Breeding Science 71: 405–416 (2021)
doi: 10.1270/jsbbs.21019

Invited Review

Targeted genome modifications in cereal crops

Hiroshi Hisano*[1)], Fumitaka Abe[2)], Robert E. Hoffie[3)] and Jochen Kumlehn[3)]

[1)] *Institute of Plant Science and Resources, Okayama University*, Kurashiki, Okayama 710-0046, Japan
[2)] *Institute of Crop Science, National Agriculture and Food Research Organization*, Tsukuba, Ibaraki 305-8518, Japan
[3)] *Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)*, D-06466 Stadt Seeland/OT Gatersleben, Germany

The recent advent of customizable endonucleases has led to remarkable advances in genetic engineering, as these molecular scissors allow for the targeted introduction of mutations or even precisely predefined genetic modifications into virtually any genomic target site of choice. Thanks to its unprecedented precision, efficiency, and functional versatility, this technology, commonly referred to as genome editing, has become an effective force not only in basic research devoted to the elucidation of gene function, but also for knowledge-based improvement of crop traits. Among the different platforms currently available for site-directed genome modifications, RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) endonucleases have proven to be the most powerful. This review provides an application-oriented overview of the development of customizable endonucleases, current approaches to cereal crop breeding, and future opportunities in this field.

Key Words: barley, CRISPR, maize, rice, TALEN, wheat, zinc-finger nucleases.

Introduction

According to the United Nations, the world population is expected to rise by 2 billion and reach 9.8 billion by 2050, which will increase the demand for food by 60% and require gains in production yield (FAO *et al.* 2018). Due to climate change and soil degradation, arable land that would otherwise support the growth of crops is being lost, thereby greatly limiting the food production chain. Crop breeding is a critical avenue for coping with these global challenges by harnessing genetic resources, such as naturally occurring and artificially generated variants, to increase yield and plant resilience against abiotic stress, pathogens, and pests.

Throughout the history of human farming, crop domestication and the development of new crop varieties have relied on spontaneous mutations in existing cultivated germplasm that led to more productive farming and/or were associated with improved product utility. In addition to passive selection of emerging traits in one crop species, targeted crossbreeding also opened the door to combining useful traits from different germplasm. However, spontaneous mutation rates are low across the genome and rarely affect a gene with potential use for crop improvement. To accelerate the discovery of agronomically relevant genetic diversity, methods aimed at inducing random mutations have been developed. Crop plants have been subjected to ionizing radiation such as X-rays, gamma-rays, or heavy ion beams, as well as chemical mutagens like ethyl methanesulfonate or sodium azide (Ahloowalia and Maluszynski 2001). To date, many induced mutant lines have been generated and incorporated into several crop breeding programs. For example, the short-straw barley (*Hordeum vulgare*) cultivar 'Diamant' was produced via gamma-ray irradiation; the causal *denso* mutation has been since introgressed into over 100 cultivars grown worldwide (Ahloowalia *et al.* 2004). However, the desired mutations are accompanied by many additional mutations randomly distributed across the genome, some of which remain due to linkage. In addition, identification of the causal gene is typically challenging, even when using segregating populations and selecting for the phenotype of interest, as the candidate interval is likely to contain more than one mutation, only one of which is causal. These problems were partially alleviated by the development of the Targeted Induced Local Lesions in Genomes (TILLING) method, which allows for the identification of mutations in a given candidate gene by reverse genetics on pooled DNA from mutant populations (McCallum *et al.* 2000). Efficient selection methods based upon DNA marker-trait associations were also developed and broadly embraced in modern plant breeding (Rasheed *et al.* 2017). In addition, advanced sequencing technologies have become indispensable for providing comprehensive genome data in all crop species

Communicated by Takeshi Nishio
Received March 1, 2021. Accepted April 13, 2021.
First Published Online in J-STAGE on August 27, 2021.
*Corresponding author (e-mail: hiroshi.hisano@rib.okayama-u.ac.jp)

(Bayer *et al.* 2020). Taking advantage of these resources, several new methods have emerged that facilitate the rapid cloning of genes of interest, even in the context of cereals with comparatively large genomes such as mapping-by-sequencing using MutMap, mutant chromosome sequencing (MutChromSeq), mutagenesis with resistance gene sequence capture (MutRenSeq), targeted chromosome-based cloning via long-range assembly (TACCA), and association genetics with resistance gene enrichment sequencing (AgRenSeq) (Abe *et al.* 2012, Arora *et al.* 2019, Bettgenhaeuser and Krattinger 2019, Sánchez-Martín *et al.* 2016, Steuernagel *et al.* 2016, Thind *et al.* 2017).

