challenging wheat production worldwide. Genetic resistance in combination with fungicide application has been the most widely employed approach to combat these fungal pathogens. Alternative approaches that could augment current practices are needed for the control of these devastating pathogens. To that end, we have recently shown that the extracellular expression of antifungal defensin MtDEF4.2 from *Medicago truncatula* confers resistance to LR. Additionally, we show that expression of this defensin also provides Type II resistance to FHB under controlled growth chamber conditions. These findings have practical applications for control of these important fungal diseases in wheat. Here, we provide details on conducting LR and FHB bioassays of transgenic wheat lines in the growth chamber.

Key words *Fusarium graminearum*, *Puccinia triticina*, *Triticum aestivum*, Defensin, Transgenic wheat, Leaf rust, Fusarium head blight

1 Introduction

Wheat is the third largest cultivated cereal crop providing one fifth of the total global calories. Many pathogens, however, adversely affect wheat productivity limiting its yield and quality. Rust diseases caused by *Puccinia* spp. and Fusarium head blight (FHB) disease caused by *F. graminearum* (*Fg*) in wheat have been ranked amongst the "Top 10" fungal pathogens underscoring their global devastation and economic impact [1]. Historically, the wheat industry has been managing these fungal diseases by planting resistant cultivars along with fungicide application. Breeding for resistance, however, has to be a continuous process to stay ahead of fast evolving hypervirulent pathogen population and changing environment. Fungicides, on the other hand, are expensive and environmentally unsafe. Thus, alternative strategies that can augment the current

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containment approaches must be explored to control these fungal diseases. To that end, we have employed a gene encoding a small cysteine-rich antifungal defensin MtDEF4.2 from *Medicago truncatula* to generate transgenic wheat for resistance to leaf rust (LR) caused by *P. triticina* (*Pt*) [2] and FHB in the growth chamber bioassays. The monocot-codon-optimized defensin gene *MtDef4.2* was cloned into a binary vector *pPZP212/ZmUbi1b::TEV::MtDef4.2::nosT* for *Agrobacterium tumefaciens* strain C58C1/pMP90 mediated transformation of two spring wheat cultivars Bobwhite (BW) and Xin Chun 9 (XC9). The generation, identification and molecular characterization of homozygous transgenic wheat lines expressing MtDEF4.2 are detailed in [2]. This chapter outlines detailed methods for phenotypic characterization of homozygous transgenic wheat lines challenged with *Pt* and *Fg* under growth chamber conditions.

2 Materials

2.1 Plant Material

Transgenic and nontransgenic wheat control lines were grown in Pro-Mix BRK soil mix in 3.55″ pots (*see* **Note 1**) at the Plant Growth Facility of the Donald Danforth Plant Science Center. Growth chamber was maintained at 16 h day–8 h night cycles, 23/16 °C day/night temperature, 50% relative humidity (RH) and light intensity of 500 μmol/m^2/s during the growth of the wheat plants. Homozygous transgenic wheat lines segregating for a single transgene locus based on the PCR segregation data were identified in T_2 generation and advanced to T_6 generation by selfing in the growth chamber [2].

Transgenic and nontransgenic wheat plants for *Pt* inoculation were planted in Metro Mix 360 (Sungro) growing medium in 3″ pots. Plants were grown in a Percival E30B growth chamber at 16 h day–8 h night cycles, 20 °C and 500 μmol/m^2/s light intensity [3].

2.2 Fungal Pathogens

Pt race MCPSS (avirulent to *Lr2a*, *Lr2c*, *Lr9*, *Lr16*, *Lr24*, *Lr11*, *Lr18*/virulent to *Lr1*, *Lr3*, *Lr26*, *Lr3ka*, *Lr17*, *Lr30*, *LrB*, *Lr10*, *Lr14a*) was chosen for its ability to partially overcome leaf rust resistance gene *Lr26* present in the wild type BW (*see* **Note 2**). *Pt* spores can be desiccated and then stored for long term at either −80 °C or in liquid nitrogen.

Previously, we have shown that MtDEF4.2 inhibits the growth of the *Fg* isolate PH-1 or NRRL 31084 (*see* **Note 3**) with an IC_{50} (concentration required for 50% growth inhibition) of 0.75–1.5 μM [4]. Based on that data we tested transgenic, nontransgenic, and control wheat lines against *Fg* PH-1 in a growth chamber bioassay. In our lab, we routinely maintain *Fg* PH-1 in CM (complete medium) per [5] (*see* **Note 4**). We recommend growing *Fg* PH-1 isolate on fresh CM plates from −80 °C stored glycerol stock.

2.3 Media

1. *Complete medium* (*CM*): per L, add 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 ml of trace element stock solution (per 100 ml, add 5 g citric acid, 5 g $ZnSO_4 \cdot 6H_2O$, 1 g Fe $(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 250 mg $CuSO_4 \cdot 5H_2O$, 50 ng $MnSO_4$, 50 mg H_3BO_3, 50 mg $Na_2MoO_4 \cdot 2H_2O$, and 95 ml distilled water, and filter-sterilize (*see* **Note 5**), 30 g sucrose, 2 g $NaNO_3$, 2.5 g N-Z Amine, and 1 g yeast extract (bacto), and bring the final volume to 990 ml and autoclave on liquid cycle for 30 min. After autoclaving let the medium cool down to <40 °C and add 10 ml of vitamin stock solution (per L, add 4 g inositol, 200 mg Ca pantothenate, 200 mg choline Cl, 100 mg thiamine, 75 mg pyridoxine, 75 mg nicotinamide, 50 mg ascorbic acid, 30 mg riboflavin, 5 mg *p*-aminobenzoic acid, 5 mg folic acid, and 5 mg Biotin) (*see* **Note 6**), and mix well (*see* **Note 7**) before pouring plates. CM plates can be stored in the dark at 4 °C for several months.
2. *Carboxymethyl cellulose* (*CMC*) *medium*: per L, add 15 g carboxymethyl cellulose (low viscosity) (*see* **Note 8**), 1 g NH_4NO_3, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 1 g yeast extract. Autoclave the medium on liquid cycle for 30 min before using.

3 Methods

3.1 Growth of Pt and Preparation of the Urediniospores Inoculum

Urediniospores (spores) are maintained on the *Pt* susceptible wheat variety Little Club [6]. Spores are taken from the −80 °C freezer and warmed to room temperature, then heat shocked at 40 °C for 10 min. Spores (25 mg) are mixed with 5 ml of Soltrol 170 and sprayed upon wheat seedlings at 2–3 leaf stage and placed overnight (16 h) in a Percival humidity chamber. Chamber settings were RH 100%, wall temperature 5 °C, and water temperature 41 °C. Plants are returned to the Percival E30B as described above. At 14 dpi, spores are collected with a Krammer-Collins vortex collector, desiccated for 3 days and stored at −80 °C.

3.2 Pt Bioassay

All transgenic lines (T_6 generation), nontransgenic BW and XC9 wheat lines were inoculated with the *Pt* race MCPSS in a growth chamber assay. Ten plants from each line were inoculated at the three-leaf stage by suspending 5 mg urediniospores into 1 ml of Soltrol 170 and spraying onto the plants using an atomizer at 40 psi. After inoculation, plants were transferred to a dew chamber with 100% RH overnight at 18 °C. Subsequently, plants are returned to the growth chamber at 16 h/8 h day/night cycle at 18 °C. The severity of rust symptoms is scored visually at 15 dpi (days post inoculation) by rating the infection-type (IT) of each line on a scale of 0–4 [7]. On this scale, IT "0" (pronounced as "naught") indicates no visible symptoms; IT ";"(pronounced as

Table 1
Leaf rust infection type scores of transgenic and nontransgenic wheat lines inoculated with *Pt* race MCPSS at 15 dpi

Line name	Average infection type (IT) scores[a]	Host reaction
BW	2	Moderately resistant
BW-A-11	Chlorosis, ;1	Highly resistant
BW-B-4	;1	Highly resistant
BW-F-10	Chlorosis, ;	Highly resistant
XC9	3	Susceptible
XC9-104-1	Fewer pustules to ;1	Highly resistant

[a]Average score of ten plants
Reproduced from Kaur et al. [2]

"fleck") indicates resistant response along with hypersensitive "flecks" and no uredinia; IT "1" is characterized by minute uredinia surrounded by distinct necrotic areas; IT "2" indicates small uredinia surrounded by chlorosis; IT "3" indicates medium-sized uredinia frequently surrounded by chlorosis and IT "4" indicates large uredinia usually without chlorosis. In addition, a "+" (plus) or "−" (minus) sign is used to indicate bigger or smaller size uredinia, respectively, within the scale. At 15 dpi, the transgenic lines were compared to their respective nontransgenic controls BW and XC9 challenged with *Pt* race MCPSS. Wild type BW had pustules that were scored as an IT of 2 indicating a moderately resistant reaction. In comparison to BW with moderately resistant IT score of 2, all three transgenic wheat lines BW-A-11, BW-B-4 and BW-F-10 showed highly resistant phenotype ranging from ; to 1 (Table 1). Compared to the nontransgenic control XC9 that was susceptible (IT score of 3), transgenic wheat line XC9-104-1 showed a highly resistant phenotype with fewer pustules to IT of 1 (Table 1). The representative leaves from each transgenic and nontransgenic wheat line showing the symptoms of rust infection at 15 dpi are shown in Fig. 1 [2].

3.3 Growth of Fg and Preparation of the Conidial Inoculum

After 3–4 days of growth on CM plates, fungal agar plugs are taken using sterile fungal needle and 1 ml pipette tip (*see* **Note 9**) and grown in 100 ml of CMC liquid medium in 250 ml washed and autoclaved conical flask. Depending on the size of the experiment, multiple 100 ml flasks are grown. The conical flasks are incubated at 28–30 °C with constant shaking at 180 rpm for 4–5 days. Typically two flasks provide sufficient inoculum to infect 50 heads at 1×10^5 macroconidia/ml concentration by point inoculation. The macroconidia are harvested by filtering the spore suspension through two layers of miracloth or cheese cloth (*see* **Note 10**) into clean 15 ml falcon tubes (*see* **Note 11**). *Fg* spore suspension in falcon tubes is

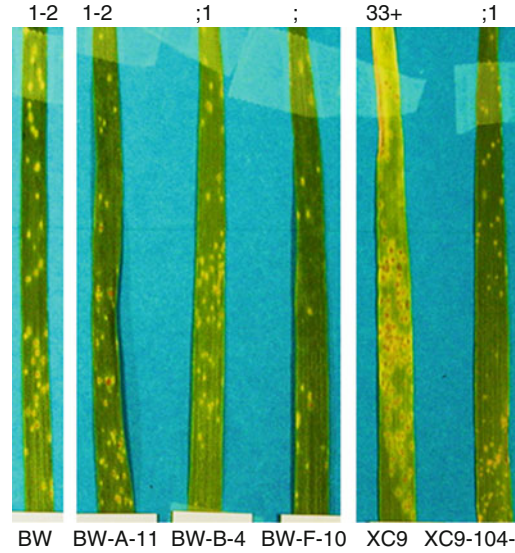

Fig. 1 Representative leaves of transgenic and nontransgenic wheat lines inoculated with *Pt* race MCPSS at 15 dpi. Leaf rust IT evaluations were done on a 0–4 scale. Reproduced from Kaur et al. [2]

centrifuged at 1503 × *g* for 10 min at room temperature (RT). Resulting pellet is washed 2× with autoclaved distilled water by centrifuging at 1503 × *g* for 5 min each at RT. After second wash, dissolve the pellet in 2 ml of autoclaved distilled water and mix well with vortexing. Use a 10 μl aliquot to count the macroconidia in hemocytometer (*see* **Note 12**) to bring the final concentration to 1×10^5 macroconidia/ml with autoclaved distilled water. Add Tween 20 at a final concentration of 0.01% as surfactant to the spore suspension before inoculations (*see* **Note 13**).

