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Precision plant breeding using genome editing technologies

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Recent advances in CRISPR/Cas genome editing enable efficient targeted modification of most crop plants, thus promising to accelerate crop improvement (Gao 2018). There are three major types of sequence-specific nucleases (SSNs): zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the CRISPR system (Boch et al. 2009; Jinek et al. 2012; Kim et al. 1996). The DNA double strand breaks (DSBs) created by SSNs are normally repaired by one of two DNA repair mechanisms: nonhomologous end joining (NHEJ) or homologous recombination (HR). Cells have the ability to detect the broken DNA and repair it either by pasting together the ends of the DNA while making tiny sequence changes at this position, or by inserting a new DNA fragment bridging the site of the cut (Symington and Gautier 2011).

Both ZFNs and TALENs recognize specific DNA sequences through protein-DNA interactions (Boch et al. 2009; Kim et al. 1996), requiring substantial protein engineering to be carried out for each DNA target site to be modified. The CRISPR system, such as CRISPR/Cas9 and CRISPR/Cas12a, is RNA-directed DNA cleavage system. The guide RNA (gRNA) binds targeted DNA via Watson-Crick base pairing and directs the Cas9/Cas12a nuclease to produce double stranded cuts (Jinek et al. 2012; Zetsche et al. 2015). The CRISPR system requires only the guide RNA sequence to be changed for each DNA target site, so it is a simple, inexpensive and versatile tool for genome editing. Another major advantage of CRISPR systems over ZFNs and TALENs is that it is easier to target multiple sites simultaneously using multiple sgRNAs while expressing a single Cas9 or Cas12a protein (Čermák et al. 2017; Zetsche et al. 2017).

CRISPR arrays in bacteria serve as an immunological memory and defense mechanism, in which the arrays are programmed to target and cut viral DNAs and so destroy any invading virus (Marraffini 2015). In 2012, the groups of Charpentier and Doudna (Jinek et al. 2012), and Siksnys (Gasiunas et al. 2012) showed that Type II CRISPR/Cas9 can be guided by crRNAs to cleave target DNA in vitro. In 2013, studies from the laboratories of Feng Zhang (Cong et al. 2013) and George Church (Mali et al. 2013) simultaneously

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showed that it was possible to use CRISPR/Cas9 to perform genome editing in mammalian cells. Since then, CRISPR has been used by many hundreds of laboratories for genome editing applications in a variety of plants and animals. In 2013, the CRISPR system was developed for use in rice, wheat, tobacco, and *Arabidopsis thaliana* by three independent groups (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013).

Ongoing crop improvement requires the constant creation and use of new allelic variants. Conventional plant breeding, i.e. cross breeding, takes many years and can only introduce traits already available in the relevant genomes. Of course mutational approaches have increased the available genetic variation by introducing random genetic changes using chemical mutagens or physical irradiation. However, mutagenesis is inherently random and non-specific, and generating and screening large numbers of mutants is challenging. By contrast, genetic transformation can introduce desired traits into elite background varieties through the transfer of exogenous genes. However, since such foreign DNA is inserted at random into the recipient genome and may have potentially harmful consequences, the commercialization of GMOs is limited by long and costly regulatory requirements, as well as public concern. Genome editing can accelerate plant breeding by allowing the introduction of precise and predictable modifications directly into an elite background (Chen et al. 2019). Genome editing using Cas9 ribonucleoprotein (RNP) preparations has been achieved in lettuce (Woo et al. 2015), wheat (Liang et al. 2017), maize (Svitashev et al. 2015) and potato (Andersson et al. 2018), and should make the new gene editing technologies more acceptable worldwide.

Gene editing can be used to create traits of value. As an example, we may consider wheat, which is hexaploid, full of duplicated sequences, and with a very large (17.1 gigabases) genome. The MLO proteins of wheat function as negative regulators of plant defense against powdery mildew disease, and wheat harbors six *MLO* alleles, making it difficult to inactivate all six genes to generate *mlo* wheat mutant lines through traditional breeding. Instead we designed a pair of TALENs that target a conserved region of *TaMLO*. We transformed the required TALEN construct into immature wheat embryos by particle bombardment and obtained a spectrum of mutation types with one, two or three mutated gene copies. When we tested the various mutants we found, as we had expected, that the number of mildew microcolonies formed on the leaves was significantly reduced in the triple mutant plants, with no fungal growth detected on the leaves of these plants (Wang et al. 2014).

Typically, CRISPR/Cas9 expression cassettes are delivered to plant cells and expressed, and proceed to cleave chromosomal target sites and produce site-specific DNA double-strand breaks, leading to genetic alterations during the repair process. The introduced CRISPR/Cas9 DNA usually becomes integrated into the plant genome, but can be segregated out in the T1 or T2 generations. The disadvantages of this conventional genome editing system include: (1) the potential for off-target effects; (2) the considerable time required to segregate integrated cassettes; (3) the fact that it is impossible to segregate integrated CRISPR cassettes in vegetatively propagated plants; and (4) the difficulty of avoiding small DNA insertions at on-target or off-target sites.

To avoid these disadvantages, we developed a simple and efficient DNA-free genome editing approach involving the use of CRISPR/Cas9 RNP. In an example of this approach, RNPs were delivered into immature wheat embryos by particle bombardment. The RNP-encoded genes were expressed transiently before being degraded by host enzymes, and the bombarded embryos produced callus cells from which seedlings were regenerated and mutants identified. The particular gene targeted was *TaGW2*, which functions in control of grain weight, and of which there are three very similar copies (*TaGW2-A1*, *-B1* and *-D1*) in bread wheat. The guide RNA sequence matched perfectly its recognition site in *TaGW2-B1* and *-D1,* but differed by a single nucleotide from its target site in *TaGW2-A1*. In parallel, we delivered the DNA plasmid pGE-TaGW2. Among the 28 mutants induced by gw2-RNPs from 640 bombarded immature embryos, we did not detect any mutations in *TaGW2-A*, while 24 of 30 *tagw2* mutants obtained using plasmid pGE-TaGW2 harbored mutations in *TaGW2-A1*. This indicates that RNP is more specific than DNA constructs in inducing mutagenesis (Liang et al. 2017), presumably because the DNA constructs persist in the transformed cells for much longer than the RNP.

To conclude, genome editing can efficiently induce targeted mutation in plant genomes and this technology offers significant potential for trait improvement

without requiring the introduction of foreign DNA. In terms of regulatory considerations, it is important that the final products created by genome editing are identical to the mutants obtained by conventional mutagenesis.

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