New Phytologist

Review

Tansley review

Towards CRISPR/Cas crops – bringing together genomics and genome editing

Author for correspondence:
David Edwards
Tel: +61 08 6488 2415
Email: Dave.Edwards@uwa.edu.au

Received: *7 April 2017*
Accepted: *31 May 2017*

Armin Scheben[1]*, Felix Wolter[2]*, Jacqueline Batley[1], Holger Puchta[2] and David Edwards[1]

[1]School of Biological Sciences and Institute of Agriculture, University of Western Australia, Perth, WA 6009, Australia; [2]Botanical Institute II, Karlsruhe Institute of Technology, Karlsruhe 76131, Germany

Contents

New Phytologist (2017)
doi: 10.1111/nph.14702

Key words: breeding, crops, CRISPR, Cas, gene targeting (GT), genome editing, targeted mutagenesis.

Summary

With the rapid increase in the global population and the impact of climate change on agriculture, there is a need for crops with higher yields and greater tolerance to abiotic stress. However, traditional crop improvement via genetic recombination or random mutagenesis is a laborious process and cannot keep pace with increasing crop demand. Genome editing technologies such as clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas) allow targeted modification of almost any crop genome sequence to generate novel variation and accelerate breeding efforts. We expect a gradual shift in crop improvement away from traditional breeding towards cycles of targeted genome editing. Crop improvement using genome editing is not constrained by limited existing variation or the requirement to select alleles over multiple breeding generations. However, current applications of crop genome editing are limited by the lack of complete reference genomes, the sparse knowledge of potential modification targets, and the unclear legal status of edited crops. We argue that overcoming technical and social barriers to the application of genome editing will allow this technology to produce a new generation of high-yielding, climate ready crops.

I. Introduction

1. Accelerating crop improvement to meet increased food demand under a changing climate

Rapid population growth and increased consumption of meat and dairy are putting pressure on agriculture to meet the rising demand for food, livestock feed and biofuels. By 2050, the global population will increase to >9 billion people and crop demand may increase by 100–110% (Tilman *et al.*, 2011). Yet at the current rate of improvement, yields of the staple crops maize, rice, wheat and soybean will increase by just *c.* 38–67% (Ray *et al.*, 2013). At the same time, climate change may lead to crop yield loss due to increased frequencies of drought, flooding and pest incidence (IPCC, 2014). In recent history, increased crop production has been achieved through improved agronomic management such as

*These authors contributed equally to this work.

the application of pesticides and fertilizer (Matson *et al.*, 1997). However, these management practises rely on finite resources, occasionally have a negative impact on the environment and are unlikely to lead to further sizeable increases in yield in many regions (Edwards, 2016). Although yields of the major crops maize, rice, wheat and soybean continue to increase in many areas, yield is beginning to stagnate in parts of Europe, Asia and Africa (Ray *et al.*, 2012). As the amount of arable land worldwide is limited and decreasing due to expanding urban areas and land degradation, improving crops to better utilize available resources and tolerate stress is an important approach complementing agronomic management. In addition, improved pest-resistant and resource-efficient crops will substantially reduce the environmental impact of agriculture by decreasing the amount of pesticide and fertilizer required for optimal yields.

Traditional breeding continues to deliver high-yielding crop varieties with enhanced traits, yet it relies on the crossing of germplasm or random mutagenesis which can take seven to 12 years to produce an improved variety (Acquaah, 2012) and is unlikely to keep pace with the predicted demand for improved crops. Although breeding efficiency can be improved using trait-linked genetic markers, the improvement of germplasm remains limited by the nontargeted nature of recombination or random mutagenesis. Transgenic genetically modified (GM) crops have delivered average yield benefits of up to 7% in industrial countries and up to 30% in developing countries (Carpenter, 2010); however, since 1995 there have been few traits broadly commercialized beside herbicide resistance and insect resistance, and strict regulation and public scepticism have slowed development of GM crops.

Over the last decade, targeted genome editing technologies have emerged, using hybrid enzymes or the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system (Jinek *et al.*, 2012). Genetic modification is performed by inducing DNA double-strand breaks (DSB) at specific genome locations and stimulating nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) to introduce specific DNA modifications into the genome. When applied to crop breeding, genome editing can rapidly generate transgene-free improved varieties. Approaches based on hybrid enzymes consisting of fused DNA-binding domains and nucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been used to edit the genomes of many crops including soybean (Curtin *et al.*, 2011) and wheat (Wang *et al.*, 2014). However, a major drawback of hybrid enzymes is the high cost and complexity of the protein engineering required for their development.

The CRISPR/Cas system is superior to hybrid enzyme approaches because of its RNA-based sequence specificity which provides high versatility and low cost, and its application can accelerate both basic research and crop improvement (Belhaj *et al.*, 2015; Bortesi & Fischer, 2015; Puchta, 2017; Scheben & Edwards, 2017). CRISPR/Cas originates from the immune system of bacteria and archaea, carrying out RNA-guided cleavage of foreign DNA such as viruses or plasmids. Current classification differentiates two classes, six types and 19 subtypes of CRISPR (Shmakov *et al.*, 2017), with the type II system of *Streptococcus pyogenes* currently most widely adopted for genome editing. The CRISPR/Cas system is generally delivered into crops via DNA expression vectors, but can also be delivered as RNA or preformed ribonucleoprotein (RNP) complexes as was first demonstrated by Woo *et al.* (2015). The Cas9 nuclease, which is the enzyme commonly used, induces a DSB at a site specified by a target sequence of 19–22 nucleotides integrated into a single guide RNA (sgRNA) (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). The target site must be followed by a three nucleotide protospacer adjacent motif (PAM) which can be bound by Cas9. For the *S. pyogenes* system, the PAM site allowing highest binding is NGG, although NAG also binds at lower efficiency. In this review, we use the specific term CRISPR/Cas9 to refer to the editing system using the Cas9 nuclease and the general term CRISPR/Cas to refer to CRISPR editing systems using any Cas nuclease (see Box 1 for definitions used in this review).

CRISPR/Cas can directly introduce mutations into elite germplasm, and in the last three years, the CRISPR/Cas system has been shown to be effective in a wide range of major crop species, including maize (Svitashev *et al.*, 2016), rice (Jiang *et al.*, 2013; Wang *et al.*, 2017) and wheat (Shan *et al.*, 2013; Wang *et al.*, 2014). Genomes of elite crop varieties can be edited in a targeted manner to produce new varieties with increased stress tolerance and nutrient-use efficiency, without being constrained by limited existing variation. In addition, the continuous improvement of elite varieties by genome editing does not introduce potentially deleterious alleles from crossing and recombination, or require time-consuming repeat backcrossing to reconstruct the elite genetic background. In this review, we propose a shift in crop improvement towards cycles of targeted genome editing of elite varieties to produce improved varieties, with advanced traits or specific adaptation to local environments, replacing the cycles of crossing or untargeted mutagenesis and selection currently employed by breeders. This new crop improvement approach provides a powerful new breeding tool, but also brings substantial new requirements for genomic information and bioinformatics. Genome editing-based crop improvement schemes rely on functional genomic information to identify editing targets including genes and regulatory regions. Cycles of editing and selection of optimal variants during field trials allow further improvement of breeding outcomes. Here, we broadly outline the requirements for genome-wide data to enable the discovery of candidate genes and regulatory regions for genome editing. We also discuss applications of genome editing and methods to improve editing specificity, multiplexing and gene regulation using CRISPR/Cas.

II. Genomic-based crop improvement before CRISPR/Cas

Many current efforts to accelerate crop breeding rely on increasing selection efficiency using marker-assisted selection (MAS) (Collard & Mackill, 2008) and genomic selection (GS) (Desta & Ortiz, 2014). MAS uses linkage disequilibrium (LD) between genetic markers and quantitative trait loci (QTL) to select plants with traits of interest for breeding programs. Although the last two decades have seen substantial advances in marker technologies, polygenic

Box 1 Definitions used in this review

Genome editing	Technique for generating site-specific insertions, deletions or substitutions in the genomes of living cellular organisms. Genome editing relies on programmable nucleases to cleave DNA, with cellular DNA repair processes inducing desired mutations. Depending on DNA repair pathway, mutations can be random or sequence-specific
Nonhomologous end-joining (NHEJ)	Error-prone DNA repair pathway that mediates direct ligation of break ends without requiring a homologous template
Homology-directed repair (HDR)	Highly accurate DNA repair pathway that mediates repair of break ends using a template homologous to the region of the break
Gene targeting	Uses HDR for site-specific induction of specific insertions, deletions or substitutions in or around genes. Can be used in genome editing to delete or add genes and regulatory regions, and to introduce point mutations
CRISPR RNA (crRNA)	Short RNA that determines the target specificity of CRISPR/Cas systems. It constitutes the variable part of the CRISPR array from which it is expressed as a pre-crRNA and processed. It consists of a constant and a variable part and associates with the DNA cleaving protein, depending on the system alone (Cpf1), in combination with tracrRNA (native type IIa system), or directly fused to tracrRNA (synthetic type IIa system)
Trans-activating crRNA (tracrRNA)	Occurs only in type IIa CRISPR/Cas systems. Binds to pre-crRNA to form double stranded RNA which is cleaved by endogenous RNaseIII in the presence of Cas9 to release functional crRNA-tracrRNA hybrids
Single guide RNA (sgRNA)	Synthetic RNA consisting of a fusion between crRNA and tracrRNA to reduce the number of RNAs that need to be expressed when using type IIa CRISPR/Cas for genome editing purposes
'Dead' Cas9 (dCas9)	Cas9 variant in which both nuclease domains (RuvC and HNH) are deactivated by point mutations, yielding a programmable DNA binding protein that can be fused to effector domains like transcriptional regulators or fluorescent proteins
Nickase	Enzyme generating a single-strand DNA break (nick). Cas9 variants with a mutated nuclease domain are available that induce nicks rather than double-strand DNA breaks
Off-target effect	Mutations induced in unintended targets during genome editing

quantitative traits are not readily amenable to MAS, and the complexity of genotype-by-environment interactions present a further challenge. In contrast to MAS, GS predicts breeding values for individual lines in a phenotyped and genotyped training population, allowing selection in breeding populations to be carried out using marker-based breeding values instead of phenotypes or gene-associated markers (Desta & Ortiz, 2014). GS can accelerate the breeding cycle by reducing the need for phenotyping breeding populations and, unlike MAS, facilitates selection of complex polygenic traits. However, marker-based approaches such as MAS and GS are limited by their reliance on markers and the available genetic diversity in the population. As our understanding of crop genomes increases, marker-based breeding approaches may transition towards approaches based on functionally characterized candidate genes. Key functional genomics resources that provide insight into gene function using reverse genetics are mutant crop populations generated with approaches such as targeting induced local lesions in genomes (TILLING) (McCallum *et al.*, 2000). TILLING is used in breeding to generate and identify novel gene variants into crops by inducing random mutations in a population and subsequently detecting mutations in target genes with oligonucleotide probes or sequencing. Backcrossing to parental, unmutagenized lines then allows breeders to capture useful variants and introgress them into elite varieties. The power of such an approach in breeding is shown, for instance, by the pod shatter resistance trait introduced into commercial canola cultivars such as Bayer IH 51 RR via a single nucleotide genic mutation (Lambert *et al.*, 2015). As breeding schemes increase their focus on candidate genes for traits, genome editing provides a more precise tool to produce novel allelic variants of these genes for agronomic assessment.