3.4 Fg *PH-1 Bioassay*

In our lab, we use a single floret inoculation method [8] to inoculate wheat plants with *Fg* PH-1 (*see* **Note 14**). This method evaluates Type II resistance to FHB that quantifies the spread of the fungus [9]. At anthesis stage, a central floret in the middle spikelet of wheat is inoculated with 10 μl of 1×10^5 macroconidia/ml spiked with 0.01% Tween 20. The inoculated spikelet is marked with a nontoxic black sharpie that helps in disease symptom assessment later on. For mock inoculations, autoclaved distilled water spiked with 0.01% Tween 20 is used. After inoculation, each wheat spike/head is covered with wet clear plastic bag (*see* **Note 15**) for maintaining high humidity for disease development. Trays (21 × 44″ length × 10.94″ width × 2.44″ depth) housing the pots with inoculated wheat plants are filled with water at the bottom as well to create high humidity. These trays are incubated in dark for 48 h after inoculation and RH is increased to 100% in the growth chamber. After 48 h, the clear plastic bags are removed

from each inoculated head, the lights are turned back on to 16 h day/8 h night photoperiod and RH is reduced to 50% in the growth chamber. Starting from day 7, FHB disease symptoms are scored at weekly interval up to 21 days post inoculation (dpi). FHB disease severity is scored as percentage of spikelets on the inoculated head with visually detectable disease symptoms i.e., bleaching on 0–100% scale. In our experience, FHB severity data at 14–15 dpi is most useful because by 21 dpi natural senescence sets in and it becomes increasingly difficult to differentiate *Fg* induced bleaching from senescence. Using this protocol, we tested transgenic wheat lines expressing MtDEF4.2 along with nontransgenic controls, BW and XC9, against *Fg* PH-1 in growth chamber assay. FHB resistant and susceptible wheat cvs. Alsen and Wheaton, respectively, were also tested during the assay. FHB severity data were analyzed using unpaired *t*-test. As shown in Table 2, transgenic wheat line BW-A-11 showed FHB severity of 15% which was significantly less compared to 24% in its corresponding nontransgenic wheat line BW. Notably, the level of FHB resistance seen in transgenic wheat line BW-A-11 was comparable to FHB resistant wheat Alsen (FHB severity of 12%). The remaining transgenic wheat lines BW-B-4, BW-F-10, and XC-104-1 did not show significant resistance to FHB compared to controls (Table 2). Susceptible wheat cv. Wheaton showed FHB severity of 50% in the same bioassay (Table 2). Representative pictures of inoculated heads are shown in Fig. 2. Mock-inoculated transgenic, nontransgenic, and control wheat lines did not show any symptoms (data not shown).

Table 2
FHB severity of transgenic, nontransgenic, and control wheat lines infected with 1×10^5 conidiospores/ml of *Fg* PH-1 at 14 dpi

Line name	No. of heads inoculated	FHB severity %
BW[a]	26	24
BW-A-11[a]	22	15*
BW-B-4[a]	22	19
BW-F-10[a]	15	28
XC9[b]	16	34
XC9-104-1[b]	15	35
Alsen[c]	24	12
Wheaton[d]	21	50

*Significantly different at $P = 0.05$ compared to nontransgenic BW using Student's *t*-test
[a]T_4 generation
[b]T_3 generation
[c]FHB resistant cv.
[d]FHB susceptible cv.

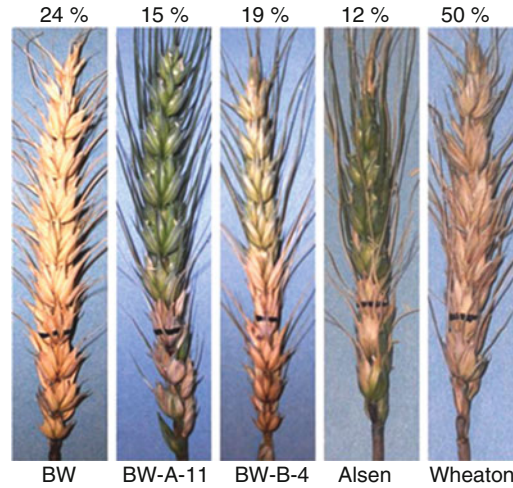

Fig. 2 Representative heads of transgenic and nontransgenic wheat lines infected with *Fg* PH-1 at 14 dpi. Severity was scored on a 0–100% scale. Alsen and Wheaton are FHB resistant and susceptible wheat cvs., respectively

4 Notes

1. Any other potting mix appropriate for growing wheat that works under suitable conditions can be used.
2. The choice of *Pt* race has to be made in accordance with the wheat genotype used to make transgenics.
3. PH-1/NRRL 31084 is a sequenced strain of *Fg*. We typically make glycerol stocks of PH-1 at 18% final concentration and store at −80 °C freezer indefinitely.
4. V8 juice agar medium can also be used to grow *Fg* PH-1.
5. This solution is stored in a capped bottle at 4 °C and 1 ml of chloroform is added to avoid contamination.
6. Sterilize this solution using 0.2 μm syringe filter and add 1 ml of chloroform for longer term storage in the dark at 4 °C.
7. We add a magnetic bead into the glass bottle containing medium before autoclaving which makes the mixing easier on the stir plate and prevents excessive bubble formation.
8. Use warm distilled water to help dissolve CMC before adding remainder of the ingredients.
9. We conduct this step in sterile laminar air flow cabinet and use bottom of autoclaved 1 ml pipette to take the plug and make sure to break each agar plug into small pieces for homogeneous sporulation.
10. Vortex the flask for 1–2 min to dislodge the spores before filtering.

11. Instead, 1.5 or 2 ml sterile eppendorf tubes can be used and centrifuged for 8 min at 2348 × *g* at RT.
12. A user friendly app for counting macroconidia is also available at https://www.hemocytometer.org/hemocytometer-app/
13. Macroconidia tend to settle down at the bottom of the tube, so be sure to tap them regularly during inoculations.
14. Make sure not to apply fungicides on wheat plants used for bioassay.
15. We typically use clear plastic sandwich bags to cover the inoculated wheat heads. Spray water inside the sandwich bag using a squirt bottle before covering the wheat head to create 100% RH.

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Chapter 18

Databases for Wheat Genomics and Crop Improvement

Yuxuan Yuan, Armin Scheben, Chon-Kit Kenneth Chan, and David Edwards

Abstract

The genomics revolution brought on by advances in high-throughput sequencing has led to the production of vast amounts of data. Databases play an essential role in storing and managing this information to make it available to researchers and crop breeders. This chapter provides an outline of how to use databases and tools for wheat genome research.

Key words Crop breeding, Genome browser, Genomics, T3 Wheat, TAGdb, *Triticum aestivum*, WheatIS

1 Introduction

The immense volumes of data generated in the last decade by high-throughput sequencing have made databases essential for storage, curation and easy accessibility of genomic data [1, 2]. The publication of assemblies of chromosome group 7 [3–5], the chromosome-sorted wheat genome [6], and a whole genome shotgun (WGS) assembly [7] have added important genomic wheat data to public databases. The Wheat Information System (WheatIS) was developed to integrate searching the diverse wheat databases. It is a web-based system for accessing multiple wheat related data resources and bioinformatics tools from a single interface. WheatGenome.info hosts part of this data and makes several tools available to access it [8]. These tools include a wheat genome browser and an associated sequence query tool. Both tools provide access to the draft wheat pangenome annotated with variation data, together enabling comprehensive genome-wide searches for sequences, regions and landmarks. The query tool TAGdb allows searching of short-read sequence data from diverse wheat cultivars

Yuxuan Yuan and Armin Scheben contributed equally to this work.

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and isolated chromosomes, providing a means to identify genes and their surrounding regions in wheat genomes.

There is a growing need to develop and apply databases and tools to assist and accelerate crop breeding. The Triticeae Toolbox (T3) (http://triticeaetoolbox.org/wheat) has been developed to collate, integrate and analyse wheat breeding information to assist in the breeding process. Here, we provide a guide on WheatIS, WheatGenome.info, TAGdb, and T3 Wheat for wheat research.

2 Materials

All databases presented in this chapter are available without restriction or passwords via the internet. They can be accessed using most standard browsers including but not restricted to: Internet Explorer, Firefox, Chrome, and Safari. As the majority of data and processing is on the server, client based access does not require a high performance computer or fast internet.

3 Methods

3.1 The Wheat Information System

WheatIS is an initiative consisting of 14 nodes working toward a single-access web-based system to integrate all wheat related data resources and bioinformatics tools. WheatIS.org serves as a main web portal offering links to major tools and databases for wheat research, and a single-access query system for searching for wheat data in different wheat databases. WheatIS features best practice guidelines for greater interoperability of data on wheat genotypes and phenotypes, providing standards for data formatting and ontologies and vocabularies. Currently WheatIS contains data from six databases: GnpIS [9], Wheat3BMine, Ensembl Plants [10], CR-EST, GBIS, and MetaCrop. These databases include annotations, gene expression data, variants, genetic markers, quantitative trait loci (QTL) and phenotype trials from different international projects on wheat. The search function allows searches for keywords, genes, markers, and QTL across all these databases in a single query. Users can apply filters to increase the specificity of the search results, which include links to the source database.

The following steps illustrate how to search for a term in WheatIS nodes.

1. Enter query term

   Select the "Search" tab and enter the name of a species, marker, trait, gene, or related term. For example, to search for data pertaining resistance to Fusarium head blight (FHB), a common disease of wheat, users may enter the term "FHB" in the search bar.