The development of genetic transformation techniques based upon Agrobacterium (*Agrobacterium tumefaciens*)-mediated or direct delivery of recombinant DNA to totipotent plant cells has opened another chapter in crop breeding history (Kumlehn and Hensel 2009). This technology makes it possible to ectopically express, overexpress, or downregulate the expression of genes of interest to achieve desirable modifications of plant traits. In addition, the ability to easily introduce transgenes into most plant backgrounds has greatly facilitated basic plant research aimed at deciphering gene function (Kumlehn *et al.* 2019). The Flavr Savr tomato (*Solanum lycopersicum*), the first genetically engineered crop, was released for commercial use in 1994. Several other genetically engineered crops have since been developed. However, their commercial use is still largely confined to herbicide- and pest-resilient varieties due to public concern about the potential but unproven human risks associated with this technology, even though each new genetically engineered crop goes through a comprehensive and costly review process prior to approval.

More recently, emerging customizable endonucleases have begun to pave the way for the introduction of mutations or predefined sequence modifications at any genomic position, followed by the removal of transgenes by simple segregation in the progeny (Koeppel *et al.* 2019). This new technology, which is commonly referred to as genome editing, has already been implemented and demonstrated to work in most crop species. Customizable endonucleases hold great promise to greatly accelerate mutation breeding in crops, either via the precise introduction of previously known allelic states conferring desired traits into advanced breeding backgrounds or by the generation of novel genetic diversity at target genes. In this review, we introduce this novel technology to readers and provide examples of its applications in the major cereal crops rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), and barley. Finally, we discuss the potential of employing customizable endonucleases for future plant breeding programs.

Principles of targeted genome modification

To ensure the survival of organisms in the face of DNA damage, their constituent cells must repair DNA breaks efficiently, which mostly relies on any one of three major repair mechanisms that are equally relevant for site-directed genome modification methods (Kumlehn *et al.* 2018, **Fig. 1**). Non-homologous end-joining (NHEJ) repair is the predominant cellular repair mechanism for DNA double-strand breaks in plants. NHEJ involves the recognition and re-ligation of free DNA ends, which is error-prone and therefore leads to insertions and deletions of usually one or a few nucleotides. NHEJ repairs the majority of events induced during site-directed mutagenesis efforts, after the customized endonuclease has targeted and cleaved the intended site of modification, although the resulting modification itself is random. Other repair mechanisms rely on sequence homology and result in more predictable repair outcomes. Even small sequence repeats located on either side of the DNA break induced by the nuclease allow for their overlapping annealing, since opposite single strands of both DNA ends are complementary along these repeats; any remaining single-stranded DNA ends are degraded before the newly assembled double strand is cured by ligation. This mechanism of microhomology-mediated end-joining (MMEJ) repair is very common in plants and produces precise, predictable deletions of one of the repeat sequences and the sequence between them. Another virtually error-free basic mechanism of DNA repair is based on homologous recombination (HR) between one damaged

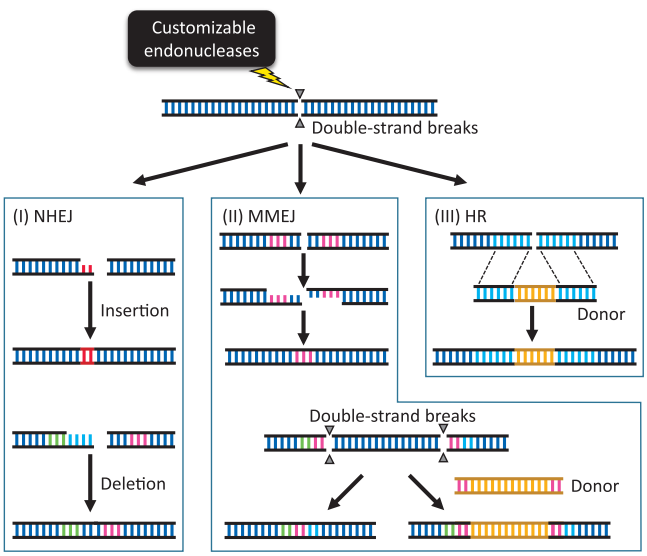

Fig. 1. Diagram summarizing the various ways in which customizable endonucleases can be used to introduce modifications into target genes. Customizable endonucleases trigger double-strand breaks in selected target sites of the plant genome. The DNA is then repaired via one of three major mechanisms, resulting in modifications at the target site: I, non-homologous end-joining (NHEJ) repair; II, microhomology-mediated end-joining (MMEJ) repair; and III, homologous recombination (HR). These mechanisms result in possible insertions, deletions, and/or substitutions of individual or multiple nucleotides. The size of the insertion or deletion is random following NHEJ-mediated DNA repair, whereas MMEJ and HR lead to predictable modifications and can thus be utilized for precise genome editing.

