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A CRISPR way for accelerating improvement of food crops

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CRISPR technology, which is widely used for plant genome editing, will accelerate the breeding of food crops beyond what was imaginable before its development. Here we provide a brief overview of CRISPR technology, its most important applications for crop improvement and several technological breakthroughs. We also make predictions of the applications of CRISPR technology to food crops, which we believe would provide the potential for synthetic biology and domestication of crops. We also discuss the implications of regulatory policy for deployment of the technology in the developing world.

Current agricultural practice is struggling to meet the level of primary productivity required to feed 10 billion people by 2050[1]. Agricultural production faces the challenge of expanding sustainably and maintaining nutritional quality under intensifying climate change. Conventional crop breeding, which depends on screening genetic variations from spontaneous mutations, chemical mutagens, physical irradiation and recombination following hybridization, is usually labour-intensive and time-consuming, and cannot keep pace with the increasing demand for food[2]. Continuous innovation in crop breeding will thus be critical to meeting these challenges and achieving sustainable food production. Recent advances in CRISPR (clustered regularly interspaced short palindromic repeats) technologies make the targeted and precise genetic manipulation of crops a reality, and can thereby accelerate the transition towards precision breeding for crop improvement[3].

Brief overview of CRISPR technology

CRISPR technology is based on RNA-programmed DNA cleavage systems that were discovered in bacteria and archaea. CRISPR–Cas9 and CRISPR–Cas12a are the best-studied and most widely used CRISPR systems[4,5] (Fig. 1a). Each system has two components: a DNA endonuclease (Cas9 or Cas12a) and an RNA molecule that confers targeting specificity, known as single-guide RNA (sgRNA) or CRISPR RNA (crRNA)[4,5] (Fig. 1a). The only prerequisite for applying CRISPR to a given target is the presence of a protospacer-adjacent motif (PAM) sequence near the site of interest. Using CRISPR for various targets thus only requires different spacer sequences; hence it is simple, rapid, efficient, inexpensive and versatile.

To take advantage of CRISPR in plant genome editing, the CRISPR reagents — in the form of DNA, RNA or ribonucleoprotein (RNP) — are delivered into plant cells (Fig. 1b) to cut the plant DNA in a predetermined sequence. To preserve genome integrity, the plant cell needs to 'repair' the break, and this leads to the introduction of different types of mutation in the targeted sequence (Fig. 1c). In cases where the break is repaired by the non-homologous end-joining (NHEJ) DNA repair pathway, small insertions or deletions of nucleotides (INDELs) can occur, with the potential to knock out the corresponding gene[6] (Fig. 1c). Alternatively, the availability of a DNA template with homologous sequences around the target site can trigger homology-directed repair (HDR), which can lead to insertion of the DNA template, thereby allowing precise gene replacement or insertions[6] (Fig. 1c).

However, making DNA breaks is not all CRISPR can do; base editing, for example, is the most recent addition to the uses of this technology[7,8] (Fig. 1d). Making use of a nicking rather than cutting Cas9, or a dead Cas9/Cas12a with one or both cutting domains deactivated, base editors have the ability to alter single target nucleotides without needing a foreign DNA template or producing DNA breaks[7,8] (Fig. 1d). So far, C–T and A–G conversions have been accomplished using base editors[7,8], provoking considerable interest in base editors involved in food crop improvement. By using a dead Cas9/Cas12a, CRISPR technology can also be used for gene regulation, epigenetic modification and chromosomal imaging and so on[3].

Applications of CRISPR for crop improvement

The application of CRISPR technology in crop improvement has so far been focused on the improved crop yields, quality and stress resistance that could be obtained by simple knockout of one or several genes that confer undesirable traits[9]. For example, knocking out *Gn1a*, *DEP1* and *GS3* in rice led to enhanced grain number, dense erect panicles and larger grain size[10]; disrupting the waxy gene *Wx1* in maize resulted in high amylopectin content with improved digestibility that has the potential to be commercialized[11]; and destroying the *MLO* allele generated powdery mildew-resistant wheat and tomato[12,13].

Recently, CRISPR-mediated gene knockout has been used to maintain heterosis[14,15], which is usually lost in subsequent generations owing to genetic segregation. In rice, a genotype named *MiMe* (Mitosis instead of Meiosis) was produced by targeting crucial genes related to meiosis, and haploid plants were also created using CRISPR technology. When *MiMe* was combined with haploidy in hybrid rice, the clonal progeny retained the genome-wide parental heterozygosity, thus demonstrating the feasibility of asexual reproduction through seed propagation in crops[14,15].