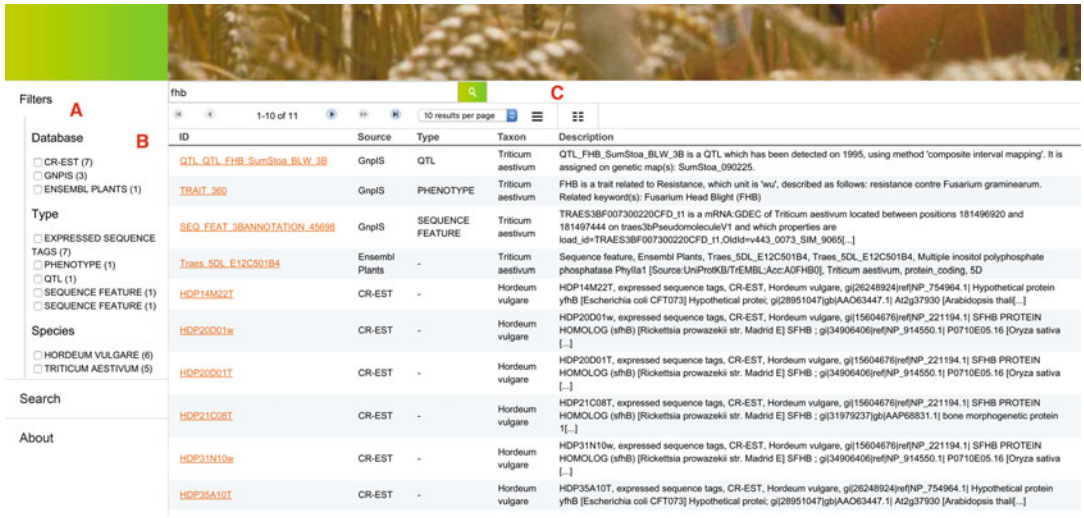


Fig. 1 Wheat Information System (WheatIS) search results for Fusarium head blight (FHB), showing optional search filters (*A*) and links to the source databases in the ID column (*B*). User can switch between a tabular view of the results (default) and a simple text view resembling a google result using the viewing options (*C*)

2. View results

   Results link to the relevant information and can be filtered by database, data type and species using the left hand column "Filters" (Fig. 1A). Hits for the query term are ordered by database (Fig. 1B). To simplify the view of the results, select the text view option (Fig. 1C). The ID tags provide a link to the original source database.

   To find QTL associated with FHB in this example, select the "QTL" tick box in the "Type" section of the filter options. This query will identify the wheat QTL "QTL_FHB_SumStoa_BLW_3B" on chromosome 3B. By following the ID link to the GnpIS database, users can find further information on this QTL including genetic loci, markers, and related publications.

Data submission via the "Submit Data" tab is carried out using the *Unité de Recherche Génomique Info* (URGI) submission utility. Access to individual tools and databases such as WheatGenome.info and T3 Wheat is provided by the "WheatIS Nodes" tab.

3.2 The Wheat Pangenome Browser

The production of genome and pangenome assemblies for bread wheat is an important step in overcoming the challenges in understanding this complex genome. A pangenomics approach is particularly important, as there is an increasing consensus that the genome of a single individual cannot accurately represent the genetic diversity of a species [11]. WheatGenome.info hosts the first draft wheat pangenome which is based on the cultivar Chinese

Spring and 16 Australian cultivars (AC Barrie, Alsen, Baxter, Chara, Drysdale, Excalibur, Gladius, H45, Kukri, Pastor, RAC875, VolcaniDDI, Westonia, Wyalkatchem, Xiaoyan 54, and Yitpi). The browser used to access this data is implemented using GBrowse2 [12] and combines a database with interactive web pages to graphically display genomic features such as the core and variable genes, predicted proteins and single nucleotide polymorphisms (SNPs).

Gene and exon annotation provide a convenient way for researchers to obtain biological information related to particular genes with agronomic traits. To reduce overrepresentation of sequences, clustering of protein sequence space based on UniRef (Uniprot Reference Clusters) is provided. UniRef is a protein sequence database composed of clustered sets of sequences from the UniProt Knowledgebase (UniProtKB) and further UniProt Archive data [13]. WheatGenome.info includes a gene database with UniRef90 functional annotation that was implemented by comparing predicted genes with the UniRef90 database. Single nucleotide polymorphisms (SNPs) provide valuable insights into wheat genome diversity [14]. WheatGenome.info hosts more than 28 million intervarietal SNPs detected between 16 Australian bread wheat cultivars across the whole wheat pangenome. These SNPs have been made publicly available and are included in the WheatGenome.info annotation data (*see* **Note 1**). While Wheat GBrowse2 does not allow searching by sequence similarity, WheatGenome.info provides a wheat genome BLAST [15] portal to perform sequence similarity queries and allow subsequent visualization in the browser [12, 16] (*see* **Note 2**).

In summary, wheat GBrowse2 is hosted at WheatGenome.info and presents the wheat genome as an integrated database containing genomic information including DNA sequence, predicted genes with UniRef90 functional annotation and intervarietal SNPs between 16 Australian wheat cultivars across the draft wheat pangenome. This genomic information is shown in the wheat genome browser as tracks displaying contigs, predicted genes, Uniref90 gene annotation and intervarietal SNPs between the wheat cultivars. The following steps illustrate how to use Wheat GBrowse2.

1. Navigate to www.wheatgenome.info and, in the "Databases" section, select "Wheat pangenome".
2. Customize the annotation tracks

   Select tracks in the "Select Tracks" tab. Under "General tracks" use the tick box to select "SNPs" to show annotation with intervarietal SNPs. Under "DNA" select the tick box for the DNA/GC track. Finally, under "Gene" select "All on" to show

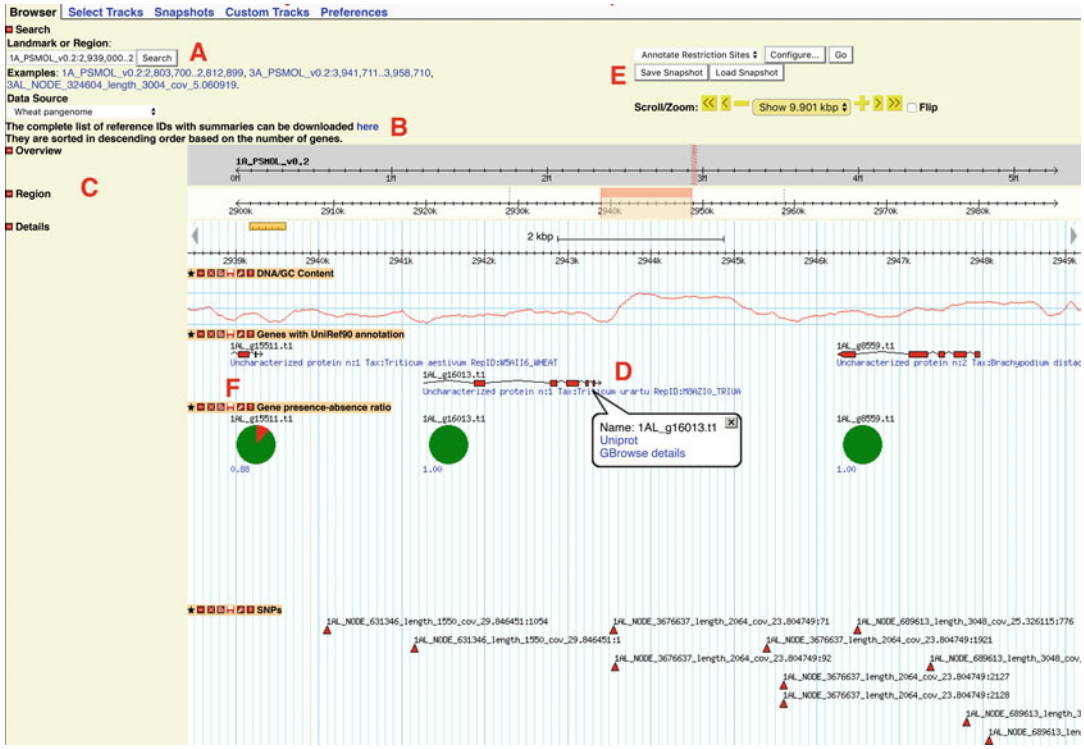


Fig. 2 A screenshot of wheat genome browser (Version 2.54; http:/appliedbioinformatics.com.au/cgi-bin/gb2/gbrowse/WheatPan/) showing various features of GBrowse2. The "Search" panel allows location of a region or landmark against the selected "Data Source" (*A*). For further analysis, a summary file containing all reference names and the number of different features in the database can be downloaded (*B*). To customize what is shown in the genome view window, the display panel (*C*) offers three optional viewing features. The "Genes with UniRef90 annotation" track has an extra feature which provides a hyperlink directly to UniProt showing the details of the annotated cluster when a feature is selected (*D*). To save a picture of the genome viewer window, users can employ the "Save Snapshot" button (*E*). Finally, all tracks can be downloaded in GFF file format using the icon bar on each track (e.g., *F*)

the gene presence–absence ratio and genes with Uniref90 annotation. Then return to the genome browser (*see* **Note 3**).

3. Search for a landmark or a region in the "Browser" tab

   In the "Search" panel enter a keyword. To search by region, input a reference name with an optional location range in the search bar. In this example we will search for the region from position 2,939,000 to position 2,948,900 on Chromosome 1A by entering 1A_PSMOL_v0.2:2,939,000..2,948,900 in the search bar (Fig. 2A). A summary file containing all reference names and the number of different features in the database can be downloaded (Fig. 2B).

4. Browse the result in the display panel of the "Browser" page

   The display panel contains three sections: "Overview," "Region," and "Details" (Fig. 2C). The "Overview" section

shows the reference from the selected data source. The "Region" section provides an enlarged view for selecting a region. Features are displayed as separate tracks in the "Details" section. All sections allow click and drag to view the preloaded regions to either side of the viewing window. Clicking on a feature will display the details of the feature, for instance the DNA sequence of a gene or the genotype information of an SNP.

View annotation details in the "Details" section. The "Genes with UniRef90 annotation" track has an extra feature, which provides a hyperlink directly to UniProt, showing the details of the annotated cluster when a feature is selected (Fig. 2D). The "Gene presence-absence ratio" track uses pie charts to show gene presence or absence in all 16 Australian wheat cultivars and Chinese Spring. Selecting a pie chart will display a web page with detailed information including cultivars with presence and absence of the gene and gene name, size and position.

5. Save current view or tracks in the "Browser" page
   Use the "Save Snapshot" button (Fig. 2E) and enter a snapshot name to save a snapshot of the current view to the "Snapshots" tab, from where the snapshot can be reloaded later or the image downloaded as a png file. To export high resolution pdf images or svg graphics, select the "File" tab at the top of the page and choose "Export as. . ." Snapshots allow users to save the current reference location and track information as a snapshot and revisit it later.

   Download the features of all tracks in GFF format using the save icon on each track (Fig. 2F).

3.3 TAGdb for Aligning Query Sequences to an Illumina Short Read Database

TAGdb is a web-based query tool hosted at WheatGenome.info for identifying Illumina paired read sequence data [17] with sequence identity to query sequences. Currently, TAGdb hosts around 20 TB of Illumina raw read data of different species, including wheat, barley and rye. Wheat data comprises sequences from 16 Australian bread wheat cultivars [18] and Chinese Spring. The Chinese Spring data includes sequence data for each chromosome arm. The input sequence is aligned with Illumina reads from one or more selected libraries, and the results visualized using a custom web interface. The identified read pair tags can be used to design oligonucleotide PCR primers for the amplification of the query region in the relevant wheat genome.

The TAGdb web page is divided into sections for email entry, query sequence input, and data source selection. In the following we provide an example of a typical TAGdb search.

1. Navigate to http://sequencetagdb.info/tagdb or select the TAGdb link at WheatGenome.info in the "Databases" section under the heading "Analysis tools".