III. Genome assembly as the starting point for genome editing

Annotated reference genome sequences underpin the targeted editing of crop genomes, and provide information on the base material for improvement. Reference genomes facilitate the discovery of functional regions implicated in agronomic traits such as genes, promoters and enhancers, and allow the design of sgRNA and alignment of sequencing reads used to validate edited positions of the genome. In 2015, there were >60 high-quality crop genomes available (Michael & VanBuren, 2015) and we can expect the decreasing cost of sequencing to soon facilitate the sequencing and assembly of all genomes of major and minor crops and their wild relatives. Transcriptomes may also play important roles as starting points for genome editing because plant genome assemblies and their annotation often remain incomplete. Genome assemblies and their annotations are likely to improve in completeness and accuracy as third-generation sequencing technologies, which generate long reads, help address previous challenges of *de novo* genome assembly limited to using short reads (Yuan *et al.*, 2017). The resequencing of crop genomes representing multiple diverse varieties is now also providing access to pangenomes, which represent the genomic diversity within a species rather than the sequence of a representative individual (Golicz *et al.*, 2016a). This

is particularly useful for the development of improved varieties because within-species differences in the presence and absence of genes can be particularly high in plants, for instance affecting almost 20% of all genes in the crop *Brassica oleracea* (Golicz *et al.*, 2016b). Pangenome references make genome-wide variation in presence/absence and copy number accessible for editing. Knowledge of within-species diversity is also important for genome editing because it enables cultivar-specific design of sgRNAs, which is necessary when the 19–22 nucleotide target or PAM site differs between cultivars. The increasing number of crop pangenome assemblies will be a valuable resource for efficient genome editing and breeding.

IV. Identifying targets for genome editing using functional genomics

Since publication of the first *Arabidopsis* transcriptome (Weber *et al.*, 2007), functional genomics has helped characterize gene function, expression and interaction in many plants. However, the functions, determinants of expression and interaction networks of most genes remain unclear and lack experimental characterization (Radivojac *et al.*, 2013). Moreover, functional sequences within the noncoding regions of the genome also remain poorly characterized, despite evidence indicating their important role in gene regulation (Haudry *et al.*, 2013). Targeted genome editing relies on detailed knowledge of gene regulation to identify targets for editing, ensure genes are only expressed in relevant tissues and make manipulation of regulatory sequences feasible.

TILLING populations are an important resource for discovering and deploying functional gene variants, and have been developed for several crop plants, including rice (Till *et al.*, 2007) and wheat (Uauy *et al.*, 2009). These populations can help identify mutations and genes associated with agronomic traits. For example, TILLING was used to identify waxy gene variants controlling starch synthase in wheat (Slade *et al.*, 2005) and mutations in sorghum leading to economically valuable acyanogenic plants (Blomstedt *et al.*, 2012). High-throughput mutation screens produced using lentiviral CRISPR-guide RNA libraries have also been used in human cells to interrogate gene function and identify enhancers (gene-distal regulatory DNA that is bound by transcription factors to enhance gene transcription) in the noncoding genome (Koike-Yusa *et al.*, 2014; Sanjana *et al.*, 2016), showing improvement over alternative approaches such as RNA interference by allowing complete target knockout and fewer off-target effects. A further line of evidence to characterize functional regions of the genome and their interactions is the increasing amount of data on the 3D structure of crop genomes and genome-wide chromatin accessibility, produced using methods such as Hi-C (Lieberman-Aiden *et al.*, 2009), assay for transposase accessible chromatin with high-throughput sequencing (ATAC-seq) (Buenrostro *et al.*, 2013) and chromatin immunoprecipitation sequencing (ChIP-seq) (Solomon *et al.*, 1988). Employing these approaches for high-resolution, genome-wide mapping of epigenetic marks and binding sites for DNA-binding proteins such as transcription factors is crucial for understanding the processes underlying transcriptional regulation and harnessing them for crop improvement. For instance, Zhang *et al.* (2012) identified 97 975 deoxyribonuclease hypersensitive sites in rice seedlings, of which 42–45% are potential regulatory elements located in intergenic regions.

Gene expression atlases with data from different tissues under different conditions are also an important resource for predicting the function of genes and the potential effects of editing. Dynamic gene expression during development and under different abiotic and biotic conditions provides insight into gene function, and assists in modelling the repercussions of genome editing throughout the life of a crop. Expression atlases have been developed for many major crops such as maize (Sekhon *et al.*, 2011) and rice (Nobuta *et al.*, 2007). Database projects integrating gene expression datasets for diverse species such as the PlexDB database (Dash *et al.*, 2012) are also providing platforms for better leveraging crop expression data. As protein–protein interaction data are poorly available for plants other than *Arabidopsis*, these expression atlases could help identification of functionally related genes by coexpression analysis (Ruprecht *et al.*, 2017). Despite these advances, the lack of functional understanding of genes remains one of the biggest bottlenecks in crop improvement using genome editing. For instance, in maize, only *c.* 1% of genes have been functionally annotated using experimental data (Andorf *et al.*, 2016). Comprehensive and integrated genomic databases including gene expression atlases together with more accurate *in silico* methods for gene function prediction based on homology and coexpression will be crucial for overcoming the gene function bottleneck.

V. Using CRISPR/Cas to improve crops

1. Improving crops by disrupting genes with CRISPR/Cas

CRISPR/Cas dramatically increases the potential to improve traits in crops compared to conventional breeding approaches. Importantly for crop improvement, homozygous pyramiding of genes of interest into elite germplasm can be carried out within a single generation (Zhang *et al.*, 2014). CRISPR/Cas is also a powerful tool for introducing heritable, trait-related mutations indistinguishable from natural allelic variants (Schaeffer & Nakata, 2015). Moreover, if the CRISPR/Cas system is transformed into the crop for delivery, as is commonly the case, plants are hemizygous for the transgenes whereas genome editing affects all alleles. This means that crossing or selfing these plants can generate transgene-free progeny, reducing the potential risks and regulatory requirements that may apply to transgenic crops.

In its simplest form, genome editing can improve crops by knocking out genes conferring undesirable traits (Table 1). This approach has been used extensively to increase pathogen resistance in crops (Andolfo *et al.*, 2016). For instance, CRISPR/Cas9 has been used to enhance resistance against blast and bacterial blight in rice by disrupting the ERF transcription factor and the *SWEET* genes, respectively (Jiang *et al.*, 2013; Wang *et al.*, 2016). The biotechnology industry is beginning to actively apply genome editing for crop improvement. In 2015, Cibus developed a transgene-free herbicide-tolerant canola using a proprietary genome editing system to carry out an amino acid exchange (Schinkel & Schillberg, 2016), with the cultivar now commercially

Table 1 Examples of genome editing studies with practical applications of genome editing for crop improvement

Crop	Editing system	Type of edit	Target gene(s)	Target traits	Results	Reference
Brassica oleracea	CRISPR/Cas9	Gene disruption	*Bolc.GA4.a*	Dwarfing and fruit dehiscence	Significant dwarfing in all transformed lines compared to wild-type and reduced fruit dehiscence	Lawrenson *et al.* (2015)
Citrus (Orange)	CRISPR/Cas9	Promotor disruption	CsLOB1 (promoter)	Citrus canker resistance	Citrus canker disease severity was decreased by 83.2–98.3% compared to the wild-type	Peng *et al.* (2017)
Cucumber	CRISPR/Cas9	Gene disruption	eIF4E	Broad virus resistance	Immunity to cucumber vein yellowing virus infection and resistance to the potyviruses zucchini yellow mosaic virus and papaya ring spot mosaic virus-W	Chandrasekaran *et al.* (2016)
Maize	CRISPR/Cas9	Promoter swap	*ARGOS8*	Drought tolerance	A total of 3.8% higher yield than the control under flowering drought stress	Shi *et al.* (2016)
Potato	TALEN	Gene disruption	*VInv*	Low concentration of reducing sugars that lead to low palatability and possible health risks	Undetectable concentrations of reducing sugars	Clasen *et al.* (2016)
Rice	CRISPR/Cas9	Promoter disruption	*OsSWEET11*, *OsSWEET14*	Bacterial blight resistance	The promoter of the blight susceptibility gene was disrupted	Jiang *et al.* (2013)
Rice	CRISPR/Cas9	Gene disruption	Gn1a, GS3, and DEP1	Grain number, grain size, panicle architecture	More grains, larger grains, denser and erect panicles	Li *et al.* (2016b)
Soybean	TALEN	Gene disruption	*FAD2*-1A, FAD2-*1B*	Low polyunsaturated fats	The amount of the monounsaturated oleic acid increased from 20% to 80% and the polyunsaturated linoleic acid decreased from 50% to under 4%	Demorest *et al.* (2016)
Tobacco	CRISPR/Cas9	Viral gene disruption	43 regions in the viral genome	Resistance to the geminivirus beet severe curly top virus	No severe leaf-curling symptoms were observed in systemic leaves, and virus accumulation in local leaves was reduced by 90–97%	Ji *et al.* (2015)
Tobacco	CRISPR/Cas9	Viral gene disruption	Six regions in the viral genome	Resistance to the geminivirus bean yellow dwarf virus	Transgenic plants expressing CRISPR–Cas reagents and challenged with geminivirus had a *c.* 5–87% reduced virus load and fewer symptoms	Baltes *et al.* (2015)
Tomato	CRISPR/Cas9	Viral gene disruption	Three regions in the viral genome	Resistance to the geminivirus Tomato yellow leaf curl virus (TYLCV)	Accumulation of viral DNA was decreased, removing or significantly attenuating symptoms of infection	Ali *et al.* (2015)
Tomato	CRISPR/Cas9	Gene disruption/ knockdown	*SP5G*	Time to harvest	Plants could be harvested 2 wk earlier than controls, with lower total yield but higher harvest index	Soyk *et al.* (2017)
Tomato	CRISPR/Cas9	Gene disruption	*RIN*	Fruit ripening (shelf life)	Delayed and permanently inhibited ripening, possibly without the side effects of conventional *rin* mutants	Ito *et al.* (2015)
Tomato	CRISPR/Cas9	Gene disruption	*SlIAA9*	Parthenocarpy (leading to seedless fruit)	Fruit development was triggered before fertilization, generating seedless tomato fruits	Ueta *et al.* (2017)
Tomato	CRISPR/Cas9	Gene disruption	*SlMlo1*	Resistance to powdery mildew	Rapid generation of a transgene-free tomato fully resistant to powdery mildew	Nekrasov *et al.* (2017)
Wheat	TALEN and CRISPR/Cas9	Gene disruption	*TaMLO-A1*, *TaMLO-B1* and *TaMLO-D1*	Resistance to powdery mildew	The number of mildew microcolonies formed on the leaves was significantly reduced against the control and no apparent fungal growth was observed on the leaves of edited plants	Wang *et al.* (2014)

available in the USA. Within five years, DuPont Pioneer aims to commercialize a high amylopectin corn with superior yield and food properties that was developed using CRISPR/Cas9 to disrupt the amylose biosynthesis gene *Wx1* (Waltz, 2016).