In crops, many agriculturally important traits are conferred by single-nucleotide polymorphisms (SNPs) or by dominant gain-of-function point mutations[16]. Such traits can now be generated by

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CRISPR-mediated base editing, which provides a new degree of precision in creating base substitutions and presents ample opportunities for crop improvement. Creating herbicide-resistant crops by base editing[17,18], for example, can improve the productivity of agricultural systems by managing weeds and preserving soil and soil moisture. Acetolactate synthase (ALS) is a key enzyme in the biosynthesis of branched-chain amino acids, in which a single amino acid substitution (ALS-P174F in wheat) confers herbicide resistance, providing an ideal target for base editing. Using A3A-PBE-mediated cytidine base editing in wheat, all six TaALS alleles could be simultaneously edited, producing nicosulfuron-resistant wheat lines[17]. The herbicide tolerance endowed by TaALS-P174 was then shown to be an efficient selective marker in wheat: by combining the TaALS-P174 editor with other editors, the co-editing events can easily be identified in medium supplemented with nicosulfuron by only selecting edits produced by other editors[18]. This selectable co-editing system improved the recovery of coupled editing events and operates without foreign DNA integration[18], which provide new options for base editing in crop breeding. In addition to wheat, herbicide-tolerant watermelon and rice have been generated by modifying ALS and a key enzyme for lipid biosynthesis, acetyl-coenzyme A carboxylase (ACC), by CRISPR-mediated cytidine and adenosine base editing, respectively[19,20].

CRISPR technology is best known for its ability to generate targeted gene knockouts. However, there are many essential genes that cause seedling lethality when knocked out, and many agriculturally important traits such as improved photosynthesis require gene overexpression[21]. CRISPR-mediated gene regulation provides solutions to these problems.

CRISPR-mediated gene regulation has so far been focused mainly on promoters implicated in gene repression, activation and epigenetic modification. This usually involves the continuing presence of foreign plasmids and leads to safety concerns when used for crop improvement. CRISPR-mediated targeted promoter mutagenesis can alleviate this concern. The best example of CRISPR-mediated gene regulation for crop improvement comes from work in tomato, where CRISPR technology was used to mutate the promoters of genes related to quantitative traits such as fruit size, inflorescence branching and plant architecture by creating a continuum of variation for tomato breeding[22]. This method can efficiently alter gene expression levels while avoiding the integration of foreign DNA. Another inventive method alters lettuce's gene expression at the translational level by targeting upstream open reading frames (uORFs), which often have negative effects on translation[23]. Increased ascorbate content was observed in lettuce after uORF editing[23]. This approach thus provides a generalizable method for manipulating the translation of mRNA without the integration of foreign DNA, and could be applied to crop improvement.

Novel technical breakthroughs for crop improvement

A prerequisite for applying CRISPR technology to crop improvement is an effective CRISPR reagent delivery system for crops (Fig. 1b). While transformation of major crops is possible, the process usually involves low efficiency and use is confined to one or two genotypes per species, which are not usually the elite cultivars (the major commercial varieties largely used in crop production). It is therefore critical to have robust, routine CRISPR reagent delivery systems for elite crop varieties in place. To this end, a recent study has shown that morphogenic regulators can be used to improve cereal transformation efficiency[24]. Moreover, it has been reported that transformation-recalcitrant elite commercial crop varieties such as inbred corn and wheat could be modified by pollenating them with pollen from a haploid inducer line harbouring a CRISPR cassette designed to generate a desired agronomic trait[25,26]. The long, laborious and complicated plant regeneration and tissue culture process could also be avoided if CRISPR reagents are delivered into plant meristems, pollen or inflorescence tissues[1]. Recently, gene-edited plants were generated through de novo meristem induction, supplying a good example of tissue culture-free plant gene editing[27].