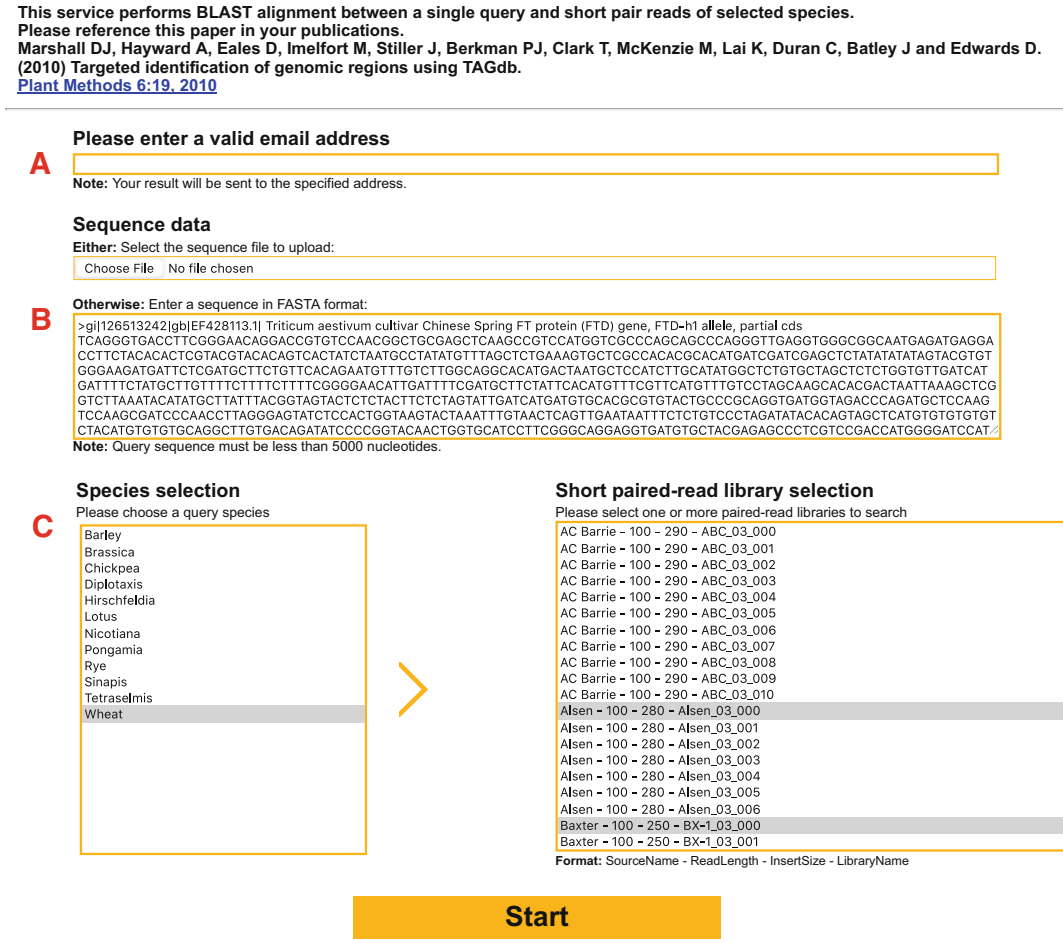


Fig. 3 The TAGdb web page layout (http:/sequencetagdb.info). (*A*) An email address is only required if you wish to receive notification of job status and a link to the results. (*B*) FASTA file input can be uploaded by the user as a file or pasted into the text box. (*C*) Users specify the species and the short read library to query

2. Input a valid email address into the top text box (Fig. 3A) to receive information on the progress of the analysis.
3. Upload target sequence FASTA file

   In the "Sequence data" section (Fig. 3B), select the "Choose file" button to upload a locally stored FASTA file or enter the query sequence manually in the text box provided (*see* **Note 4**). In this example, insert the sequence of the public wheat flowering time protein (FTD) gene (GenBank accession EF428113).
4. Select query species and database

   In the "Species selection" panel (Fig. 3C), select "Wheat" from the available Illumina short read databases.

   The "Short paired-read library selection" panel will list Australian bread wheat cultivars and the reference genome cultivar Chinese Spring. Select the Australian cultivar libraries Alsen_03_000 and BX-1_03_000 (*see* **Note 5**).

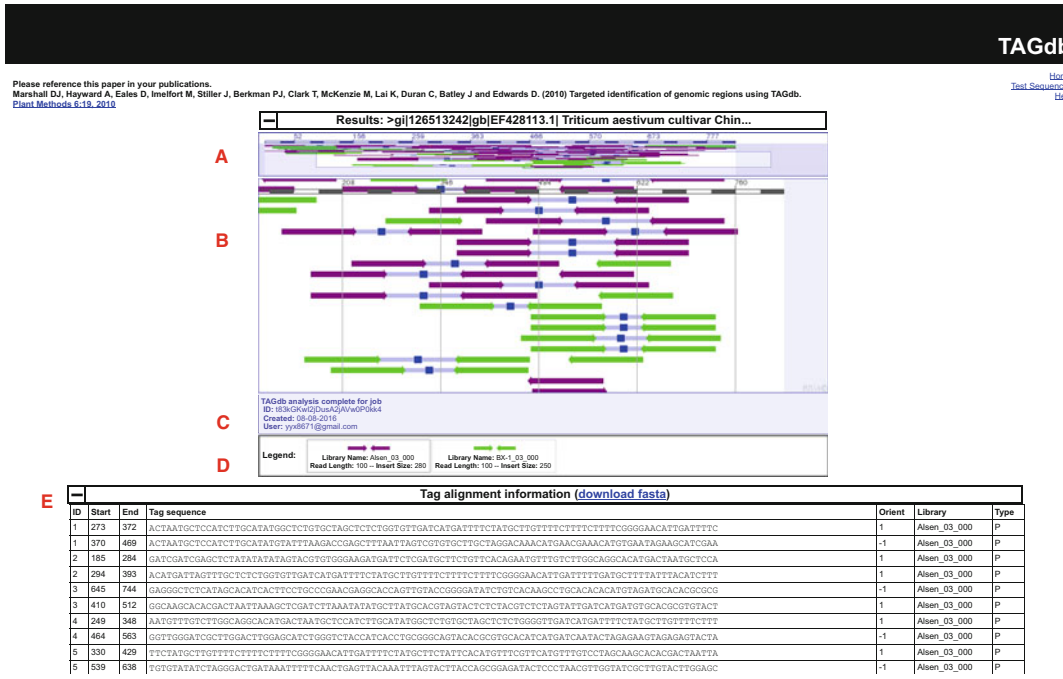


Fig. 4 Example of a TAGdb alignment result for wheat. The *top panel* shows an overview of aligned reads (*A*). Below is a more detailed view of the aligned reads, which are displayed as *single* or *paired colored arrows* (*B*). Details of aligned reads are shown in the *lower panel* when users hover over the reads with the cursor (*C*). The *bottom panel* displays the color legend for the reads (*D*). To provide users with more details of the whole alignment shown, the alignment is also provided in tabular form (*E*)

5. Submit the TAGdb query by selecting the "Start" button. An email with a unique reference number and a link to check the job status will be automatically sent the email address indicated before. After completion of the search, a second email is sent with a link to the results (*see* **Note 6**).
6. On the TAGdb results page, the upper panel displays an overview of the alignment of short reads matching the query (Fig. 4A). The enlarged window below presents a more detailed view of the alignment (Fig. 4B). Users can click and drag the enlarged window to move to a different position in the alignment. Moving the cursor over the arrows shows details of an aligned read in the lower third section of the page (Fig. 4C). The next section shows the colour legend of the arrows (Fig. 4D), while the lowest panel (Fig. 4E) presents all the alignment information as a table. Users can download this information by selecting "download fasta" to save the alignment result locally.

3.4 T3 Wheat Databases for Wheat Breeding

T3 Wheat hosts data on 334 phenotype trials, 53 genotype trials and 49 breeding programs [19]. The phenotypic data originates from greenhouse trials and field nurseries grown across North America. T3 Wheat includes data from genotyping by sequencing (GBS) studies, with over 3.5 million GBS markers and over 163 million data points. T3 Wheat provides functionalities to search for and combine datasets for download or undertake online analysis using a suite of tools based on the statistical programming language R [20].

The T3 Wheat portal offers four main tabs to interact with the hosted databases. On the homepage, the "Data submission" button (Fig. 5A) directs users to a page where they can submit their own germplasm, phenotypic, and genotypic data. In the "Search Trials" panel (Fig. 5B), users can find wheat phenotype trials, filtering by breeding program, trait, year and experiment in the corresponding dropdown boxes. The web page displays the trials matching user criteria, and allows users to select trials to view and download trial details and results. When plot data is available for phenotypic trials, heat maps and numeric maps of traits by field position can be displayed via a link at the bottom of the page.

The other three main menu bars allow the user to find and combine genotypic and phenotypic datasets ("Select"), carry out various analyses on selected data ("Analyze") and download data hosted by T3 Wheat in various output formats for further downstream analysis ("Download"). In the "Select" menu bar, ten options for data selection are offered. The "Wizard" option is

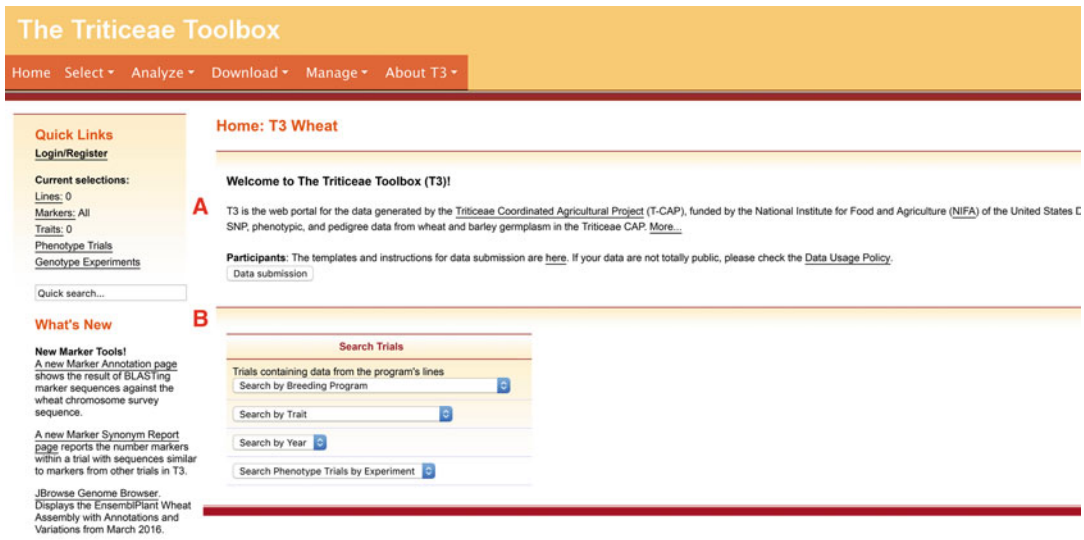


Fig. 5 The T3 Wheat homepage with data submission (*A*) and search (*B*) utilities

newly developed, allowing users to search and combine lines or phenotypes from the same breeding program, or with similar traits [19]. Using this function, T3 enables users to define panels of germplasm for later analysis or download (*see* **Notes 7** and **8**), as shown in more detail in the example below. The "Markers" menu bar gives users three options to select specific markers: searching by name, selecting by map positions or selecting by genotyping platform and experiment.