2. Precise gene modifications

Although disrupting genes can confer agronomically important traits, most traits can only be improved by precise gene modifications. These precise alterations can be achieved by gene targeting (GT), which refers to HDR-mediated site-specific transgene integration or point mutations. GT can be performed by providing a template for DSB repair via HDR containing desired modifications at the same time as inducing the break. However, GT is still a challenge in plants, because NHEJ remains the dominant pathway of DSB repair in plants (Puchta, 2005), even when a repair template is available. A further challenge is that many crops still lack efficient transformation and regeneration procedures (Altpeter *et al.*, 2016). Although isolated protoplasts can be transformed at high efficiency and allow GT (Townsend *et al.*, 2009), efficient regeneration of plants from protoplasts is difficult or not yet possible for many crops, particularly monocotyledons including wheat, rice, barley and sorghum (Eeckhaut *et al.*, 2013). Recently, a highly efficient and widely applicable transformation approach for monocot species was reported (Lowe *et al.*, 2016). The enhanced efficiency is based on overexpression of maize *Baby boom* (*Bbm*) and *Wuschel2* (*WS2*) genes after *Agrobacterium*-mediated transformation of immature embryos, leading to a growth stimulation of transformed tissue compared to nontransformed tissue. Furthermore, Lowe *et al.* successfully transformed alternative target tissues such as embryo slices from mature seeds with this approach. This is especially useful as supplying immature embryos for classical transformation is highly time and cost intensive and embryo slices from mature seeds can be supplied in much larger quantities due to automated preparation.

A GT method for crops that are recalcitrant to efficient transformation is termed *in planta* GT (Fauser *et al.*, 2012). Here, the CRISPR/Cas9 construct and the repair template are stably transformed into the plant genome and the nuclease not only cuts in the target, but also excises the repair template, activating it for HDR. Thus, GT events can happen during the life cycle of the plant, and upon entering the germline can be harvested as seeds in the next generation, so, in principle, a single successful transformation event is sufficient. Using this method, Schiml *et al.* (2014) achieved site-specific insertion in *Arabidopsis*.

The limitation of reduced amounts of HDR template in transformed cells can be addressed by employing geminiviral replication mechanisms to increase repair template copy numbers (Baltes *et al.*, 2014). Using this approach, it was possible to achieve efficient GT via *Agrobacterium*-mediated transformation of cotyledons or leaf explants and subsequent plant regeneration in tomato (Cermak *et al.*, 2015). Biolistic transformation is another method that enables delivery of larger amounts of repair template compared to *Agrobacterium*-mediated transformation (Altpeter *et al.*, 2016). Via biolistic transformation and regeneration of immature embryos, site-specific gene modifications and insertions were demonstrated in maize (Svitashev *et al.*, 2016), soybean (Li *et al.*, 2015) and rice (Sun *et al.*, 2016). Gil-Humanes *et al.* (2017) combined viral replication with biolistic transformation of wheat cells and achieved multiplexed GT at all three homeoalleles, albeit without regeneration of edited plants.

Instead of HDR, NHEJ also can be harnessed for precise genome editing. For example, gene replacements and insertions were demonstrated in the rice *5-enolpyruvylshikimate-3-phosphate synthase* gene (*EPSPS*) by targeting two adjacent introns and providing a repair template with point mutations in the intermediate exon via biolistic transformation (Li *et al.*, 2016a). Using the more dominant NHEJ repair pathway can help to overcome the challenge of inefficient gene modification when relying on HDR. Finally, precise 'base editing' is now possible without cleaving double-stranded DNA, by using a cytidine deaminase enzyme (Komor *et al.*, 2016). When fused to Cas9, it can target a specific site and effects the conversion of cytidine to uridine within a window dependent on the fusion protein linker, leading to the substitution of cytosine (C) with thymine (T), or guanine (G) with adenine (A). Using Cas9 nickase (an enzyme variant that induces single-stranded breaks or 'nicks') to cleave the nonedited strand strongly enhances efficiency compared to inactive Cas9 by promoting replacement of the nonedited base. Zong *et al.* (2017) successfully applied this technique in rice, wheat and maize with a high editing efficiency of up to 40% in transgenic plants. Using the XTEN linker to link Cas9 and cytidine deaminase, they observed an editing window covering 7nt of the protospacer.

3. Beyond Cas9: broadening the toolbox with Cpf1

Cpf1 (CRISPR from *Prevotella* and *Francisella* 1; recently renamed 'Cas12a' by Shmakov *et al.*, 2017), another DNA cleaving enzyme from a class II CRISPR system, is now also available for genome editing in plants. There are several key differences between Cas9 and Cpf1 (Fig. 1). First, unlike Cas9, Cpf1 does not require transactivating CRISPR RNA (tracrRNA) (Zetsche *et al.*, 2015). Instead, it cleaves precursor crRNA (pre-CRISPR RNA) autonomously and associates with mature crRNA alone to cleave target DNA. Thus, only the short crRNA (*c.* 42 nt) is required without the need for a long chimeric RNA as is the case for Cas9. This decreases the size of the construct that is delivered into plant cells, which can be advantageous when multiplexing on a large scale. Furthermore, the pre-crRNA processing ability of Cpf1 can be harnessed to enhance multiplexing by constructing a polycistronic gene consisting of tandem repeats of a direct repeat alternating with target-specific spacers (Wang *et al.*, 2017; Zetsche *et al.*, 2017). Second, Cpf1 recognizes a T-rich PAM upstream instead of downstream of the target sequence (Zetsche *et al.*, 2015). This provides a new range of target sites across plant genomes, particularly in promoter regions that are naturally AT-rich and are often difficult to target using the G-rich Cas9 PAM. Third, Cpf1 cleaves each complementary strand of DNA at different sites, spaced five nucleotides apart, leading to sticky ends instead of the blunt ends produced by Cas9. This could prove useful because it might enhance the efficiency of genomic rearrangements such as precise chromosomal deletions or somatic crossovers. Although

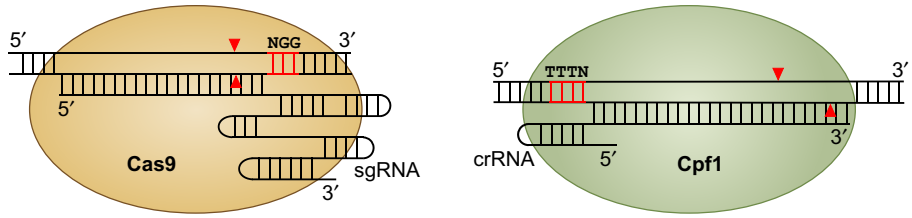

Fig. 1 Key differences between the DNA-cleaving nucleases clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) and CRISPR from *Prevotella* and *Francisella* 1 (Cpf1). Cpf1 recognizes a T-rich protospacer adjacent motif (PAM) (nucleotides highlighted in red) upstream of the target sequence and generates a staggered cut (indicated by red triangles) further from the PAM site than Cas9, leaving a sticky end. In addition, it associates with CRISPR-RNA (crRNA) alone, without trans-activating crRNA (tracrRNA) or a synthetic chimeric RNA. Lastly, the target sequence of Cpf1 is longer, usually 23–25 bp as opposed to the 20 bp required for Cas9.

blunt ends generated by two Cas9 cuts flanking the fragment targeted for deletion rarely spontaneously rejoin, compatible sticky ends generated by two Cpf1 cuts might have a higher likelihood of joining due to Watson–Crick base-pairing.

Three Cpf1 orthologues have been successfully tested in plants: Cpf1 from *Francisella novicida* (FnCpf1), *Lachnospiraceae bacterium* ND2006 (LbCpf1) and *Acidaminococcus* sp. BV3L6 (AsCpf1). Direct comparisons have shown that LbCpf1 has higher efficiency than AsCpf1 and FnCpf1 (Tang *et al.*, 2017; Wang *et al.*, 2017). Importantly, correct processing of the crRNA ends seems to be crucial for high Cpf1 activity in plants: Tang *et al.* (2017) dramatically improved Cpf1 efficiency by flanking the crRNA with ribozymes leading to a release of crRNAs without deleterious extensions such as the poly-U from polymerase III termination or the required 'G' for the initiation by the U6 promoter. They also demonstrated that Cpf1 has potential for transcriptional repression in plants. By fusing nuclease deficient Cpf1 to a repression domain, greater repression was achieved than previously with 'dead' Cas9 (dCas9) based fusions.

4. Chromosomal rearrangements and recombination control

CRISPR/Cas can enable large-scale chromosomal rearrangements and provides opportunities for control of meiotic recombination. The ability to precisely control recombination would open a new dimension of possibilities to the plant breeder. Although breaking genetic linkage between genes for beneficial and adverse traits is an obstacle for plant breeding, maintaining linkage between genes for beneficial traits is desirable. Both can be achieved by control of chromosomal translocations. By induction of a DSB on two different chromosomes, reciprocal exchanges of chromosome arms can be achieved (Pacher *et al.*, 2007) (Fig. 2a). Similarly, linkage can be broken by inducing artificial crossovers (Sadhu *et al.*, 2016). Induction of two DSBs on the same chromosome can lead to chromosomal deletions and inversions (Lee *et al.*, 2012; Zhou *et al.*, 2014; Ordon *et al.*, 2016) (Fig. 2b). These chromosomal inversions can prevent meiotic recombination between homologues to stabilize linkage of positive traits (Puchta, 2016). It should be noted that Cpf1 may prove to be an efficient tool for these NHEJ-based genomic rearrangements due to its ability to generate compatible overhangs. Alternatively, precise targeted recombination between parental genomes can be achieved by CRISPR/Cas-induced manipulation of meiotic recombination. Using meiosis specific expression, DSBs can be induced during meiosis at specific sites, or effector proteins involved in initiation of meiotic recombination such as SPO11 can be targeted to specific sites to guide meiotic recombination, as has been shown in yeast (Peciña *et al.*, 2002).

VI. Improving the efficiency of the CRISPR/Cas system

1. CRISPR/Cas efficiency in plants

Initial demonstration of the functionality of the CRISPR/Cas9 system in plant cells was carried out in 2013 (Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013) and the demonstration of stable inheritance of induced mutations followed one year later (Feng *et al.*, 2014). The technology was rapidly adopted and targeted mutagenesis for disruption of gene function is now a routine procedure. Numerous approaches to further enhance the efficiency of the system have been demonstrated, including the choice of the promoter to drive Cas9 (Wang *et al.*, 2015; Yan *et al.*, 2015; Eid *et al.*, 2016) and improved design of the sgRNA scaffold (Dang *et al.*, 2015). In the latter approach, the duplex part of the sgRNA was extended, mimicking the natural system, and the continuous stretch of Ts was mutated, as these function as a termination signal for RNA-Polymerase III, which commonly drives sgRNA expression. Concerning the choice of the target site, in contrast to the situation in animals, there are no robust tools for prediction of sgRNA efficiency in plants, and it is advisable to test sgRNA efficiencies in a transient protoplast system before engaging in the time- and cost-intensive procedure of transgenic plant production (Ishida *et al.*, 2007).

2. Genome editing specificity

Early investigations of CRISPR/Cas9 genome editing specificity found a relatively high off-target potential in human cells (Fu *et al.*, 2013) and extensive analyses of cleavage efficiency on mismatched targets with base specific resolution are available (Hsu *et al.*, 2013). In plants, potential off-target sites identified by bioinformatics approaches have been sequenced but showed no off-target cleavage (Li *et al.*, 2013; Nekrasov *et al.*, 2013, 2017; Zhou *et al.*, 2014; Peterson *et al.*, 2016; Ueta *et al.*, 2017). Feng *et al.* (2014) demonstrated high specificity in plants as whole genome sequencing of CRISPR/Cas9-induced mutants could not detect any off-target effects.