Although NHEJ is the predominant DNA break repair mechanism in eukaryotes, many desirable traits can only be obtained in crops by the precise insertion or replacement of DNA segments. Base editing provides a new method for base substitutions[7,8]; however, it is so far limited to C–T and A–G conversions. Recently, a ground-breaking genome editor 'prime editing' that can directly write new genetic information into a specified DNA site has been developed, greatly expanding the scope and capabilities of genome editing[28] (Fig. 1e). In the prime editing system, Cas9 protein has been engineered to be a nickase fused to a reverse transcriptase, and the sgRNA is replaced by a prime editing guide RNA (pegRNA), which contains an sgRNA for target site recognition and an RNA template specifying the DNA sequence to be inserted into the target genome[28] (Fig. 1e). Prime editing can realize targeted insertions, deletions and all 12 types of point mutation in human cells without requiring DNA breaks or donor DNA templates[28]. We hope that similar applications in crop plants won't be too far behind.

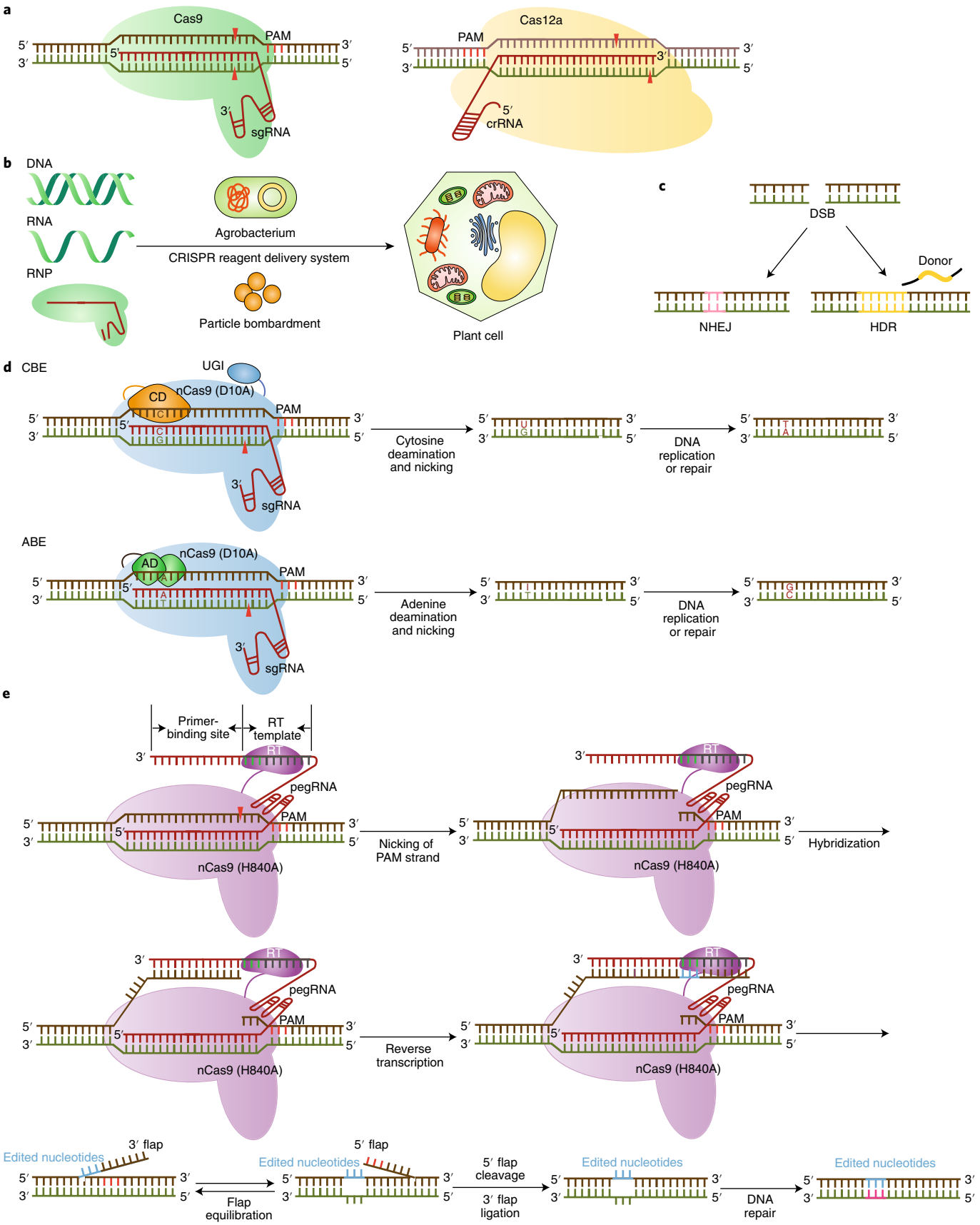
Future prospects

CRISPR technology has demonstrated potential for crop improvement, whereas plant synthetic biology and crop domestication are new areas where CRISPR technology could contribute greatly to the improvement of food crops. However, the first obstacle to the implementation of CRISPR technology for food crops is the construction of worldwide regulatory frameworks for gene-edited crops.