In the "Analyze" menu bar, 17 options for different analyses of phenotypic and genotypic data are provided. All options require users to have saved data in their current selection. If users have not selected lines, markers, traits, phenotype trials, genotype experiments, and a genetic map, these can be specified in the "Select" menu. Here we focus on a genome-wide association study (GWAS) to find genotypic markers significantly linked to phenotypic traits. GWAS is carried out using the R package "rrBLUP" [21]. Further analyses made available by T3 Wheat have been described in detail in [19]. In the following we describe how to use the T3 Wheat "Genome Association and Prediction" tool for GWAS.

1. Navigate to https://triticeaetoolbox.org/wheat/
2. In the menu of the "Analyze" bar on the top of the main page, select "Genome Association and Prediction".
3. Users then choose between three analysis types: genome-wide association by consensus genotype, genome-wide association by single genotype experiment, and genomic prediction. In this example we demonstrate how to carry out a GWAS by consensus genotype; the individual steps for each analysis, however, are similar.

   Before starting the analysis, users must follow the hyperlink to the Wizard tool to select a set of lines, trials, and a trait of interest. For this example, select the breeding program "Agriculture and Agri-Food Canada, Manitoba", the year 2012, the trial "SW-AMPanel_2012_Bozeman" and the trait "flowering date" (Fig. 6A).
   Select all 250 lines to be included in the analysis, and click on the "Save current selection" button.
4. Select a genetic map

   Navigate to the "Select" menu bar and select "Genetic map" (Fig. 6B). The "Genetic Map" option provides nine physical and genetic maps to use in analyses.
   Click the "Calculate markers in map for selected lines" button and, when the calculation is complete, select a genetic map with a high number of available markers. In this case, select the 90K Infinium array.
5. Set analysis parameters and start job

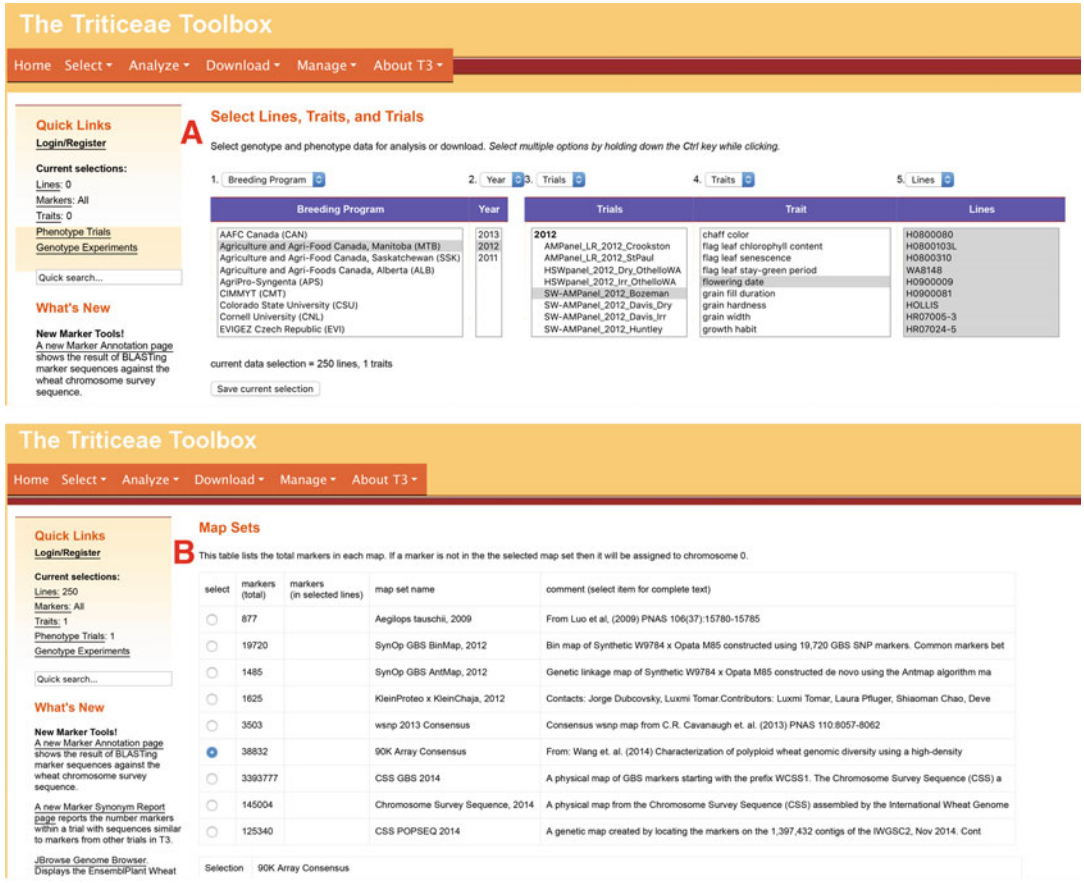


Fig. 6 Using the T3 Wheat Wizard to select lines, traits, and trials (*A*), and the genetic map menu to select one of the nine maps with sufficient markers for analysis in the selected lines (*B*)

After selecting and saving all required information, return to the "Genomic Association and Prediction" page (Fig. 7).

First, filter the lines and markers based on the minor allele frequency (MAF) and the amount of missing data with the "Filter Lines and Markers" button, in this case using the default values. Then set the number of principal components to include as fixed effects and the method for control of potentially confounding effects of population structure. Here, we set three fixed effects, and use the faster EMMAX method to control for population structure (*see* **Note 9**).

To start the analysis, click the "Analyze" GWAS button (Fig. 7). If T3 Wheat estimates that the analysis will take over 2 min, the estimated time required is shown and a "Check results" button is provided to check if the analysis is complete.

6. View and export results

After job completion, the page displays the results, including a scree-plot of the principal component eigenvalues, a *Q-Q* plot

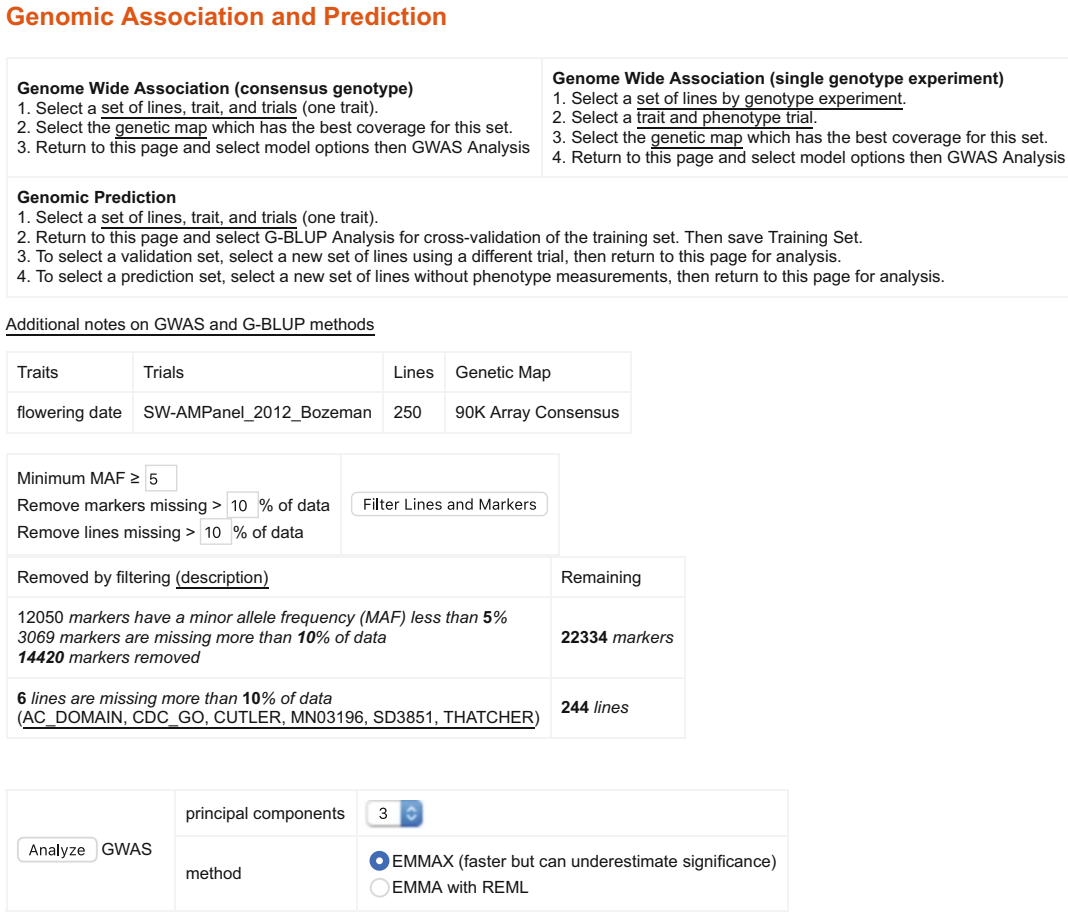


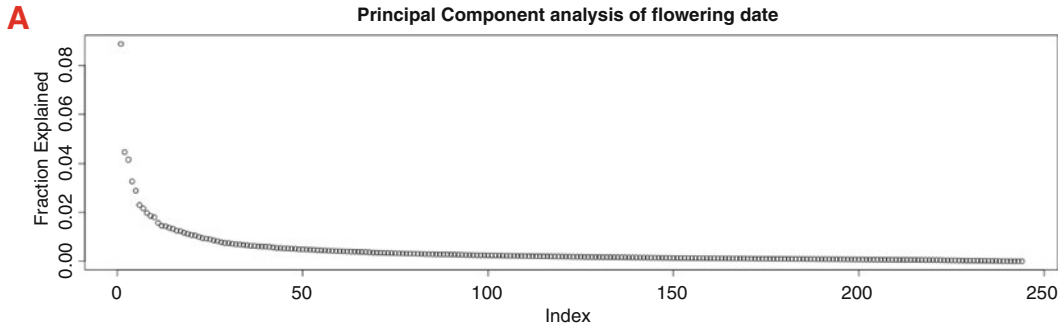
Fig. 7 T3 Wheat filter and analysis parameters for genome-wide association study

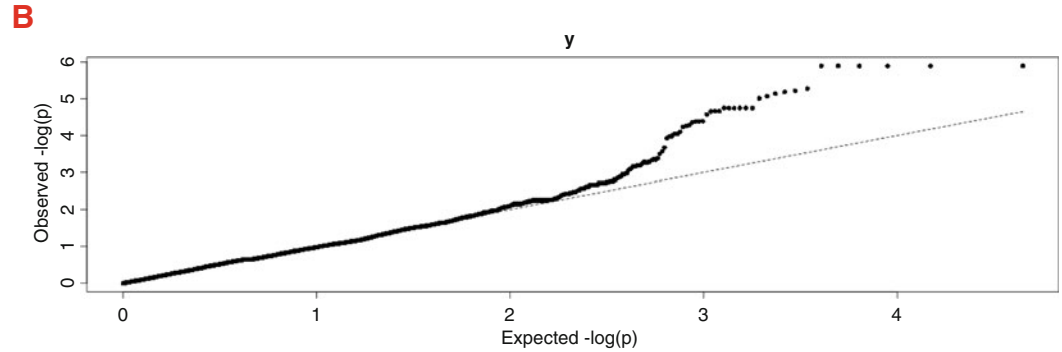
of observed versus expected −log10 *p*-values and a Manhattan plot (Fig. 8). The five markers with the highest negative log probability of association are also shown, with links to external resources providing more information on these markers.