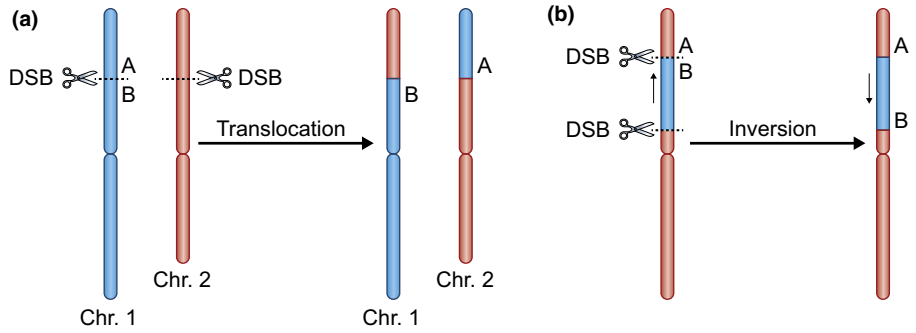

Fig. 2 Approaches to control recombination with chromosomal rearrangements using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas). Genetic linkage between traits A and B can be broken by (a) chromosome arm exchanges or (b) intrachromosomal inversions (vertical arrows indicating sequence orientation).

However, off-target cleavage has been reported in rice, soybean and maize, mainly occurring in gene paralogues with almost identical sequences to the targets (Shan *et al.*, 2013; Jacobs *et al.*, 2015; Svitashev *et al.*, 2015; Li *et al.*, 2016b).

Editing specificity can be increased using computational sgRNA selection, protein engineering, RNA modifications and improved delivery systems (Tycko *et al.*, 2016). The development and optimization of bioinformatics tools for designing highly specific sgRNA and detecting the entire repertoire of potential off-target editing sites allows substantial increases in target specificity (Rousseau *et al.*, 2009; Heigwer *et al.*, 2014; Montague *et al.*, 2014). Prediction of highly specific genome-wide sgRNAs with minimized off-target effects in six crops and two further plant species indicated that 67.9–96.0% of transcripts have at least 10 specific sgRNA designs, with the exception of maize where only 30% of transcripts allow 10 or more sgRNA designs (Xie *et al.*, 2014). This exception is likely due to the large genome size and ancient polyploidy of maize, suggesting polyploid crops with large genomes such as wheat may also prove challenging to edit. Nevertheless, wheat homeoalleles have been successfully edited simultaneously (Wang *et al.*, 2014), and in some cases the targeting of multiple related genes may be advantageous (Lawrenson *et al.*, 2015).

Further solutions addressing the issue of off-target activity include the use of truncated sgRNAs (Fu *et al.*, 2014) and paired nickases (Ran *et al.*, 2013). The paired nickase approach makes use of the D10A mutant of Cas9. Here, one of the two nuclease domains, RuvC, is inactivated, converting the nuclease to a nickase that induces single strand breaks. Two nicks are then induced in close proximity, ultimately resulting in a mutagenic DSB. Nicks at potential off-target sites are repaired with high fidelity via base excision repair and are thus not mutagenic (Fauser *et al.*, 2014). The system has already been used successfully in *Arabidopsis* (Schiml *et al.*, 2014, 2016), rice (Mikami *et al.*, 2016), and maize (Wolter *et al.*, 2017). The dosage of Cas9 and sgRNA delivered also affects off-target editing, with higher enzyme concentrations increasing off-target cleavage (Hsu *et al.*, 2013; Pattanayak *et al.*, 2013). For instance, Ranganathan *et al.* (2014) used the weaker H1 promoter to express sgRNAs, lowering off-target effects. Off-target cleavage is also affected by the delivery method. Recently, efficient genome editing was achieved in rice and wheat when Cas9 and gRNA were delivered as pre-assembled RNP complexes into immature embryos (Svitashev *et al.*, 2016; Liang *et al.*, 2017). Compared to conventional DNA delivery, levels of on-target mutagenesis were comparable (up to 9% of regenerated plants), whereas off-target mutagenesis was drastically reduced and only detectable by amplicon deep sequencing. Finally, based on insights from the structure of Cas9 in complex with target DNA and sgRNA (Nishimasu *et al.*, 2014), engineered modifications of Cas9 with dramatically enhanced specificity in human cells were developed (Kleinstiver *et al.*, 2016; Slaymaker *et al.*, 2016).

3. Multiplexing the CRISPR/Cas system

The nature of the CRISPR/Cas system renders it highly amenable for multiplexing approaches, targeting multiple sites in the genome simultaneously. Although it is possible to use identical or multiple different promoters (Xing *et al.*, 2014; Ma *et al.*, 2015; Wang *et al.*, 2015), this approach is only feasible for a small number of guides as efficiency drops with increasing construct size. Xie *et al.* (2015) used the endogenous tRNA-processing system to express multiple sgRNAs from a single synthetic gene consisting of multiple repeats of sgRNA and tRNA. Upon cleavage by the endogenous tRNA-processing RNases, the individual sgRNAs are released (Fig. 3). Using this approach, a maximum of eight genes have so far been edited simultaneously (Xie *et al.*, 2015). Interestingly, Cas9 activity at the individual targets was only slightly reduced when eight genes were targeted compared to when only two or four genes were targeted, indicating low enzyme saturation. Concentration of free Cas9 was thus not a major limitation even when Cas9 was distributed among eight sgRNAs, which suggests that it is possible to target more sites simultaneously, although this has not been demonstrated. The tRNA-based approach has also been successfully applied in maize (Qi *et al.*, 2016). Moreover, Tang *et al.* (2016) developed a multiplexing system where sgRNAs are expressed from the same PolII promoter that drives expression of Cas9, along with a self-cleaving hammerhead ribozyme. After transcription, Cas9 and sgRNAs are separated by ribozyme cleavage sites, releasing functional Cas9 and sgRNAs. Recently, the endogenous multiplexing capacity of Cpf1 was harnessed in plants, with a single polymerase III promoter used to generate a transcript consisting of several units each composed of a direct repeat of the crRNA and a target sequence. Cpf1 recognizes the direct repeat

sequence and cleaves upstream of the stem loop, releasing functional mature crRNAs (Wang *et al.*, 2017).

4. Using CRISPR/Cas as a site-specific effector

Although the enzyme Cas9 has mainly been used for DNA cleavage, it is a versatile tool. Cas9 can be transformed into a DNA binding protein by mutating its two nuclease domains (Jinek *et al.*, 2012). By fusing the enzyme to an effector domain, it can then be used to guide diverse enzymatic functions to any specific site in the genome (Gilbert *et al.*, 2013) (Fig. 4). The effector domain can also be fused to an RNA binding protein that can interact with an aptamer sequence integrated into the sgRNA (Konermann *et al.*, 2015). CRISPR/Cas9 can thus be employed not only to edit the genome, but also to control expression of specific genes (Lowder *et al.*, 2015) and induce sequence-specific epigenetic modifications (Hilton *et al.*, 2015; Thakore *et al.*, 2015). Interestingly, Cpf1 also appears to be a potent tool for effector fusions, as was shown for transcriptional repression in *Arabidopsis* (Tang *et al.*, 2017).

The availability of various Cas9 orthologues offers the possibility of bringing genome editing to a new level. Because different Cas9 orthologues only interact with their species-specific sgRNA (Steinert *et al.*, 2015), diverse effects can be performed in the same cell simultaneously. For instance, one orthologue could guide transcriptional activators to one set of genes, whereas another orthologue guides transcriptional repressors to a different set of genes. In this way, the outcome of a specific DSB induced by a third orthologue could be influenced by manipulation of the protein machinery involved in the processing of the break, for example suppressing NHEJ while enhancing HDR for gene replacement (Puchta, 2016).

VII. An emerging new breeding process

1. Breeding schemes using rapid cycles of genome editing

Since the first domestication of crops 10 000 years ago (Doebley *et al.*, 2006), humans have relied on nontargeted approaches such as spontaneous mutation, random mutagenesis and genetic recombination to generate diversity for selecting improved crops. Selection from the genetic diversity generated in this way and subsequent fixation of desirable agronomic traits remain the cornerstone of crop breeding. Although transgenic plants form a notable exception to the nontargeted production of diversity within the current breeding process, these crops suffer from a lack of public acceptance and are limited in the scope of improvement they can offer by incorporating novel genes that often have individual and specific effects such as herbicide resistance (Daniell, 2002). With rapid advances in crop genome sequencing, functional genomics and CRISPR/Cas efficiency, we expect a novel crop breeding process to play and increasingly important role (Fig. 5). As progress in sequencing technologies and genome assembly algorithms provide more high-quality genome assemblies, and genome editing improves with regard to delivery and editing precision, the limiting factor in large-scale crop improvement programs using genome editing will be deciding what to edit. When an integrated knowledge of functional genomics in plants can be incorporated into crop breeding schemes based on genome editing, we believe a fundamental shift in how crops are improved will follow.

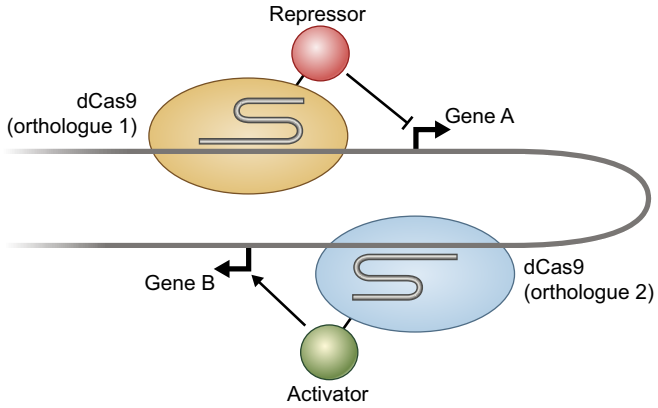

Fig. 4 Utilizing clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas) to regulate gene expression. Catalytically inactive 'dead' Cas9 (dCas9) is fused to activator or repressor domains and guided to the promoter region of regulated genes. Using multiple Cas9 orthologues, both functions can be performed simultaneously.

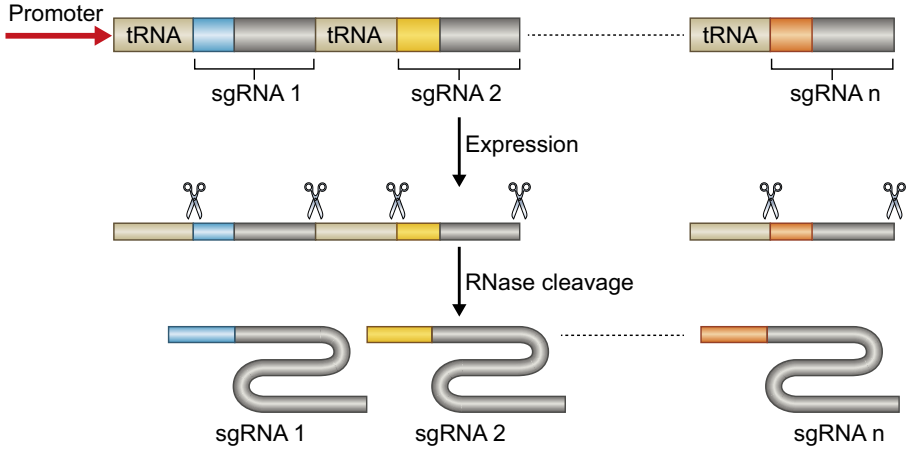

Fig. 3 Multiplexing using the endogenous tRNA-processing system. Endogenous ribonucleases recognize and cleave the 5′ and 3′ ends of the tRNA sequence (shown in light brown) on the primary transcript, releasing functional single guide RNAs (sgRNAs) composed of a unique targeting sequence (shown in blue, yellow and orange) and a scaffold sequence (shown in grey).