CRISPR technology shows potential for plant synthetic biology. The term plant synthetic biology refers to the (re-)design of biological components and systems that do not already exist in plants. Plant synthetic biology can revolutionize agriculture when given the chance[29]. In a broad definition, any new crop traits obtained by CRISPR technology can be classified in the plant synthetic biology category. More specifically, CRISPR may be an ideal tool for plant synthetic biology by eliminating or adjusting host sequences, inserting non-host genes and regulating the transcription or translation of host or non-host genes. Here, using CRISPR-mediated multiplexing and trait stacking as the best examples, we demonstrate its application in crop synthetic biology. We also highlight the recent directed evolution of plant proteins in situ achieved by CRISPR technology.

Multiplexing and trait stacking in crop breeding. In plants, any individual agronomic trait depends on complex gene regulatory networks,

Fig. 1 | CRISPR technology used for plant genome editing. a, CRISPR–Cas9 (left) and CRISPR–Cas12a (right) systems. **b**, CRISPR reagents, in the form of DNA, RNA or RNP, are delivered into plant cells. The two methods widely used for plant transformation are Agrobacteria- and particle bombardment-mediated transformation. **c**, CRISPR-mediated gene knockout, insertion and replacement. **d**, CRISPR–Cas9-mediated base editing. nCas9 (D10A) is fused to CD or AD, and the complex will convert cytosine (C) or adenine (A) in the targeting region to uracil (U) or inosine (I), respectively, causing C–T or A–G substitutions. **e**, CRISPR–Cas9-mediated prime editing. nCas9 (H840A) is fused to a reverse transcriptase (RT) pegRNA that contains an sgRNA and an RNA template will replace sgRNA. The nCas9–RT complex will write new DNA sequences into the targeted genome. ABE, adenine deaminase-mediated base editing; AD, adenine deaminases; CBE, cytidine deaminase-mediated base editing; CD, cytidine deaminase; DSB, double-strand break; nCas9 (D10A), a Cas9 nickase that has a D10A mutation and can cleave only the DNA strand complementary to the sgRNA; nCas9 (H840A), a Cas9 nickase that has a H840A mutation and can cleave only the DNA strand with PAM sequence; UGI, uracil glycosylase inhibitor.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Cas9
PAM
sgRNA
Cas12a
crRNA
b
DNA
RNA
RNP
Agrobacterium
CRISPR reagent delivery system
Particle bombardment
Plant cell
c
DSB
Donor
NHEJ
HDR
d
CBE
UGI
nCas9 (D10A)
CD
Cytosine deamination and nicking
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Adenine deamination and nicking
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RT template
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pegRNA
nCas9 (H840A)
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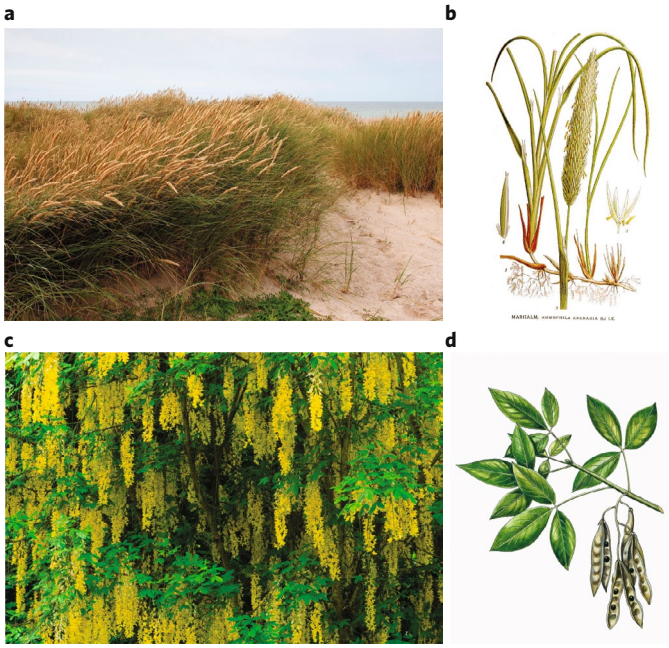