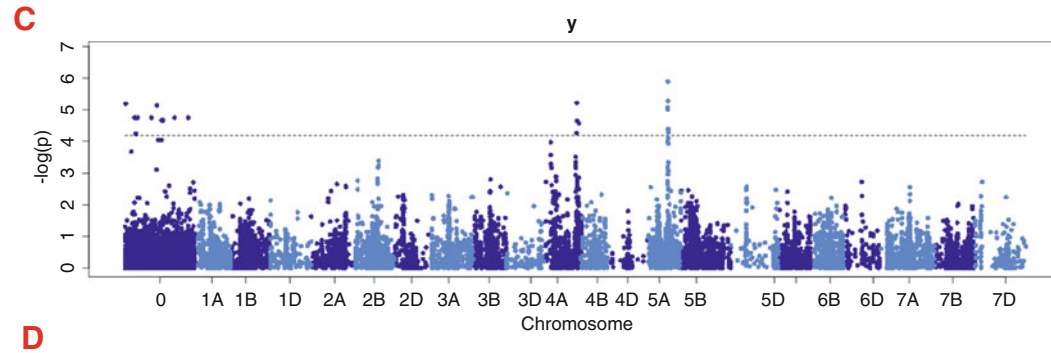
GWAS results can be exported as csv files via a hyperlink below the Manhattan plot. A kinship matrix of the lines analysed can also be exported.

4 Notes

1. Wheat Gbrowse2 has different wheat syntenic build versions (v0.1 and v2.0) of chromosome group 7. This is important because the cultivars contributing to the SNP track vary between these versions, with the earlier version containing less SNP information. SNPs in chromosome group 7 v2.0







D

marker	chrom	pos	value	external link (resource name)
BS00022071_51	5A	9054.441952	5.89011637630936	5AL_2686861 (IWGSC CSS) IWGSC_CSS_5AL_scaff_2686861:1956-2056 (Ensembl) UNK:828099570..828099670 (Triticeae Toolbox)
BS00031117_51	5A	9054.441952	5.89011637630936	5AL_2756379 (IWGSC CSS) IWGSC_CSS_5AL_scaff_2756379:3710-3810 (Ensembl) UNK:849864228..849864328 (Triticeae Toolbox)
IACX448	5A	9054.441952	5.89011637630936	5AL_2756379 (IWGSC CSS) IWGSC_CSS_5AL_scaff_2756379:5145-5245 (Ensembl) UNK:849865663..849865763 (Triticeae Toolbox)
Kukri_c9411_676	5A	9054.441952	5.89011637630936	
Excalibur_c23354_187	5A	9130.338364	5.89011637630936	5D:131823405-131823505 (Ensembl) 5D:131823405..131823505 (Triticeae Toolbox)

Fig. 8 Consensus genotype GWAS results using the T3 Wheat "Genomic Association and Prediction" tool. (**a**) Scree plot of principal component eigenvalues. (**b**) *Q-Q* plot showing observed GWAS negative log probability of association values against expected values. (**c**) Manhattan plot of GWAS. The *dotted horizontal line* indicates the cutoff for a false discovery rate (FDR) of 0.05. Unmapped markers are binned in a chromosome 0. (**d**) Highest scoring five markers with chromosome location, position, negative log probability of association value, and links to marker details

and the wheat pangenome were called using all 16 Australian cultivars.

2. The wheat genome BLAST GBrowse2 does not allow users to obtain results via email, which means closing the web page of an unfinished job will prevent the results being reported. However, users can bookmark the results page and retrieve results up to 5 days after job completion.
3. Users can visualize further features in the wheat genome browser by uploading customized tracks through the "Custom Tracks" tab. The upload accepts various file formats such as General Feature Format (GFF), Browser Extensible Data (BED), and Sequence Alignment/Map (SAM) files for displaying different types of information.
4. Query sequences in TAGdb must have a header in FASTA format and be less than 5000 nucleotides long.
5. One or more of these short paired-read libraries can be selected from the "Short paired-read library selection" panel. The naming format for the short paired-read libraries is *SourceName-ReadLength-InsertSize-LibraryName*.
6. To enhance the alignment speed and efficiency of TAGdb, it is advisable to submit multiple jobs, particularly if more than ten short read libraries are being queried.
7. The T3 Wheat datasets can vary greatly with regard to the amount of missing data. It is therefore advisable to take this into account when combining diverse datasets and filtering them for missing data, because this may inadvertently lead to a sampling different from that initially intended.
8. Optionally, T3 Wheat users can register for a free account to allow them to save current data selections from previous sessions.
9. To correct for sample structure by accounting for relatedness between individuals in GWAS, efficient mixed-model association (EMMA) uses high-density markers to model the phenotype distribution. The T3 Wheat GWAS offers two flavours of this method: EMMA and EMMA eXpedited (EMMAX). In EMMA, variance components are calculated for each marker being tested. EMMAX, on the other hand, makes the simplifying assumption that the effect of each SNP on the trait is small, and thus conducts only one variance component calculation, without any markers in the model. EMMAX is thus far less computationally demanding than EMMA, at the cost of potentially underestimating significance when individual markers have large effects. Although it is therefore advisable to use EMMA when markers with large effects are expected, this can increase the runtime of the analysis by magnitudes.

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Chapter 19

High-Density SNP Genotyping Array for Hexaploid Wheat and Its Relatives

Amanda J. Burridge, Mark O. Winfield, Alexandra M. Allen, Paul A. Wilkinson, Gary L.A. Barker, Jane Coghill, Christy Waterfall, and Keith J. Edwards

Abstract

A lack of genetic diversity between wheat breeding lines has been recognized as a significant block to future yield increases. Wheat breeding and prebreeding strategies are increasingly using material from wheat ancestors or wild relatives to reintroduce diversity. Where molecular markers are polymorphic between the host and introgressed material, they may be used to track the size and location of the introgressed material through generations of backcrossing. To generate markers for this purpose, sequence capture targeted resequencing was carried out for a range of wheat varieties, wheat relatives, and wheat progenitors. From these sequences, putative SNPs were identified and used to generate the Axiom® Wheat HD array. A selection of varieties representing a selection of elite wheat breeding material, progenitor species, and wild relatives were used to validate the array. The procedures used are described here in detail.

Key words Wheat, *Triticum aestivum*, SNPs, Genotyping, Array

1 Introduction

Hexaploid wheat (*Triticum aestivum*) is a globally important crop being the staple food for humans and livestock. Breeding efforts during the Green Revolution resulted in substantial yield increases that saw average yield double from 1.4 to 2.8 t/ha [1]. This yield increase has not been sustained and all countries are experiencing yield stagnation [1]. Recent breeding efforts have employed genomic technologies to develop faster and more accurate breeding strategies. These may also be used to identify potentially useful novel alleles outside of the elite wheat gene pool such as landrace varieties, wheat relatives, and progenitor species.

Wheat is derived from the hybridization of diploid *Aegilops tauschii* with tetraploid wild emmer, *Triticum turgidum* ssp. *dicoccoides* [2–4]. Since this hybridization event, early domestication

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subjected crops to strong selection pressure and often inbreeding, resulting in a reduced level of genetic diversity in modern wheat varieties compared to their wild ancestors [5]. This lack of genetic diversity is often cited as a limiting factor in the production of high yielding and stress resistant varieties [6–8]. It is possible to introduce novel genetic diversity into elite breeding lines through the introgression of material from wheat ancestors or wild relatives [9], a process employed by some prebreeding programs. Where molecular markers are polymorphic between the host and introgressed material, they may be used to track the size and location of the latter through generations of backcrossing.

Molecular markers are also widely used as a research tool to analyze existing populations. Within wheat, SNP based markers have been used to carry out genome-wide associate studies to elucidate the regions of the genome associated with disease resistance [10] and grain yield [11], for example.

Sequence capture targeted resequencing was carried out for a range of wheat varieties and for wheat relatives and progenitors considered to be a potential source of novel variation suitable for introgression into wheat. From these sequences, a large number of putative SNPs between different varieties of hexaploid wheat and between hexaploid wheat and related species were identified [12]. The Axiom® Wheat HD array was designed to validate these SNPs and to map introgressions within the hexaploid wheat genome. A selection of diploid, tetraploid, and hexaploids varieties, representing elite wheat breeding material, progenitor species, and wild relatives, was used to validate the array (Table 1). The procedures described in depth in this chapter cover the method used to genotype these accessions using the Wheat HD array.

The markers, associated sequence, and genotype information relating to the Axiom® Wheat HD array have been made available through an interactive website. www.cerealsdb.uk.net.

2 Materials

2.1 Plant Material

For this study, plants of bread wheat and wheat relatives (Table 1) were grown in a peat-based soil in single pots at 15–25 °C with a day–night cycle of 16 h light and 8 h dark. An approximately 8 cm section of the first true leaf was collected from plants 6 weeks post-germination and stored at −80 °C prior to DNA extraction.

2.2 Reagents and Solutions

1. RNase A (Thermo Scientific).
2. Isopropanol.
3. DNA Extraction Buffer: 200 ml 1 M NaCl, 25 ml 2 M Tris–HCl pH 7.5, 50 ml 10% SDS, 50 ml 0.5 M EDTA per litre.

Table 1
Wheat species and wheat relatives used on the 820K HD Array

Accession	Genome
Hexaploid	
108 *Triticum aestivum* elite varieties	ABD
27 *Triticum aestivum* landrace accessions	ABD
1 *Thinopyrum intermedium*	SJJ
133 Avalon × Cadenza mapping population	ABD
64 Savannah × Rialto mapping population	ABD
60 Synthetic × Opata mapping population	ABD
32 Deletion lines	
20 Nullisomic lines	
Tetraploid	
8 *Triticum turgidum* ssp. *Durum*	AB
1 *Triticum timopheevii*	AG
1 *Aegilops peregrina* (*Ae. variabilis*)	SU
Diploid	
1 *T. monococcum* ssp. *aegilopoides* (*T. urartu*)	A
11 *Ae. tauschii* accessions	D
1 *Thinopyrum elongatum*	E
1 *Thinopyrum bessarabicum*	J
1 *Secale cereale*	R
1 *Aegilops speltoides*	S
1 *Aegilops markgrafii* (*Ae. caudata*)	T
1 *Amblyopyrum muticum* (*Ae. mutica*)	T
Decaploid	
1 *Thinopyrum ponticum*	JJJJsJs

4. 25 bp ladder (TrackIt, Invitrogen).

The following solutions were made, according to guidelines given in *Axiom® 2.0 Assay Manual Workflow User Guide Rev3*, using reagents supplied by Affymetrix upon the purchase of the Axiom array.

5. Denaturation Master Mix: 400 μl "Axiom 2.0 Denat Soln" 10×, 3.6 ml DNAse-free water (*see* **Note 1**).
6. Neutralization Solution: "Axiom Neutral Soln" use as supplied.