Conventional breeding schemes generate genetic variants via recombination or untargeted mutagenesis, applying cycles of backcrossing, gene pyramiding, pedigree breeding or recurrent selection to introduce novel traits into elite varieties. As these approaches do not allow precise control of the genetic material introgressed, they can lead to unpredicted effects such as the introduction of deleterious material genetically linked to desirable traits via linkage drag. For this reason, current breeding and selection schemes are laborious and require multiple rounds of crossing and selection to generate improved varieties. By contrast, genome editing provides an unparalleled level of control over the mutation process. For instance, Ito *et al.* (2015) point out that a deletion in the fruit-ripening regulator *RIN* used in conventional tomato breeding likely spans the regulatory region of a neighbouring gene, interfering with development of flowers and the pedicel abscission zone, which connects fruits to the main body of the plant. The authors disrupted the *RIN* gene using genome editing to delay fruit ripening without affecting neighbouring genes, illustrating the application of precise mutagenesis.

In order to generate diverse populations for selection, future breeders can apply genome editing of candidate genes and regulatory regions instead of relying on genetic recombination, random mutagenesis or somatic hybridization. By generating a comprehensive range of novel allelic variants aimed at modifying a specific trait, laboratory trials and subsequent multi-environment field trials will allow trait optimization, rather than just trait improvement. To optimize traits, the multiplexing capability of the CRISPR/Cas system could be leveraged to harness epistasis (interaction between genes) and redundant pathways, which often present obstacles to conventional breeding. Under this new breeding process based on cycles of genome editing and selection, breeding outcomes are more predictable, and as there is no requirement to remove unfavourable alleles, the production of improved varieties is faster.

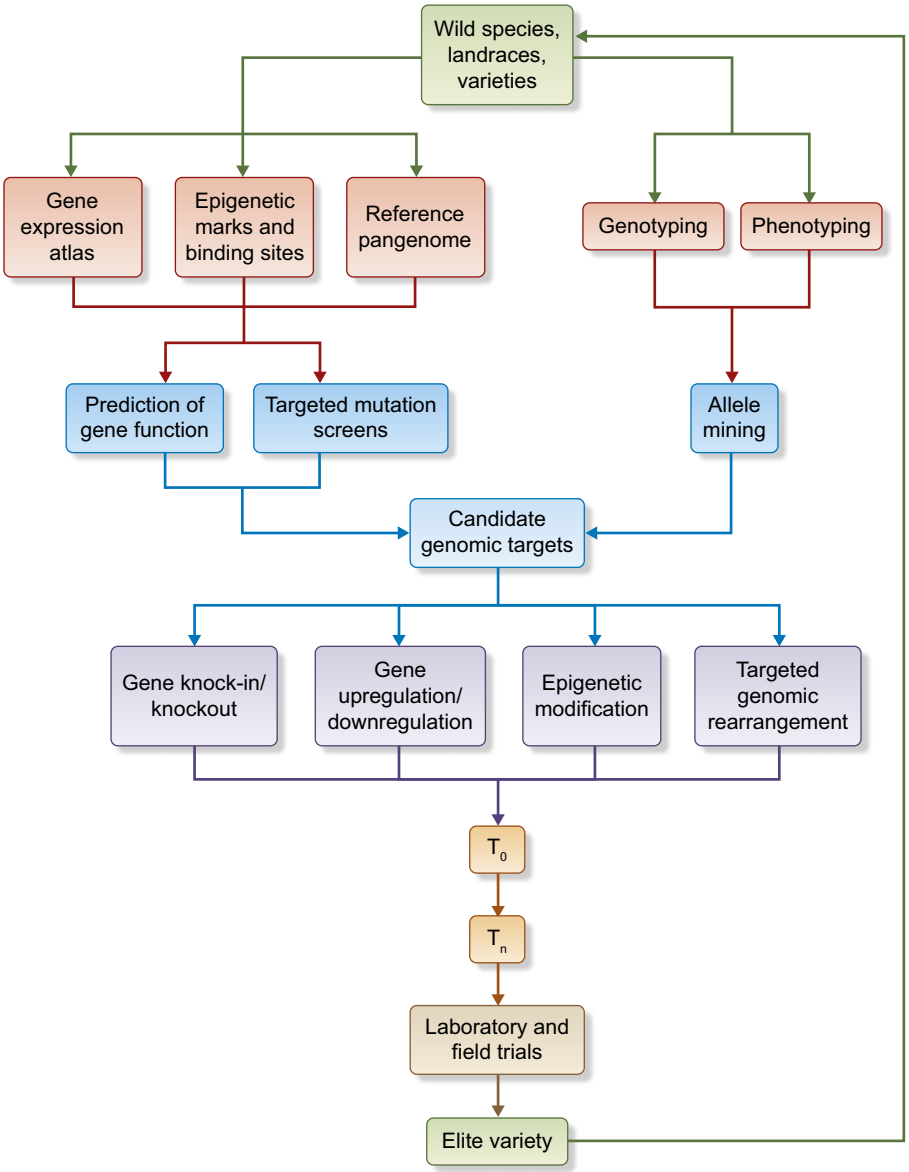

Fig. 5 Breeding scheme for crop improvement using genomic, transcriptomic and phenomic resources together with genome editing. By integrating various types of data on crop germplasm (wild species, landraces and varieties), traits can be associated with genomic regions to help identify candidate genomic targets for editing. Genome editing is followed by crossing unwanted regions of the genome such as the CRISPR/Cas genes out of the first-generation transformant (T_0). Optimization of novel allelic variants is carried by selecting the best performing variants in multi-environment field trials. Cycles of editing and selection allow generation of novel elite varieties that can be added to crop germplasm resources.

Genome editing represents a progression from genomics-assisted breeding approaches based on genetic markers such as MAS and GS. By generating novel allelic variants that directly affect traits, genome editing overcomes the imprecision resulting from the use of markers linked to traits but not directly influencing them. Rather than genomic tools assisting in the breeding process, they will be able to play a more pivotal role as they become the means of identifying and generating variation. Furthermore, genome editing surpasses earlier GM approaches that introduced genes for herbicide resistance and pest resistance into staple crops, because it allows both the introduction of novel genes in precise genomic positions and can modify the sequence and regulation of existing genes. Although we expect GM and marker-assisted approaches such as MAS and GS to offer further benefits to breeders in the coming years, these approaches will be superseded by genome editing as our functional knowledge of crop genomes grows.

2. Applications of genome editing beyond simple traits

During the history of crop breeding, many agronomic traits have been continuously selected in major crops and may now only allow incremental improvement using current breeding methods. In particularly, simple monogenic traits that are easily targeted for breeding may no longer be efficient targets for further improvement as many have been optimized through traditional methods. Most recent applications of genome editing in crop improvement discussed in this review have focused on disrupting one or several genes linked to an agronomic trait, commonly pest resistance. This

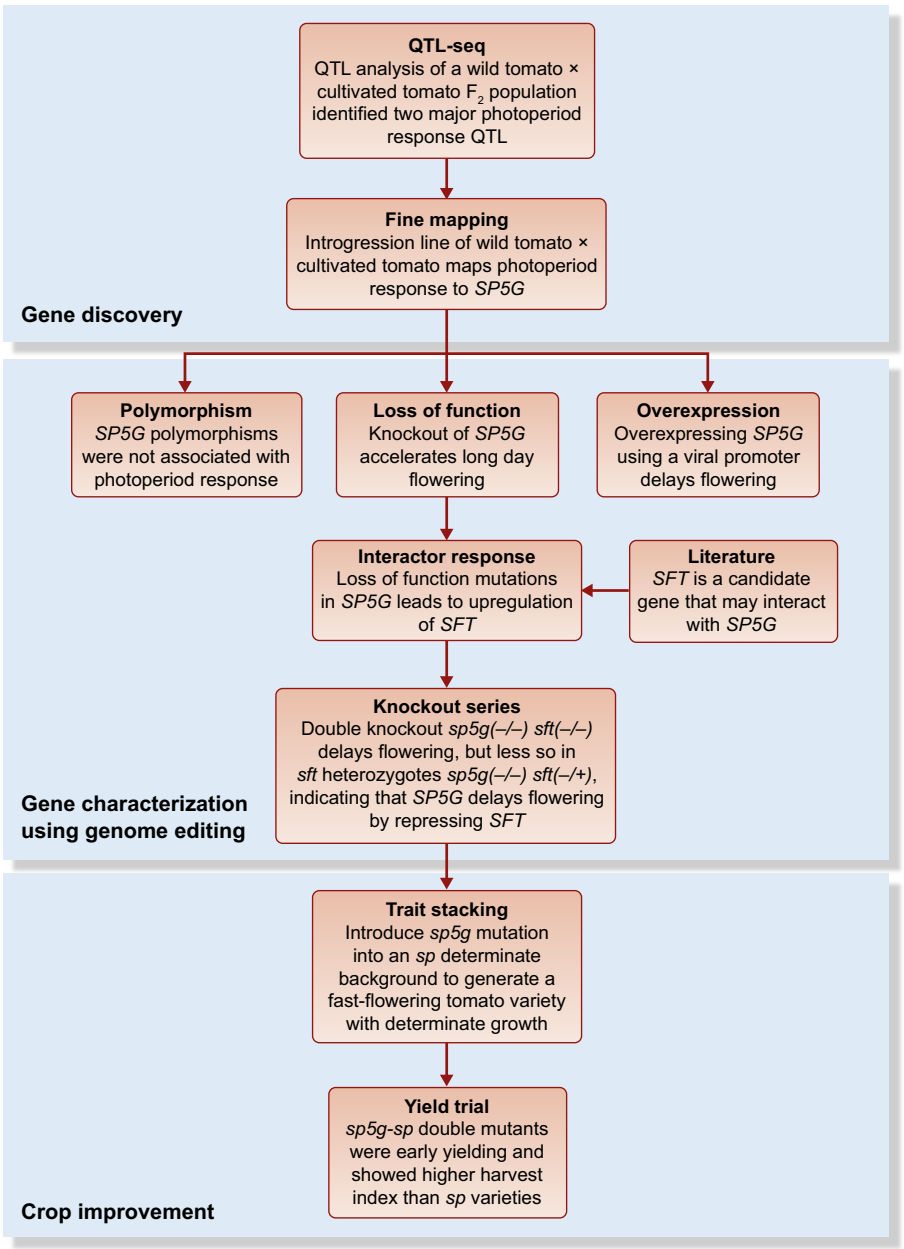

Fig. 6 Crop improvement strategy used by Soyk *et al.* (2017) to identify, characterize and edit the flowering repressor *SP5G*, and introduce the quantitative trait earliness of yield into an elite cultivated tomato variety. Quantitative trait loci sequencing (QTL-seq) and fine mapping identified *SP5G*, and genome editing and expression analysis characterized its function as a repressor of the florigen gene *SFT*. Genome editing was then used to disrupt *SP5G* in a determinate background, stacking the determinate growth conferred by a mutation in the *SP* gene and the earliness of yield caused by disruption of *SP5G*.

outcome may also be achieved using conventional breeding, albeit less efficiently. Genome editing, however, has the potential to target complex traits using combinations of gene editing, editing of regulatory elements and genomic rearrangement. As CRISPR/Cas multiplexing allows useful edits to be made simultaneously, multiple traits can be stacked in a new variety within a single generation. This provides a key advantage over conventional breeding methods, which rely on recombination over multiple generations or random simultaneous mutation events to stack traits. Genome editing will be most effectively used for crop improvement when it is used to generate traits that cannot be arrived at through nonspecific mutations or the simple addition of a transgene.