Fig. 2 | Accelerated domestication of wild plants could broaden the diversity of food crops. a,**b**, Converting resilient plants into food: *Ammophila arenaria* (**a**) is an example of a perennial grass that is extremely resilient and tolerates draught, salt and cold and has edible but slender seeds (**b**). Guided by our knowledge of domestication genes in grain crops, CRISPR modification of homologous genes in wild grasses has the potential to turn these into high-yielding crops. **c**,**d**, Converting toxic trees into food: *Laburnum* sp. (**c**) is an example of a nitrogen-fixing tree with prolific but toxic seeds (**b**). It is therefore grown only as an ornamental plant. By using CRISPR technology to neutralize toxin transporters, trees might in the future become sustainable providers of nutritious food. Credit: Blickwinkel / Alamy Stock Photo (**a**); Bilder ur Nordens Flora, Stockholm (**b**); David Cobb / Alamy Stock Photo (**c**); DE AGOSTINI PICTURE LIBRARY // gettyimages (**d**)

and trait improvement therefore tends to involve multiple genes. For example, to increase ribulose 1,5-bisphosphate carboxylase–oxygenase (Rubisco) content to improve photosynthesis in maize, three genes, encoding the Rubisco large (*LS*) and small (*SS*) subunits and Rubisco assembly factor 1 (*RAF1*), respectively, must be edited at the same time[30]. At present, CRISPR technology surpasses any other genome editing tools in its ability to manipulate multiple genes simultaneously. Trait stacking, also known as gene stacking, refers to integrating two or more genes into a predetermined location for co-segregation. For example, clustering of nucleotide-binding leucine-rich repeat (NLR)-encoding disease resistance (R) genes together usually shows stronger efficacy in plants[31]; and 'stacks' of linked genes that control tolerance and/or resistance to drought, salt and other abiotic stresses can be assembled. Once constructed, the trait stacks could be used in different crop species. Using CRISPR technology, the trait stacks could be integrated at a predetermined locus that should have no or negative relevance for crop improvement, providing plants with new traits. Furthermore, when the molecular mechanism underlying a given trait has been uncovered, the respective gene can be added to a pre-existing module by CRISPR, forming an enhanced trait stack for crop improvement.

CRISPR-mediated directed evolution in crops. Directed evolution is a method that can modify proteins (or nucleic acids) in a user-defined direction. It can increase genetic diversity, identify novel traits and accelerate trait improvement[32]. However, it is usually performed by constructs in bacteria or yeast, which can cause some subsequent stability and activity issues in plants. Saturation mutagenesis is a common technique used for directed evolution, and because CRISPR-mediated saturated mutagenesis is highly suitable for creating genetic variants, it is now possible to carry out directed evolution of plant proteins in situ.

Two groups have recently used CRISPR technology for directed evolution in rice: one took advantage of the ability of CRISPR technologies to achieve targeted mutagenesis to evolve the spliceosome component, SF3B1, and several in-frame mutants with resistance to splicing inhibitors were created[33]; the other made use of CRISPR-mediated base editing to generate in-frame mutations modifying ACC, and both known and novel herbicide resistance variants were obtained[34]. In our opinion, CRISPR-mediated saturation mutagenesis could be used to evolve any desired plant protein provided a proper selection method is available. If this 'faster and cheaper' evolution method is used to optimize the function of metabolic enzymes for traits such as crop yield, quality and resistance, it should accelerate crop improvement.

CRISPR technology can accelerate crop domestication. Plant domestication is a time- and labour-intensive process involving altering a plant from its wild state to a new form that can serve human needs. Thousands of years ago, ancient farmers initiated the domestication of all major crops, including rice, wheat and maize. However, our ancestors used only a limited number of progenitor species during the domestication process, and simply selected plants with improved traits such as high yield and ease of breeding, culture, harvest and storage, resulting in the loss of genetic diversity and reduced nutritional value and taste of our current food crops. Increasing current crop diversity is one of the most powerful approaches for promoting sustainable agricultural systems, and the domestication of neglected, semi-domesticated or wild crops would increase such diversity.

Recently, CRISPR technology has been used to domesticate wild tomato, *Solanum pimpinellifolium*, which is remarkably stress tolerant but is defective in terms of fruit production[35,36]. In one study, six loci that are important for yield and productivity were targeted, and the engineered lines displayed increased fruit size, fruit number and fruit lycopene accumulation[35]. Another study used CRISPR to modify coding sequences, *cis* regulatory regions and uORFs of genes associated with day-length sensitivity, shoot architecture, flower/fruit production and ascorbic acid synthesis, and the desirable traits were successfully introduced into wild tomatoes[36].