7. Amplification Master Mix: 26 ml "Axiom 2.0 Amp Soln" and 578 µl "Axiom 2.0 Amp Enzyme" (*see* **Note 1**).
8. Fragmentation Master Mix: 6 ml "Axiom 10× Frag Buffer," 1.35 ml "Axiom Frag Diluent," and 131 µl "Axiom Frag Enzyme" (*see* **Note 1**).
9. Precipitation Master Mix: 26 ml "Axiom Precipitation Solution 1" and 218 µl "Axiom Precipitation Solution 2" (*see* **Note 1**).
10. Resuspension Buffer: "Axiom Resusp Buffer" use as supplied.
11. Hybridization Master Mix: 7.8 ml "Axiom Hyb Buffer," 55.6 µl "Axiom Hyb Solution 1," and 1 ml "Axiom Hyb Solution 2" (*see* **Note 1**).
12. Stain 1 Master Mix: 22.2 ml "Axiom Wash A," 463 µl "Axiom Stain Buffer," 231 µl "Axiom Stain 1A," 231 µl "Axiom Stain 1B." Prepared soon before use, store away from light.
13. Stain 2 Master Mix: 11.1 ml "Axiom Wash A," 231 µl "Axiom Stain Buffer," 115.6 µl "Axiom Stain 2A," and 115.6 µl "Axiom Stain 2B." Prepared soon before use, store away from light.
14. Stabilization Master Mix: 10.3 ml DNAse-free water, 1.16 ml "Axiom Stabilize Diluent," and 144.8 µl "Axiom Stabilize Soln" (*see* **Note 1**).
15. Ligation Master Mix: 7.3 ml "Axiom Ligate Buffer," 1.45 ml "Axiom Ligate Soln 1," 348 µl "Axiom Ligate Soln 2," 1.16 ml "Axiom Probe Mix 1," 1.16 ml "Axiom Probe Mix 2," and 174.4 µl "Axiom Ligate Enzyme." Prepared immediately prior to use.

2.3 Consumables

1. Deep well 96-well plates (at least 2.2 ml).
2. PCR machine suitable 96-well plates.
3. Good quality plate seals.
4. Precast 4% agarose eGel (Thermo Fisher Scientific).
5. Qiagen purification kit (Qiagen).

2.4 Equipment

1. Multichannel pipettes (8 or 16 channel); P20, p200, and p1200 suggested.
2. Vortex.
3. Centrifuge capable of up to 2250 RCF.
4. Ovens; at least two, but three would be ideal to prevent cross-contamination.
5. Freezer set to −20 °C.
6. Mircroplate reader with 260 nm (A260) optic module.
7. Microplate shaker.
8. UV Transilluminator.

3 Methods

3.1 Array Design

This protocol is for the predesigned Wheat HD array, however other custom arrays may be designed and genotyping performed in the same manner. As well as custom designs from sequence data, it is possible to generate new arrays from a combination of existing probes. For the Wheat HD array, smaller arrays have already been designed selecting only the probes most useful in wheat breeding [13] and in wide crosses with wild relatives [14]. When designing an array, take care in choosing the probes to include. Redesigns are expensive so it is worthwhile including multiple probes on important targets. Fortunately, there is a good conversion of probes between genotyping platforms enabling the use of prevalidated probes.

3.2 DNA Extraction

1. Prior to use, store extraction buffer for 1 h in a preheated (65 °C) oven.
2. Take an approximately 8 cm section of the first true leaf (*see* **Note 2**) and immediately freeze using liquid nitrogen. Store at −20 °C prior to DNA extraction.
3. Using a mortar and pestle, grind frozen leaf tissue into a fine powder. Add small quantities of liquid nitrogen throughout to keep the leaf tissue frozen and brittle.
4. Transfer tissue to a solvent-safe, 15 ml centrifuge tube and add 5 ml of DNA extraction buffer.
5. Invert several times and then incubate at 65 °C for 20 min; invert once more after the first 10 min.
6. Allow the centrifuge tube to cool to room temperature and add 5 ml phenol. Invert several times and centrifuge at 3900 RCF for 20 min.
7. Using a large volume pipette, carefully transfer the top aqueous phase to a fresh solvent-safe, 15 ml centrifuge tube. Add 3 ml chloroform, invert several times and centrifuge at 3900 RCF for 10 min.
8. As before, carefully transfer the top aqueous phase to a fresh solvent-safe 15 ml tube. Add 3 ml isopropanol, invert several times and centrifuge at 3900 RCF for 20 min.
9. Carefully invert over the sink to remove the waste isopropanol solution; pellet should appear pale white and clean. Allow the centrifuge tube to remain inverted on lab roll for approximately 10 min.
10. When there is no longer an odour from the residual isopropanol, add 200 μl water and vortex to suspend the pellet.

3.3 RNase Treatment

1. Preheat an oven or water bath to 37 °C.
2. Quantify samples using a fluorometric method such as Qubit and, where there is great variation in concentration, dilute to a uniform 50 ng/μl.
3. Add 1 μl of RNase to 150 μl of 50 ng/μl DNA. Briefly vortex and centrifuge each sample and incubate at 37 °C for 50 min.
4. Cool to room temperature and continue to DNA purification.

3.4 DNA Purification

Samples are purified using the protocol suggested by the QIAquick PCR purification kit (QIAquick Spin Handbook, 2008) (*see* **Note 3**).

1. Add 750 μl of Buffer PB to 150 μl sample DNA at 50 ng/μl and transfer to the QIAquick column. Centrifuge for 60 s at 14,100 RCF and discard the waste solution.
2. Add 750 μl of Buffer PE to the QIAquick column and centrifuge for 60 s at 14,100 RCF. Discard the waste solution and centrifuge again for 60 s at 14,100 RCF without any additional buffer.
3. Replace the bottom component of the QIAquick column with a microcentrifuge tube. Cut the hinge lid from the microcentrifuge tube before use to prevent it causing friction in the centrifuge.
4. Add 30 μl of Buffer EB directly to the bottom of the QIAquick column and allow to stand for 60 s before a final centrifugation for 60 s at 14,100 RCF.

3.5 Sample Preparation

The Wheat HD Axiom array exists as a two array set (550491 and 550492). The following stages must be carried out simultaneously for two 96-well sample plates with identical sample layouts (*see* **Notes 4** and **5**).

1. Quantify samples and dilute to a uniform 10 ng/μl (*see* **Note 6**).
2. Carefully transfer 20–22 μl of 10 ng/μl DNA to each well. Ensure that the same sample has the same location in each plate (A and B) (*see* **Note 7**).
3. Briefly centrifuge to spin down samples.

3.6 Amplification

1. Preheat the oven to 37 °C (*see* **Note 7**).
2. Using a multichannel pipette, carefully add 20 μl of the Denaturation Master Mix to each well (*see* **Note 8**). Set a timer for 10 min. Seal the plates well, vortex to mix, and centrifuge briefly to bring down any solution from the sides of the well. Allow the plates to sit at room temperature for the remaining time.

3. At the end of 10 min, use a multichannel pipette to add 130 µl of the supplied neutralization solution ("Axiom Neutral Soln") to each well. Work in the same row and plate order as previously. Seal the plates well, vortex to mix, and centrifuge to spin down solute.
4. Carefully add 230 µl Amplification Master Mix to each well. Seal the plates well, vortex to mix, and centrifuge to spin down solute. Place in the preheated 37 °C oven and allow to incubate for 23 h. This incubation time can be increased if difficulty in extracting sample resulted in less than the recommended starting amount of DNA being used.

3.7 Fragmentation

1. Preheat one oven to 65 °C and another to 37 °C (*see* **Note 9**).
2. At the end of the 23 h amplification incubation, transfer both sample plates to the 65 °C for 20 min to stop the amplification reaction.
3. After 20 min transfer both sample plates to the 37 °C oven for 45 min.
4. Using a multichannel pipette, carefully add 57 µl of the Fragmentation Master Mix to each well. Set a timer for 30 min. Seal the plates well, vortex to mix, and centrifuge to spin down solution before returning to the 37 °C oven.
5. Be prepared to add 19 µl of the supplied "Stop Solution" to each well after exactly 30 min. Using a multichannel pipette, work in the same row and plate order as previously. Seal the plates well, vortex to mix, and centrifuge to spin down prior to precipitation.

3.8 Precipitation

1. Add 240 µl of the Precipitation Master Mix to each well. Seal the plates well, vortex to mix, and spin down. The samples should now be pale blue in colour.
2. Using a large volume multichannel pipette, add 600 µl of isopropanol to each well. To thoroughly mix, carefully aspirate and dispense several times until the solution in each well is uniformly cloudy.
3. Use lab roll to remove any excess liquid from the top of the plate to prevent contamination between wells. Seal the plates well and incubate at −20 °C for 16–24 h.

3.9 Resuspension

1. Prechill a centrifuge to 4 °C and one post-amplification oven to 37 °C.
2. Centrifuge both plates simultaneously at 2250 RCF for 40 min. Pale blue-white pellets should be visible in each well.
3. Remove the seal from the sample plate and carefully invert over the sink to remove the waste isopropanol solution. The pellets

should not dislodge if the plate is inverted with a smooth action; take care not to knock or shake the plate as this could result in pellet loss.

4. Allow the plates to remain inverted on lab roll for 5 min. The tissue will rapidly become saturated, so it is recommended to replace the tissue several times.
5. After 5 min, move sample plates to the 37 °C oven to dry for a further 20 min. Where possible, do not exceed 20 min drying time as this will affect the resuspension of the pellet.
6. Remove any excess liquid from the top of the plate with lab roll and add 35 μl of the supplied resuspension buffer ("Axiom Resusp Buffer") to each well.
7. Seal well and use a plate shaker to shake the plates for 10 min. This can be extended as required to fully resuspend the pellets. Occasionally, the addition of an extra 5–10 μl of resuspension buffer may be required if the pellet remains present after 20 min.
8. When fully suspended, spin down both plates and transfer the contents to a 96-well, PCR plate. Add 80 μl of the Hybridization Master Mix to each well. Seal well, vortex to mix, and spin down.
9. As this stage, it is possible to remove a small amount from each well to confirm the success of the amplification and fragmentation reactions. This is recommended as repeated sample preparation is preferable to poor array data.
10. The hybridization plates can be safely stored at −20 °C until the sample quality has been established.

3.10 Quality Control

To confirm the successful amplification and fragmentation of sample DNA, whole plate quantification is suggested using an optical plate reader. All, or a substantial subset, of the samples may be run on a gel to confirm fragment size.