Genome editing could be used to increase the efficiency of the key CO_2 fixing enzyme Rubisco and thus improve photosynthesis efficiency and yield (Sharwood, 2017). An ambitious goal is to increase photosynthesis efficiency in C_3 plants such as rice by making their photosynthesis pathways more similar to those of C_4 plants such as maize. C_4 Rubisco has a faster carboxylation rate and thus higher efficiency than C_3 Rubisco, but is less specific and requires higher CO_2 concentrations, which C_4 plants provide via carbon concentration mechanisms. Important candidate genes for improving photosynthesis efficiency using genome editing are thus genes encoding for Rubisco and for carbon concentration mechanisms. Rubisco consists of large subunits and small subunits, encoded by the *rbcL* gene in the chloroplast genome and the *RbcS* multi-gene family in the nucleus. Catalytic switches responsible for faster carboxylation in C_4 plants are known, for instance the large subunit Met-309-Ile substitution (Whitney *et al.*, 2011). A recent study found 88 candidate genes likely involved in the C_4 differentiation process including genes encoding carbon shuttle enzymes and key transporters such as *PEPC*, *PPDK*, *NADP-ME* and *OMT*, and the less well-known *TPT* and *NHD-BASS2* (Huang *et al.*, 2017). Although converting crops from C_3 to C_4 photosynthesis will be a challenge, genome editing could help overcome this challenge using multiplexed gene replacement to alter and optimize photosynthesis components. To circumvent the challenge of chloroplast transformation, genes could be encoded in the nuclear genome together with genes encoding transit peptides and membrane transporters for transfer into the chloroplast.

Another application of genome editing is biofortification of staple crops to improve human health. When pathways to accumulate micronutrients do not occur in a crop or do not allow micronutrient accumulation in edible parts of the crop, biofortification is generally not possible using conventional breeding because the changes required in the genome are too complex. Crop metabolic pathways generally involve suites of genes, for example, the pathway for production of the cancer-preventive glucoraphanin includes 13 genes (Mikkelsen *et al.*, 2010). Although inserting transgenes encoding metabolic pathways has been a key approach in biofortification, altering endogenous pathways with genome editing may provide better micronutrient accumulation and control, as endogenous genes and regulators are likely better optimized to function together. For instance, the beta-carotene (vitamin A precursor) pathway engineered into 'Golden Rice' using the two key transgenes *psy*and *crtI* (Ye *et al.*, 2000) could potentially be achieved using editing of the endogenously occurring beta-carotene pathway.

Drought is an important driver in crop yield loss but enhancing drought tolerance is challenging because of the complex networks involved in regulating drought response (Hu & Xiong, 2014). An important target for increasing drought tolerance is the phytohormone ABA, which inhibits growth and helps regulate plant stress response. The discovery of 14 genes encoding receptors that bind to ABA and activate ABA signalling, *PYR1* and 13 related *PYR1-like* genes (*PYL*) (Park *et al.*, 2009), provides candidates for genome editing to adjust the ABA pathway to reduce yield loss under drought while maintaining plant growth during nondrought periods. Furthermore, a recent review listed 15 genes that are known to increase drought stress survival, biomass or yield in various crops during field trials (Mickelbart *et al.*, 2015). Although there are likely to be trade-offs between drought tolerance genes, stacking combinations of edits of these genes may lead to overall improvements in drought tolerance.

3. Domesticating new crops using genome editing

The majority of calories consumed by humans are derived from only 20 crops, with rice, wheat and maize contributing the largest proportion to the global food supply (Massawe *et al.*, 2016). Over the past decades, the improvement of these major crops has been a priority, and has been followed by an increasing homogenization of food production. However, improvement of major crops may not always be the most efficient approach for generating high-yielding, climate resilient crops. Although monogenic traits have been targeted by breeders with considerable success, altering complex polygenic traits such as abiotic stress tolerance remains challenging. To overcome this difficulty, wild species or minor crops with a more favourable genetic background for these traits could be rapidly domesticated using genome editing.

Recent publications have emphasized the potential for crop domestication using genome editing, listing candidate domestication genes involved in traits including seed shattering, growth architecture and flowering time (Osterberg *et al.*, 2017; Zsögön *et al.*, 2017). For example, in a pioneering study, Soyk *et al.* (2017) characterized and edited the flowering repressor *SP5G* in tomato, reducing time to harvest by two weeks (Fig. 6). By fine-mapping a QTL region linked to photoperiod response, *SP5G* was found to cause delayed flowering during long days. Using genome editing to knockout combinations of *SP5G* and the dosage-dependent flowering activator *SFT*, the authors showed that *SP5G* is a likely repressor of *SFT*, making *SP5G* a candidate for helping to domesticate tomato relatives and other species by enabling them to grow in more northerly latitudes.

The ongoing domestication of numerous wild plants could be accelerated by targeting orthologues of domestication genes such as *SP5G*. For instance, in the distant Australian rice relative weeping grass (*Microlaena stipoides*), a potential crop that is resistant to a range of abiotic stresses, chemical mutagenesis of the rice orthologues *qSH1* and *sh4* decreased seed shattering (Shapter *et al.*, 2013). Pennycress (*Thlaspi arvense*), a common weed in Eurasia and North-America, could be domesticated into a valuable,

cold-tolerant oilseed crop if traits such as oil quality and seed pod shatter could be improved (Sedbrook *et al.*, 2014). Progenitors of staple crops such as the maize progenitor teosinte (*Zea mays* ssp. *parviglumis*), wild emmer wheat (*Triticum dicoccoides*) and common wild rice (*Oryza rufipogon*) could also be candidates for domestication as they may contain valuable adaptations lost during domestication bottlenecks. Genome editing was recently used in the potential rubber crop *Taraxacum koksaghyz* to disrupt an expected antagonist of rubber production (Iaffaldano *et al.*, 2016). As more genomes of wild species and minor crops are sequenced, it will become easier to identify orthologous domestication genes known from well-studied plants that can be targeted with genome editing. If candidate domestication genes are known and the challenges of epistasis and gene dosage can be overcome, genome editing in wild or minor crops will help substantially expand the crop germplasm pool.

4. Regulation of genome edited crops

Strict regulation and nationwide bans, particularly in Europe, have slowed the commercial development and widespread adoption of GM crops since they were first introduced in 1995. Research and development of nontransgenic genome edited crops may be similarly slowed if regulatory authorities treat them as GM crops. In contrast to transgenic GM crops, genome edited crops can be indistinguishable from crops produced by conventional breeding. To differentiate between conventional mutagenesis breeding and genome editing, legislation may therefore attempt to regulate the process of crop improvement rather than the result. However, advances in the delivery of genome editing machinery such as the use of RNP complexes, which do not require even temporary introduction of foreign DNA into the genome, may rapidly undermine legislation regulating the crop improvement process (Wolter & Puchta, 2017). The European Court of Justice is due to decide in 2018 whether genome edited crops should be regulated as GM crops. The status of genome edited crops in many other countries remains unclear, as legislation lags behind scientific innovation. Regulation of genome edited crops must address rapid changes in the technology and distinguish between transgenic GM crops and nontransgenic genome edited crops. Canada provides an example of a pragmatic approach to biotechnology regulation by evaluating plants on a case-by-case basis with a focus on novel traits rather than the breeding process (Canadian Food Inspection Agency, 2016).

VIII. Conclusion

Genome editing technology is likely to play an important role in addressing growing global crop demand in the face of population growth and predicted climate change. Genome editing can facilitate the production of crops with higher yield, improved nutrient content, resistance to pests and tolerance of abiotic stress by accelerating crop improvement schemes and increasing their effectiveness, and this can support food security in developed as well as developing countries. The main hurdles that remain to be overcome to establish a new genome editing process for crop improvement are the assembly of high-quality pangenome references, the systematic inference of candidate editing sites using functional genomics, the improved delivery of genome editing systems and the reduction of off-target editing. For this new crop improvement process to be successful, the accumulation and integration of knowledge in genomics, transcriptomics, phenomics and biotechnology will be essential. The regulation of genome edited crops must also be clarified to support the development of this technology and gain consumer acceptance. Despite the remaining technical and social challenges, just four years after the CRISPR/Cas9 system was first applied to edit a plant genome, large-scale crop improvement using genome editing is on the verge of becoming a reality.

Acknowledgements

Armin Scheben was supported by an IPRS awarded by the Australian government. This work was supported by the Australian Research Council (project nos. LP160100030, LP140100537, LP130100061 and LP130100925). We thank three anonymous reviewers for comments that helped improve the manuscript. We are also grateful to Maxim Kapralov for comments on Rubisco.

References

Acquaah G. 2012. *Principles of plant genetics and breeding*. Chichester, UK: Wiley-Blackwell.

Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz MM. 2015. CRISPR/Cas9-mediated viral interference in plants. *Genome Biology* **16**: 238.

Altpeter F, Springer NM, Bartley LE, Blechl AE, Brutnell TP, Citovsky V, Conrad LJ, Gelvin SB, Jackson DP, Kausch AP *et al.* 2016. Advancing crop transformation in the era of genome editing. *Plant Cell* **28**: 1510–1520.

Andolfo G, Iovieno P, Frusciante L, Ercolano MR. 2016. Genome-editing technologies for enhancing plant disease resistance. *Frontiers in Plant Science* **7**: 1813.

Andorf CM, Cannon EK, Portwood JL, Gardiner JM, Harper LC, Schaeffer ML, Braun BL, Campbell DA, Vinnakota AG, Sribalusu VV *et al.* 2016. MaizeGDB update: new tools, data and interface for the maize model organism database. *Nucleic Acids Research* **44**: D1195–D1201.

Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF. 2014. DNA replicons for plant genome engineering. *Plant Cell* **26**: 151–163.

Baltes NJ, Hummel AW, Konecna E, Cegan R, Bruns AN, Bisaro DM, Voytas DF. 2015. Conferring resistance to geminiviruses with the CRISPR-Cas prokaryotic immune system. *Nature Plants* **1**: 15145.

Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. 2015. Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology* **32**: 76–84.

Blomstedt CK, Gleadow RM, O'Donnell N, Naur P, Jensen K, Laursen T, Olsen CE, Stuart P, Hamill JD, Moller BL *et al.* 2012. A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production. *Plant Biotechnology Journal* **10**: 54–66.

Bortesi L, Fischer R. 2015. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances* **33**: 41–52.

Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. 2013. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nature Methods* **10**: 1213–1218.