DNA region and the corresponding and intact sequence of the sister chromatid or a homologous chromosome, resulting in longer stretches of identical sequences than in MMEJ. HR may be harnessed to introduce a foreign DNA fragment as long as it is flanked by regions with homology to the region surrounding the site of DNA cleavage. While HR is essential for meiosis, the underlying enzymes are comparatively rarely active in somatic plant cells. In addition, the sister chromatids that are predominantly recruited as native repair templates are exclusively present in the G2 phase of the cell cycle. These limitations contribute to the current difficulties in implementing HR in plant genetic engineering and for precise genome editing in crop plants.

Site-directed genome modification is initiated by the cleavage of a given target sequence in genomic DNA by customizable endonucleases. Several effective endonucleases are available: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) endonucleases. Meganucleases, such as I-SceI, I-CreI, and I-DmoI, include a transit peptide for nuclear localization, a site-specific double-stranded DNA-binding domain of 12 to 40 nucleotides, and an endonuclease domain to cleave DNA. Although meganucleases have high target sequence specificity, the small number of DNA-binding sequences identified makes it difficult to design a derivative that targets other sequences of interest, unlike ZFNs, TALENs, or Cas endonucleases (see below). These nucleases are typically deployed in plant cells by introducing a transgene including a promoter that drives their expression and is recognized by RNA polymerase II, such as the cauliflower mosaic virus (CaMV) 35S or *UBIQUITIN* promoters. ZFNs and TALENs are chimeric proteins composed of a DNA-binding domain fused to a nuclease domain derived from the restriction enzyme FokI. The DNA-binding domain of ZFNs is composed of three to six zinc-finger modules derived from DNA-binding transcription factors. Each zinc-finger module specifically recognizes a unique nucleotide triplet. By contrast, the DNA-binding domain of TALENs consists of approximately 20 modules of four basic types, each with specificity to one of the four nucleobases A, C, G, or T (Gurushidze *et al.* 2014). This DNA-binding principle was adapted for biotechnological use from transcription activator-like effectors produced and delivered by plant pathogenic bacteria to bind to specific host genomic sequences to manipulate gene expression in infected cells. Due to differences in the architecture of the modules, the design and construction of TALENs is more straightforward compared to ZFNs (Budhagatapalli *et al.* 2016, Hensel and Kumlehn 2019). Since FokI only cleaves DNA as a dimer, two versions of ZFNs or TALENs are typically expressed, with each monomer binding to a DNA sequence on either side of the intended cleavage site and separated by an appropriate distance to direct endonucleolytic cleavage.

Cas endonucleases were originally discovered as components of microbial CRISPR-Cas immune systems. SpCas9 isolated from the bacterium *Streptococcus pyogenes* is currently the most widely used Cas endonuclease for biotechnological purposes due to its high cleavage efficiency within a physiologically relevant range of temperatures in a variety of hosts. The deployment of Cas endonucleases in microbial cells relies on a single-stranded guide RNA (gRNA) derived from the same native immune system that targets a DNA sequence that is recognized as foreign and must therefore be eliminated. The gRNA can be customized to target any sequence of interest for Cas-dependent cleavage. In most SpCas9 applications, the gRNA is a single-stranded chimeric RNA harboring two parts whose native predecessors were independent molecules: the CRISPR RNA (crRNA), which includes ~20 bp of customizable sequence that binds to the DNA target motif via complementary base-pairing, and trans-activating CRISPR RNA (tracrRNA) scaffold, which binds to the Cas endonuclease. In general, polymerase II-type promoters are used to drive the expression of the genes encoding Cas endonucleases, while polymerase III-type promoters from small non-coding RNAs are mainly used to express gRNAs. A Cas endonuclease target motif is not confined to the sequence bound by the gRNA 5′ end via complementary base-pairing, as it must also include a short binding site for the Cas endonuclease. In the case of SpCas9, this so-called protospacer-adjacent motif (PAM) consists of two guanine nucleobases preceded by any nucleobase (i.e., NGG). This general pattern must therefore be given some thought when searching for appropriate Cas endonuclease target motifs within the host genome. Several powerful online platforms are now available that greatly facilitate the identification of target sequences within genes of interest based on experimental considerations such as the Cas endonuclease variant and the host species used (e.g., WU-CRISPR, http://crisprdb.org/wu-crispr/; CRISPR-P 2.0, http://crispr.hzau.edu.cn/CRISPR2; CRISPOR, http://crispor.tefor.net; and CRISPR Guide RNA Design, https://www.benchling.com/crispr).