1. Transfer 3 μl from each well of the hybridization plates to new 96-well "Dilution plate" and dilute with 33 μl of DNase-free water. Seal well, vortex to mix, and spin down.
2. Further dilute the Dilution Plate samples 1:10 with DNase-free water and use an OD260 optical plate reader to quantify all samples simultaneously. Methods may vary depending on the plate reader used. After multiplication by the dilution factor of 120, the plate median DNA should be over 1000 μg. If the plate median is considerably below this, poor genotyping will result.
3. Further dilute the dilution plate samples 1:40 with gel loading dye and load 20 μl sample per well onto a precast eGel (4%

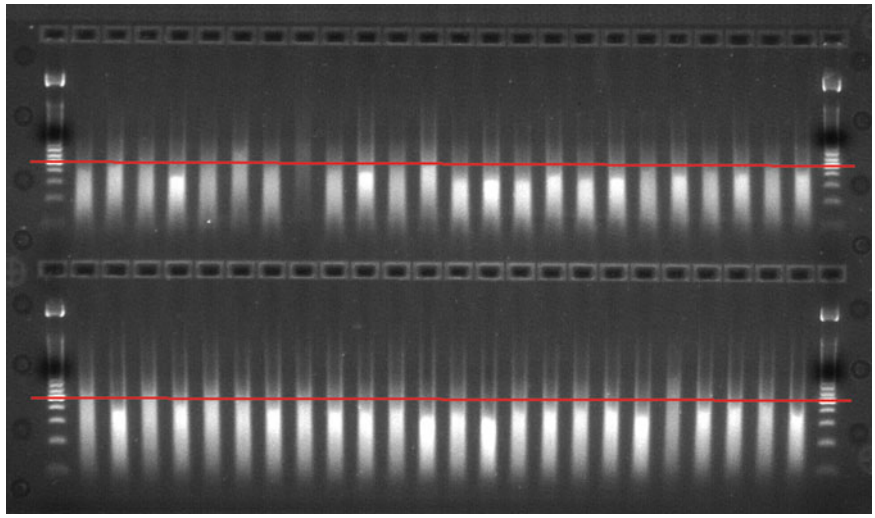

Fig. 1 Agarose gel showing amplified samples. The *red line* indicates a fragment size of 125 bp. After a successful fragmentation, the DNA should be below this point at a range of 125–25 bp

agarose, double-comb cassette). This allows for 48 samples per gel minimizing gel to gel variation.

4. Add 15 μl of a 25 bp ladder (1:10 dilution TrackIt, Invitrogen) to the flanking wells of each row.
5. Run gel using the preset EG protocol for 22 min and visualize immediately using a UV transilluminator. The post-fragmentation size should be between 125 and 25 bp (Fig. 1). Larger fragments will negatively affect the hybridization to the array.

3.11 Denaturation

The following steps relate to the Gene Titan equipment (*see* **Note 10**). Sample denaturation is not to be carried out for both plates simultaneously. The Gene Titan software will indicate when plate B should be loaded (approximately 8 h after plate A). It is recommended that plate B is stored at −20 °C until required.

1. Defrost the sample plate and allow the appropriate array to acclimatize to room temperature.
2. Centrifuge sample plate and using a PCR machine with a hot-lid function, denature at 95 °C.
   - The suggested protocol is as follows:
     - 95 °C 10 min
     - 48 °C 3 min
     - 48 °C hold
3. The sample plate is ready to be transferred to the Gene Titan after the 3 min 48 °C step. A hold step is used to maintain the

sample plate at 48 °C should any delays or technical difficulties be encountered.

4. Using a multichannel pipette, carefully transfer the contents of each well to a Gene Titan readable "Hybridization Tray" and load into the Gene Titan and the appropriate array following the on-screen instructions.

3.12 Gene Titan Reagent Preparations

1. Make up the Stabilization Master Mix, Ligation Master Mix, and both Stain 1 and 1 Master Mix as described in Subheading 2.2 immediately prior to use. As the Ligation Master Mix involves the least stable enzyme it is suggested that this is left until last.
2. Use a static gun to remove potential charge on all Gene Titan readable stain trays and lids. Failure to remove static will lead to reagent adhering to the lid and being lost from the tray.
3. Using two stain trays for Stain 1 and one stain tray for the other reagents, add 105 μl of the Master Mix to each well. Ensure that no bubbles are present as this will interfere with the application of the reagents onto the array.

3.13 Data Analysis

Data analysis is carried out using the proprietary but freely available "Axiom Analysis Suite" software.

1. Select the correct array type from the drop-down menu. The Wheat HD array has an A and B option which are genotyped separately. Take care to upload .CEL files from the corresponding A or B plate (*see* **Note 11**).
2. Select the correct analysis and prior files. These will vary with array but may also vary within projects. Genotype calls are generated by the Axiom GT1 clustering algorithm which uses the observed data and specified priors file to do so. The default Wheat HD priors (Axiom_BristolW_A.r2.AxiomGT1 and Axiom_BristolW_B.r2.AxiomGT1) were designed based on the clustering behavior of 475 wheat and wheat relative lines.
3. Use the "Import CEL files" option to select the appropriate files. The Gene Titan will generate a number of files, some are intermediary and some will only become relevant during troubleshooting. The files required for generating genotype data are the .CEL files, each of which represents one sample. It is possible to combine .CEL files from multiple projects as long as the array type remains the same; for example, .CEL files generated using the Wheat HD array cannot be genotyped simultaneously with the Wheat Breeders Array. For accurate clustering, at least 96 samples are required in one project.
4. Adjust the Threshold Configurations for the project. Defaults exist for some levels of ploidy, but changes should be made to

suit the needs of the project. For example, the default call rate threshold is 97%, but for a set containing both wheat and wheat relatives, a call rate threshold of 80% may be used. The Wheat HD array contains a range of probes which are polymorphic between different material of different ploidy (elite hexaploid, wheat relatives, and landraces). As such, there is no wheat accession which could result in a 100% call rate and so the cutoff for a "good" call rate is lowered.

5. Select the output folder and batch name then select "Run Analysis." The analysis may take some time to run, depending on the number and size of the files.
6. The genotypes are exported in a file called "AxiomGT1.calls.txt", each row of which represents an SNP on the array and each column a sample. The genotypes are encoded numerically (Table 2).

The software uses a number of clustering properties to classify the SNPs into performance categories (Fig. 2) (*see* **Note 12**).

1. Poly High Res: Polymorphic and codominant. Three clusters consisting of one heterozygous group and two homologous groups. Each cluster will contain at least two samples.
2. No Minor Hom: Polymorphic and dominant. Two clusters, one heterozygous group and one homologous group.
3. Off-Target Variant (OTV): Four clusters consisting of one heterozygous group and two homologous groups.
4. Mono High Res: Monomorphic single cluster.
5. Call Rate Below Threshold: The SNP call rate is below the defined threshold while other cluster properties are above threshold.
6. Other: One or more of the cluster properties are below threshold.

Table 2
Numerical called given in the AxiomGT1.calls.txt file and their genotype equivalent

Axiom genotype	
−1	Null
0	AA
1	AB
2	BB

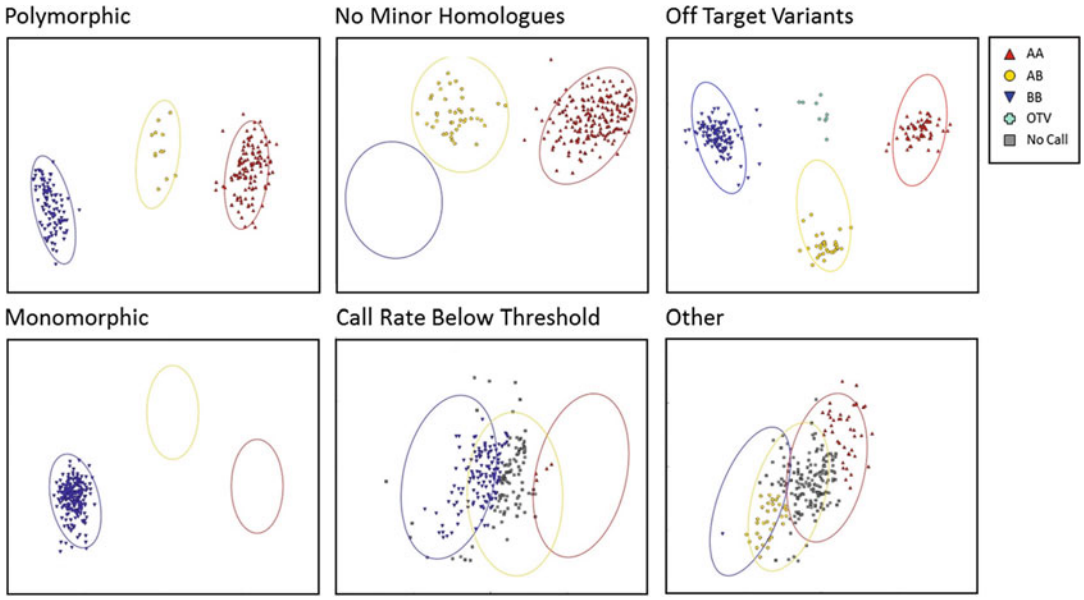

Fig. 2 Examples of the six performance categories into which the Axiom Analysis Suite software can place probes

4 Notes

1. These solutions should be prepared prior to use, not made in advance and stored.
2. Different accessions and tissues types may be used depending on the research interest of the investigator. As the Wheat HD array was designed to accommodate diverse breeding material (Table 1), it is possible to use a range of wheat related species on the same array.
3. RNAase treatment and purification of samples are carried out to minimize any potential interference from impurities in the sample. In practice, we have found the protocol to be very robust and generate unambiguous genotype scores for untreated DNA extracts. Note, however, that if RNAase treatments are omitted, then quantification by spectrophotometer such as Nanodrop may overestimate the amount of DNA present.
4. Use two 2.2 ml deep-well 96-well plates labeled "A" and "B" to ensure sample preparation is carried out in the same order each time.
5. The Wheat HD sample preparation involves two sample plates and the ability to balance a centrifuge at every step. If you are using a single-plate array, it is recommended that you prepare

Table 3
Plate weight required to balance sample plate at each stage of sample preparation

Sample preparation stage	Additional weight
Empty plate	–
After denaturation solution	–
After neutralization solution	15 g
After amplification master mix	37 g
After fragmentation master mix	42 g
After fragmentation stop solution	42 g
End of precipitation	124 g
Resuspension	c. 5 g but varies with pellet size

Weights are additional to the base weight of the plate which will vary with manufacturer

the appropriate balances in advance to prevent delays in the protocol (Table 3).

6. The protocol is quite robust and may tolerate samples outside of the 20–22 μl of 10 ng/μl parameters if necessary.
7. During the preparation of sample plates, it is possible to make a mistake resulting in nonidentical sample plate layouts. As long as each sample is present on each plate (A and B) the correct sample locations may be recorded on the Gene Titan sample submission sheet and the resulting data will still be correct. Unlike many other genotyping methods, a negative control is not required to generate an accurate genotype call. In fact, where negative controls are included, the genotyping software will identify this as a poor quality sample and remove it from the analysis.
8. For all stages involving both samples plates, work quickly and methodically. Pipette in the same plate and column order each time to reduce differences in incubation times between samples.
9. Where possible, carry out pre- and post-amplification steps in separate locations and using separate pipettes to prevent contamination of future preamplified samples.
10. This Gene Titan is unique to the Axiom array, so is not as ubiquitous as the other items of equipment used throughout the method. It may be necessary to transport the prepared sample plate to another facility which has access to a Gene Titan. Ensure that the sample plates are well sealed and frozen for transit.
11. The clustering algorithm determines genotype more accurately where all three clusters are observed. If data from multiple

Wheat HD arrays are to be compared, better results will be obtained by analysing all the "A" .CEL files and all the "B" .CEL files simultaneously rather than attempting to compare outputs for multiple projects.

12. The SNP performance classification is based on traits such as SNP call rate and cluster spread which can vary depending on the samples used. An SNP may be assigned a different performance category depending on the set of samples used. *See* Fig. 2 for an example of each performance category.

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