Canadian Food Inspection Agency. 2016. *Plants with novel traits*. [WWW document] URL http://www.inspection.gc.ca/plants/plants-with-novel-traits/eng/1300137887237/1300137939635 [accessed 8 May 2017].

Carpenter JE. 2010. Peer-reviewed surveys indicate positive impact of commercialized GM crops. *Nature Biotechnology* **28**: 319–321.

Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF. 2015. High-frequency, precise modification of the tomato genome. *Genome Biology* **16**: 232.

Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A. 2016. Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Molecular Plant Pathology* **17**: 1140–1153.

Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, Tibebu R, Davison S, Ray EE, Daulhac A *et al.* 2016. Improving cold storage and processing traits in potato through targeted gene knockout. *Plant Biotechnology Journal* **14**: 169–176.

Collard BC, Mackill DJ. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B* **363**: 557–572.

Curtin SJ, Zhang F, Sander JD, Haun WJ, Starker C, Baltes NJ, Reyon D, Dahlborg EJ, Goodwin MJ, Coffman AP *et al.* 2011. Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. *Plant Physiology* **156**: 466–473.

Dang Y, Jia G, Choi J, Ma H, Anaya E, Ye C, Shankar P, Wu H. 2015. Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. *Genome Biology* **16**: 280.

Daniell H. 2002. Molecular strategies for gene containment in transgenic crops. *Nature Biotechnology* **20**: 581–586.

Dash S, Van Hemert J, Hong L, Wise RP, Dickerson JA. 2012. PLEXdb: gene expression resources for plants and plant pathogens. *Nucleic Acids Research* **40**: D1194–D1201.

Demorest ZL, Coffman A, Baltes NJ, Stoddard TJ, Clasen BM, Luo S, Retterath A, Yabandith A, Gamo ME, Bissen J *et al.* 2016. Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. *BMC Plant Biology* **16**: 225.

Desta ZA, Ortiz R. 2014. Genomic selection: genome-wide prediction in plant improvement. *Trends in Plant Science* **19**: 592–601.

Doebley JF, Gaut BS, Smith BD. 2006. The molecular genetics of crop domestication. *Cell* **127**: 1309–1321.

Edwards D. 2016. The impact of genomics technology on adapting plants to climate change. In: Edwards D, Batley J, eds. *Plant genomics and climate change*. New York, NY, USA: Springer, 173–178.

Eeckhaut T, Lakshmanan PS, Deryckere D, Van Bockstaele E, Van Huylenbroeck J. 2013. Progress in plant protoplast research. *Planta* **238**: 991–1003.

Eid A, Ali Z, Mahfouz MM. 2016. High efficiency of targeted mutagenesis in *Arabidopsis* via meiotic promoter-driven expression of Cas9 endonuclease. *Plant Cell Reports* **35**: 1555–1558.

Fauser F, Roth N, Pacher M, Ilg G, Sanchez-Fernandez R, Biesgen C, Puchta H. 2012. In planta gene targeting. *Proceedings of the National Academy of Sciences, USA* **109**: 7535–7540.

Fauser F, Schiml S, Puchta H. 2014. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant Journal* **79**: 348–359.

Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang D-L, Wang Z, Zhang Z, Zheng R, Yang L *et al.* 2014. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **111**: 4632–4637.

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology* **31**: 822–826.

Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature Biotechnology* **32**: 279–284.

Gasiunas G, Barrangou R, Horvath P, Siksnys V. 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences, USA* **109**: E2579–E2586.

Gil-Humanes J, Wang Y, Liang Z, Shan Q, Ozuna CV, Sanchez-Leon S, Baltes NJ, Starker C, Barro F, Gao C *et al.* 2017. High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. *Plant Journal* **89**: 1251–1262.

Gilbert LA, Larson MH, Morsut L, Liu ZR, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA *et al.* 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**: 442–451.

Golicz AA, Batley J, Edwards D. 2016a. Towards plant pangenomics. *Plant Biotechnology Journal* **14**: 1099–1105.

Golicz AA, Bayer PE, Barker GC, Edger PP, Kim H, Martinez PA, Chan CK, Severn-Ellis A, McCombie WR, Parkin IA *et al.* 2016b. The pangenome of an agronomically important crop plant *Brassica oleracea*. *Nature Communications* **7**: 13 390.

Haudry A, Platts AE, Vello E, Hoen DR, Leclercq M, Williamson RJ, Forczek E, Joly-Lopez Z, Steffen JG, Hazzouri KM *et al.* 2013. An atlas of over 90,000 conserved noncoding sequences provides insight into crucifer regulatory regions. *Nature Genetics* **45**: 891–898.

Heigwer F, Kerr G, Boutros M. 2014. E-CRISP: fast CRISPR target site identification. *Nature Methods* **11**: 122–124.

Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA. 2015. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature Biotechnology* **33**: 510–517.

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li YQ, Fine EJ, Wu XB, Shalem O *et al.* 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology* **31**: 827–832.

Hu HH, Xiong LZ. 2014. Genetic engineering and breeding of drought-resistant crops. *Annual Review of Plant Biology* **65**: 715–741.

Huang P, Studer AJ, Schnable JC, Kellogg EA, Brutnell TP. 2017. Cross species selection scans identify components of C_4 photosynthesis in the grasses. *Journal of Experimental Botany* **68**: 127–135.

Iaffaldano B, Zhang YX, Cornish K. 2016. CRISPR/Cas9 genome editing of rubber producing dandelion *Taraxacum kok-saghyz* using *Agrobacterium rhizogenes* without selection. *Industrial Crops and Products* **89**: 356–362.

IPCC, Barros VR, Field CB, Dokke DJ, Mastrandrea MD, Mach KJ, Bilir TE, Chatterjee M, Ebi KL, Estrada YO, Genova RC *et al.*, eds. 2014. *Climate Change 2014: impacts, adaptation, and vulnerability. Part B: regional aspects.* Contribution of Working Group II to the fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge, UK & New York, NY, USA: Cambridge University Press.

Ishida Y, Hiei Y, Komari T. 2007. *Agrobacterium*-mediated transformation of maize. *Nature Protocols* **2**: 1614–1621.

Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Toki S. 2015. CRISPR/Cas9-mediated mutagenesis of the *RIN* locus that regulates tomato fruit ripening. *Biochemical and Biophysical Research Communications* **467**: 76–82.

Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA. 2015. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology* **15**: 16.

Ji X, Zhang HW, Zhang Y, Wang YP, Gao CX. 2015. Establishing a CRISPR-Cas-like immune system conferring DNA virus resistance in plants. *Nature Plants* **1**: 15 144.

Jiang WZ, Zhou HB, Bi HH, Fromm M, Yang B, Weeks DP. 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Research* **41**: e188.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**: 816–821.

Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng ZL, Joung JK. 2016. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**: 490–495.

Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera Mdel C, Yusa K. 2014. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nature Biotechnology* **32**: 267–273.

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**: 420–424.

Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H *et al.* 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**: 583–588.

Lambert B, Denolf P, Engelen S, Golds T, Haesendonckx B, Ruiter R, Robbens S, Bots M, Laga B. 2015. Omics-directed reverse genetics enables the creation of new productivity traits for the vegetable oil crop canola. *Procedia Environmental Sciences* **29**: 77–78.

Lawrenson T, Shorinola O, Stacey N, Li C, Ostergaard L, Patron N, Uauy C, Harwood W. 2015. Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biology* **16**: 258.

Lee HJ, Kweon J, Kim E, Kim S, Kim JS. 2012. Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. *Genome Research* **22**: 539–548.

Li M, Li X, Zhou Z, Wu P, Fang M, Pan X, Lin Q, Luo W, Wu G, Li H. 2016b. Reassessment of the four yield-related genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in rice using a CRISPR/Cas9 system. *Frontiers in Plant Science* **7**: 377.

Li J, Meng X, Zong Y, Chen K, Zhang H, Liu J, Li J, Gao C. 2016a. Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. *Nature Plants* **2**: 16139.

Li J-F, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J. 2013. Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology* **31**: 688–691.

Li Z, Liu Z-B, Xing A, Moon BP, Koellhoffer JP, Huang L, Ward RT, Clifton E, Falco SC, Cigan AM. 2015. Cas9-guide RNA directed genome editing in soybean. *Plant Physiology* **169**: 960–970.

Liang Z, Chen KL, Li TD, Zhang Y, Wang YP, Zhao Q, Liu JX, Zhang HW, Liu CM, Ran YD *et al.* 2017. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Communications* **8**: 14261.

Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO *et al.* 2009. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* **326**: 289–293.

Lowder LG, Zhang D, Baltes NJ, Paul JW, Tang X, Zheng X, Voytas DF, Hsieh T-F, Zhang Y, Qi Y. 2015. A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiology* **169**: 971–985.

Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho MJ, Scelonge C, Lenderts B, Chamberlin M, Cushatt J *et al.* 2016. Morphogenic regulators *Baby boom* and *Wuschel* improve monocot transformation. *Plant Cell* **28**: 1998–2015.

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y *et al.* 2015. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Molecular Plant* **8**: 1274–1284.

Massawe F, Mayes S, Cheng A. 2016. Crop diversity: an unexploited treasure trove for food security. *Trends in Plant Science* **21**: 365–368.

Matson PA, Parton WJ, Power AG, Swift MJ. 1997. Agricultural intensification and ecosystem properties. *Science* **277**: 504–509.

McCallum CM, Comai L, Greene EA, Henikoff S. 2000. Targeted screening for induced mutations. *Nature Biotechnology* **18**: 455–457.

Michael TP, VanBuren R. 2015. Progress, challenges and the future of crop genomes. *Current Opinion in Plant Biology* **24**: 71–81.

Mickelbart MV, Hasegawa PM, Bailey-Serres J. 2015. Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nature Reviews Genetics* **16**: 237–251.

Mikami M, Toki S, Endo M. 2016. Precision targeted mutagenesis via Cas9 paired nickases in rice. *Plant and Cell Physiology* **57**: 1058–1068.

Mikkelsen MD, Olsen CE, Halkier BA. 2010. Production of the cancer-preventive glucoraphanin in tobacco. *Molecular Plant* **3**: 751–759.

Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. 2014. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Research* **42**: W401–W407.

Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S. 2013. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotechnology* **31**: 691–693.

Nekrasov V, Wang C, Win J, Lanz C, Weigel D, Kamoun S. 2017. Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports* **7**: 482.

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O. 2014. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**: 935–949.

Nobuta K, Venu RC, Lu C, Belo A, Vemaraju K, Kulkarni K, Wang WZ, Pillay M, Green PJ, Wang GL *et al.* 2007. An expression atlas of rice mRNAs and small RNAs. *Nature Biotechnology* **25**: 473–477.

Ordon J, Gantner J, Kemna J, Schwalgun L, Reschke M, Streubel J, Boch J, Stuttmann J. 2016. Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome editing toolkit. *Plant Journal* **89**: 155–168.

Osterberg JT, Xiang W, Olsen LI, Edenbrandt AK, Vedel SE, Christiansen A, Landes X, Andersen MM, Pagh P, Sandoe P *et al.* 2017. Accelerating the domestication of new crops: feasibility and approaches. *Trends in Plant Science* **22**: 373–384.

Pacher M, Schmidt-Puchta W, Puchta H. 2007. Two unlinked double-strand breaks can induce reciprocal exchanges in plant genomes via homologous recombination and nonhomologous end joining. *Genetics* **175**: 21–29.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TFF *et al.* 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071.