Orphan crops, such as sweet potato, groundnut, cassava, banana and quinoa, are locally important crops that have good nutritional attributes and adaptations. However, despite their great potential for improving food and nutrition security, the undesirable characteristics (such as low yield, sprawling growth and fruit drop,) prevent orphan crops from wider cultivation. CRISPR technology, which is cheap, fast, precise and capable of editing multiple sites and modifying gene regulation, provides a powerful method for accelerating the domestication of orphan crops. It was recently used to target genes that control plant architecture, flower production and fruit size in groundcherry, a semi-domesticated orphan crop, and the modified plants showed improved domestication traits[37].

Our major grain crops are all annual. Wheat, for example, needs to be sown every year as an annual crop — the process requires the soil to be disturbed and exposed to erosion, which renders the shallow root systems inefficient in terms of water and nutrient uptake, especially at the beginning of the growth season. This has become a major cause of groundwater pollution by nitrate leaching. The domestication of perennial grain crops with deep root systems would be a major step towards more sustainable agriculture that is more efficient in taking up nutrients and water, can adapt to climate change and store carbon underground (Fig. 2a,b). Attempts to turn wheat into a perennial by hybridization with perennial wild grasses have so far proven unsuccessful[38]. An alternative strategy

would be the direct domestication of wild perennial grasses such as *Thinopyrum intermedium*, which is related to wheat[39]. This process can be accelerated by using CRISPR technology to directly target genes that are homologous to domestication genes of wheat, which are already well characterized[40].

Forests play a key role in mitigating the atmospheric CO_2 increases from anthropogenic emissions. Seeds of trees are often big and have high lipid and protein contents. Therefore, expanding the repertoire of trees that can be used for food production would be one way to counter climate change while enhancing food security (Fig. 2c). However, plants typically protect themselves by synthesizing toxic secondary metabolites, which can accumulate to high levels in seeds (Fig. 2d), limiting their use as food. For example, wild almond trees accumulate the bitter and toxic cyanogenic diglucoside amygdalin. The domestication of almond was only possible following the selection of a 'sweet' mutant deficient in the synthesis of amygdalin[41]. As toxins that accumulate in seeds are often produced in other parts of the plant and subsequently transported to seeds by specialized transport proteins, one smart strategy involves neutralizing specific metabolite transporters by CRISPR technology to produce plants that have 'sweet' seeds but are still 'bitter' and protected in other parts[42].

Once the genes controlling the domestication are known, we believe it is possible to engineer desirable traits into any crops by CRISPR technology as soon as their reference genomes are available and efficient tissue culture and transformation methodologies are developed, which could contribute considerably to increasing global food security.

The regulatory landscape regarding gene-edited crops. Although CRISPR technology has great potential for revolutionizing molecular precision breeding, its ultimate deployment in crops will depend on how the use of gene-edited crops will be regulated. This question is difficult to answer, as different countries have different regulatory frameworks and the majority of countries are ambiguous. The existing regulatory frameworks for conventional genetically modified organisms (GMOs) may give us some clues about the attitudes of regulatory authorities. Existing regulatory frameworks are generally of one of two types: process-based regulatory frameworks, which focus on the techniques used to create new crop plant varieties, and product-based regulatory frameworks, which focus on the risks posed by the final food products. Because CRISPR technology can generate products with greater similarity to those arising spontaneously, or produced through physical or chemical mutagenesis, it is not subject to GMO regulation in countries with product-based regulatory systems, but is in countries with process-based regulatory systems.

Regulatory frameworks were created to protect the environment and to address public safety concerns. However, as the safety issues have been assessed for genetically modified (GM) crops, process-based regulation is questionable in terms of its scientific rigour and the proportionality of its precautions, and can become obstacle to innovations in plant breeding. Fortunately, countries with process-based regulatory systems have provided timely solutions that permit the exclusion of some types of plant product from the scope of GMO regulations.

To simplify the outcomes of CRISPR technology, changes produced by genome editing can be classified into three types[43]: site directed nuclease-1 (SDN-1), NHEJ-mediated small sequence changes, involving no foreign DNA template (CRISPR-mediated base editing products should also be classified into this type); SDN-2, HDR with a small nucleotide template, which generates one or several nucleotide (up to 20) changes; SDN-3, HDR with a long template and usually leads to a gene or other DNA sequence to be introduced to the DNA break. We will give several examples of regulations affecting gene-edited crops in various countries.