Pattanayak V, Lin S, Guilinger JP, Ma EB, Doudna JA, Liu DR. 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology* **31**: 839–843.

Peciña A, Smith KN, Mézard C, Murakami H, Ohta K, Nicolas A. 2002. Targeted stimulation of meiotic recombination. *Cell* **111**: 173–184.

Peng A, Chen S, Lei T, Xu L, He Y, Wu L, Yao L, Zou X. 2017. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant Biotechnology Journal* **10**: 1011–1013.

Peterson BA, Haak DC, Nishimura MT, Teixeira PJPL, James SR, Dangl JL, Nimchuk ZL. 2016. Genome-wide assessment of efficiency and specificity in CRISPR/Cas9 mediated multiple site targeting in *Arabidopsis*. *PLoS ONE* **11**: e0162169.

Puchta H. 2005. The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *Journal of Experimental Botany* **56**: 1–14.

Puchta H. 2016. Using CRISPR/Cas in three dimensions: towards synthetic plant genomes, transcriptomes and epigenomes. *Plant Journal* **87**: 5–15.

Puchta H. 2017. Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. *Current Opinion in Plant Biology* **36**: 1–8.

Qi W, Zhu T, Tian Z, Li C, Zhang W, Song R. 2016. High-efficiency CRISPR/Cas9 multiplex gene editing using the glycine tRNA-processing system-based strategy in maize. *BMC Biotechnology* **16**: 58.

Radivojac P, Clark WT, Oron TR, Schnoes AM, Wittkop T, Sokolov A, Graim K, Funk C, Verspoor K, Ben-Hur A *et al.* 2013. A large-scale evaluation of computational protein function prediction. *Nature Methods* **10**: 221–227.

Ran FA, Hsu PD, Lin C-Y, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y *et al.* 2013. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**: 1380–1389.

Ranganathan V, Wahlin K, Maruotti J, Zack DJ. 2014. Expansion of the CRISPR-Cas9 genome targeting space through the use of H1 promoter-expressed guide RNAs. *Nature Communications* **5**: 4516.

Ray DK, Mueller ND, West PC, Foley JA. 2013. Yield trends are insufficient to double global crop production by 2050. *PLoS ONE* **8**: e66428.

Ray DK, Ramankutty N, Mueller ND, West PC, Foley JA. 2012. Recent patterns of crop yield growth and stagnation. *Nature Communications* **3**: 1293.

Rousseau C, Gonnet M, Le Romancer M, Nicolas J. 2009. CRISPI: a CRISPR interactive database. *Bioinformatics* **25**: 3317–3318.

Ruprecht C, Vaid N, Proost S, Persson S, Mutwil M. 2017. Beyond genomics: studying evolution with gene coexpression networks. *Trends in Plant Science* **22**: 298–307.

Sadhu MJ, Bloom JS, Day L, Kruglyak L. 2016. CRISPR-directed mitotic recombination enables genetic mapping without crosses. *Science* **352**: 1113–1116.

Sanjana NE, Wright J, Zheng K, Shalem O, Fontanillas P, Joung J, Cheng C, Regev A, Zhang F. 2016. High-resolution interrogation of functional elements in the noncoding genome. *Science* **353**: 1545–1549.

Schaeffer SM, Nakata PA. 2015. CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. *Plant Science* **240**: 130–142.

Scheben A, Edwards D. 2017. Genome editors take on crops. *Science* **355**: 1122–1123.

Schiml S, Fauser F, Puchta H. 2014. The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in *Arabidopsis* resulting in heritable progeny. *Plant Journal* **80**: 1139–1150.

Schiml S, Fauser F, Puchta H. 2016. Repair of adjacent single-strand breaks is often accompanied by the formation of tandem sequence duplications in plant genomes. *Proceedings of the National Academy of Sciences, USA* **113**: 7266–7271.

Schinkel H, Schillberg S. 2016. Genome editing: intellectual property and product development in plant biotechnology. *Plant Cell Reports* **35**: 1487–1491.

Sedbrook JC, Phippen WB, Marks MD. 2014. New approaches to facilitate rapid domestication of a wild plant to an oilseed crop: example pennycress (*Thlaspi arvense* L.). *Plant Science* **227**: 122–132.

Sekhon RS, Lin HN, Childs KL, Hansey CN, Buell CR, de Leon N, Kaeppler SM. 2011. Genome-wide atlas of transcription during maize development. *Plant Journal* **66**: 553–563.

Shan QW, Wang YP, Li J, Zhang Y, Chen KL, Liang Z, Zhang K, Liu JX, Xi JJ, Qiu JL *et al.* 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology* **31**: 686–688.

Shapter FM, Cross M, Ablett G, Malory S, Chivers IH, King GJ, Henry RJ. 2013. High-Throughput sequencing and mutagenesis to accelerate the domestication of *Microlaena stipoides* as a new food crop. *PLoS ONE* **8**: e82641.

Sharwood RE. 2017. Engineering chloroplasts to improve Rubisco catalysis: prospects for translating improvements into food and fiber crops. *New Phytologist* **213**: 494–510.

Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE. 2016. *ARGOS8* variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnology Journal* **15**: 207–216.

Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI *et al.* 2017. Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology* **15**: 169–182.

Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D. 2005. A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology* **23**: 75–81.

Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. 2016. Rationally engineered Cas9 nucleases with improved specificity. *Science* **351**: 84–88.

Solomon MJ, Larsen PL, Varshavsky A. 1988. Mapping protein-DNA interactions *in vivo* with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. *Cell* **53**: 937–947.

Soyk S, Muller NA, Park SJ, Schmalenbach I, Jiang K, Hayama R, Zhang L, Van Eck J, Jimenez-Gomez JM, Lippman ZB. 2017. Variation in the flowering gene *SELF PRUNING 5G* promotes day-neutrality and early yield in tomato. *Nature Genetics* **49**: 162–168.

Steinert J, Schiml S, Fauser F, Puchta H. 2015. Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. *Plant Journal* **84**: 1295–1305.

Sun Y, Zhang X, Wu C, He Y, Ma Y, Hou H, Guo X, Du W, Zhao Y, Xia L. 2016. Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. *Molecular Plant* **9**: 628–631.

Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A. 2016. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nature Communications* **7**: 13274.

Svitashev S, Young JK, Schwartz C, Gao HR, Falco SC, Cigan AM. 2015. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiology* **169**: 931–945.

Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q *et al.* 2017. A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nature Plants* **3**: 17018.

Tang X, Zheng X, Qi Y, Zhang D, Cheng Y, Tang A, Voytas DF, Zhang Y. 2016. A single transcript CRISPR-Cas9 system for efficient genome editing in plants. *Molecular Plant* **9**: 1088–1091.

Thakore PI, D'Ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM, Reddy TE, Crawford GE, Gersbach CA. 2015. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nature Methods* **12**: 1143–1149.

Till BJ, Cooper J, Tai TH, Colowit P, Greene EA, Henikoff S, Comai L. 2007. Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biology* **7**: 19.

Tilman D, Balzer C, Hill J, Befort BL. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences, USA* **108**: 20260–20264.

Townsend JA, Wright DA, Winfrey RJ, Fu FL, Maeder ML, Joung JK, Voytas DF. 2009. High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* **459**: 442–445.

Tycko J, Myer VE, Hsu PD. 2016. Methods for optimizing CRISPR-Cas9 genome editing specificity. *Molecular Cell* **63**: 355–370.

Uauy C, Paraiso F, Colasuonno P, Tran RK, Tsai H, Berardi S, Comai L, Dubcovsky J. 2009. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biology* **9**: 115.

Ueta R, Abe C, Watanabe T, Sugano SS, Ishihara R, Ezura H, Osakabe Y, Osakabe K. 2017. Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Scientific Reports* **7**: 507.

Waltz E. 2016. CRISPR-edited crops free to enter market, skip regulation. *Nature Biotechnology* **34**: 582.

Wang FJ, Wang CL, Liu PQ, Lei CL, Hao W, Gao Y, Liu YG, Zhao KJ. 2016. Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the erf transcription factor gene *OsERF922*. *PLoS ONE* **11**: e0154027.

Wang M, Mao Y, Lu Y, Tao X, Zhu J-k. 2017. Multiplex gene editing in rice using the CRISPR-Cpf1 system. *Molecular Plant* **10**: 1011–1013.

Wang YP, Cheng X, Shan QW, Zhang Y, Liu JX, Gao CX, Qiu JL. 2014. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology* **32**: 947–951.

Wang Z-P, Xing H-L, Dong L, Zhang H-Y, Han C-Y, Wang X-C, Chen Q-J. 2015. Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in *Arabidopsis* in a single generation. *Genome Biology* **16**: 144.

Weber APM, Weber KL, Carr K, Wilkerson C, Ohlrogge JB. 2007. Sampling the *Arabidopsis* transcriptome with massively parallel pyrosequencing. *Plant Physiology* **144**: 32–42.

Whitney SM, Sharwood RE, Orr D, White SJ, Alonso H, Galmes J. 2011. Isoleucine 309 acts as a C_4 catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) carboxylation rate in *Flaveria*. *Proceedings of the National Academy of Sciences, USA* **108**: 14 688–14 693.

Wolter F, Edelmann S, Kadri A, Scholten S. 2017. Characterization of paired Cas9 nickases induced mutations in maize mesophyll protoplasts. *Maydica* **62**: 1–11.

Wolter F, Puchta H. 2017. Knocking out consumer concerns and regulator's rules: efficient use of CRISPR/Cas ribonucleoprotein complexes for genome editing in cereals. *Genome Biology* **18**: 43.

Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, Kim H, Kim S-G, Kim S-T, Choe S, Kim J-S. 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology* **33**: 1162–1164.

Xie K, Minkenberg B, Yang Y. 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences, USA* **112**: 3570–3575.

Xie K, Zhang J, Yang Y. 2014. Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops. *Molecular Plant* **7**: 923–926.

Xing H-L, Dong L, Wang Z-P, Zhang H-Y, Han C-Y, Liu B, Wang X-C, Chen Q-J. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology* **14**: 327.

Yan L, Wei S, Wu Y, Hu R, Li H, Yang W, Xie Q. 2015. High-Efficiency genome editing in *Arabidopsis* using YAO promoter-driven CRISPR/Cas9 system. *Molecular Plant* **8**: 1820–1823.

Ye XD, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287**: 303–305.

Yuan Y, Bayer PE, Batley J, Edwards D. 2017. Improvements in genomic technologies: application to crop genomics. *Trends in Biotechnology* **35**: 547–558.

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A *et al.* 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**: 759–771.

Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS *et al.* 2017.

Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nature Biotechnology* **35**: 31–34.

Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N *et al.* 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnology Journal* **12**: 797–807.

Zhang WL, Wu YF, Schnable JC, Zeng ZX, Freeling M, Crawford GE, Jiang JM. 2012. High-resolution mapping of open chromatin in the rice genome. *Genome Research* **22**: 151–162.

Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. 2014. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Research* **42**: 10903–10914.

Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu JL, Wang D, Gao C. 2017. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology* **35**: 438–440.

Zsögön A, Cermak T, Voytas D, Peres LEP. 2017. Genome editing as a tool to achieve the crop ideotype and *de novo* domestication of wild relatives: case study in tomato. *Plant Science* **256**: 120–130.