The United States uses product-based regulation and the United States Department of Agriculture (USDA) exempts CRISPR-edited plants such as anti-browning mushrooms and Waxy corn, whose endoderm starch consists almost exclusively of amylopectin, from regulations covering GMOs[11]. Early in 2017, the USDA proposed a rule for regulating gene-edited crops: products that contain deletions of any size (SDN-1), or single base-pair substitutions (SDN-2) would be exempt from regulation[44].

Argentina also employs product-based regulation and offers a good example of national legislation on plant breeding innovations. In 2015, the country issued a regulation for products of 'New Breeding Techniques' and provided regulatory criteria for gene-edited crops[45]. In 2018, Argentina established a regulatory classification for gene-edited crops: products generated by SDN-1 are not GMO; no regulatory criteria were issued for those generated by SDN-2; crops modified by SDN-3 were classified as GMOs[46].

Recently, Brazil, Chile and Colombia have established similar regulations, so the regulatory classifications for gene-edited crops in these countries will be consistent with those of Argentina.

Canada also applies product-based regulation; intriguingly, the Canadian Food Inspection Agency uses 'novelty' as a trigger for regulatory assessment. Products of targeted mutagenesis tools such as SDN-1 and SDN-2 will therefore be free of assessment and regulation, provided they are not defined as novel.

Japan employs process-based regulations for conventional GM crops; however, it is becoming a leader in the introduction of gene-edited crops. In August 2018, the Japanese Environment ministry committee recommended that gene-edited crops resulting from elimination of gene function (SDN-1) should no longer be considered under GMO regulation. In March 2019, the Japanese advisory panel recommended that gene-edited crops be regulated as conventional crops and gene-edited foods be sold to consumers without safety evaluations[47]. These recommendations are waiting to be formally adopted by the Ministry of Health, Labour and Welfare.

Australia uses process-based regulation; however, amendments are allowed so that regulation remains up-to-date, relevant and commensurate with risk. To clarify the regulatory status of gene-edited crops, a technical review of Gene Technology Regulations was initiated in 2016, and newly proposed amendments were introduced: products developed from SDN-1 would not be GMOs, while those developed by SDN-2 and SDN-3 would be considered GMOs[48]. These amendments are awaiting government approval.

China applies process-based regulation and strictly limits the production of conventional GM crops. Rules for gene-edited crops have not yet been established in China and gene-edited food has not been commercialized. At the same time, the Chinese government provides considerable financial support to gene-editing research and we hope that China will regulate gene-edited crops in a manner similar to that of Japan.

The European Union likewise has a process-based regulation and following a decision by the European Court of Justice on 25 July 2018: any use of CRISPR technology to modify a plant will result in a product being classified as a GMO[49]. This ruling was anticipated as nucleic acid sgRNA molecules will always be required when using CRISPR. A new political decision by the European Commission will be required before genome-edited crops can be exempted from being classified as GMOs in the European Union.

It is clear that the high level of regulatory uncertainty and differences between countries represent a bottleneck in harnessing CRISPR technology for crop improvement. Scientists all over the world should work together and exert some pressure in this arena. For example, a science-based regulatory framework for gene-edited crops was proposed by scientists from China, Germany and the United States[50]. Gratifyingly, in November 2018, 13 nations — the United States, Canada, Argentina, Australia, Brazil, Colombia, the Dominican Republic, Guatemala, Honduras, Jordan, Paraguay, Uruguay and Vietnam — issued a joint statement supporting agricultural applications of precision biotechnology. This statement

represents a good beginning in the construction of worldwide regulatory frameworks to enable innovation in agriculture.

Concluding remarks

CRISPR technology has been widely used in plant genome editing and has great potential for precision breeding; the system is subject to some technical limitations, but they are not the main obstacles to its application in food crops. The real challenge lies in the market: are consumers willing to choose gene-edited products? In this situation it is important to spread reliable information about CRISPR technology to gain public trust. Despite the difficulties, we believe that CRISPR technology will play an important role in realizing sustainable agriculture.

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Y.Z. drafted the first version of the manuscript. M. Pribil revised the manuscript, and C.G. and M. Palmgren designed and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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