Establishment of targeted genome modification in cereals

Among cereal crops, three types of nucleases mentioned above (meganucleases, ZFNs, and TALENs) were used to generate targeted genome modifications prior to the advent of Cas endonucleases. The first example of targeted genome modification using customizable endonucleases was by D'Halluin *et al.* (2008), who succeeded in inserting the 35S promoter upstream of a promoterless herbicide resistance transgene using a meganuclease in maize. The following year, Shukla *et al.* (2009) reported the first use of ZFNs for site-directed mutagenesis at the maize *INOSITOL-1,3,4,5,6-PENTAKISPHOSPHATE 2-KINASE 1* (*IPK1*) gene as well as site-directed DNA insertion of a *PHOSPHINOTHRICIN ACETYLTRANSFERASE* (*PAT*) gene at the same locus. For

TALENs, Li *et al.* (2012) were the first to successfully modify the promoter sequence of the bacterial leaf blight susceptibility gene *SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER 14* (*OsSWEET14*) in rice.

Cas9 endonuclease-based targeted genome modification was first achieved in rice and wheat by Shan *et al.* (2013), Miao *et al.* (2013), and Feng *et al.* (2013), who used SpCas9 to target genes associated with chlorophyll biosynthesis and thus with easily scorable phenotypes: *PHYTOENE DESATURASE* (*PDS*), *CHLOROPHYLL A OXYGENASE 1* (*CAO1*), and *YOUNG SEEDLING ALBINO* (*YSA*).

Besides SpCas9, endonucleases derived from other CRISPR systems have also been co-opted for targeted genome modification. For example, Endo *et al.* (2016), Tang *et al.* (2017b), and Yin *et al.* (2017) induced mutations by expressing the Cas12a (CRISPR from Prevotella and Francisella 1 or Cpf1) endonuclease in rice. In contrast to Cas9, Cas12a functions with a native single crRNA. Like several other types of Cas endonucleases, Cas12a broadened the choice of possible target sequences because it requires a T-rich PAM (5′-(T)TTV-3′) that resides upstream of the nucleotides bound by the gRNA. Unlike Cas9, Cas12a results in staggered cleavage of the target sequence.

A single nucleotide substitution method called base editing has also been developed. Base editing relies on Cas derivatives (so-called nickases) that cleave only one of the two DNA strands, after which a fused cytidine deaminase or adenosine deaminase domain converts a single nucleobase. For example, Li *et al.* (2018) expressed a Cas9-adenosine deaminase fusion in rice and wheat and introduced T/A to C/G conversions in target genes.

More recently, another approach for precise genome modifications called prime editing was developed, based upon chimeric fusion proteins between a Cas9 nickase and a reverse transcriptase. Such proteins are coupled with gRNAs carrying a 3′ extension that acts as a primer to specify the replacement of particular nucleobases within the target sequence (Anzalone *et al.* 2019). This method was successfully implemented in rice and wheat among other plants (Hua *et al.* 2020, Lin *et al.* 2020).

Highly versatile, precise genome editing can be achieved using homology-based approaches in which customizable nucleases are used along with a repair template that carries DNA fragments with homology to the target region while also harboring the desired modified sequence to be incorporated at the genomic target site (**Fig. 1**). Proof-of-concept studies demonstrated precise genome editing using meganucleases and TALENs in barley, although these initial examples were confined to the modification of previously inserted transgenes (Budhagatapalli *et al.* 2015, Watanabe *et al.* 2015). Begemann *et al.* (2017) subsequently used the Cas12a endonuclease to perform site-directed DNA insertions in rice, while Gil-Humanes *et al.* (2017) developed a site-directed DNA insertion method using the replication system of wheat dwarf virus combined with the Cas9 endonuclease, which resulted in increased frequency of knock-in of foreign DNA at a genomic target region in wheat.

Another novel approach that also largely alleviates the need for stable transformation of the host species was reported by Budhagatapalli *et al.* (2020), who pollinated wheat plants with pollen from Cas9-gRNA-transgenic maize plants, resulting in haploid wheat plants that could be subjected to whole-genome duplication to produce a homozygous mutant and transgene-free wheat. This method was demonstrated using a panel of five diverse genotypes including durum wheat (*Triticum durum*) and common wheat.

Targeted genome modifications may not require the introduction of a transgene at all, relying instead on preassembled Cas9 or Cas12a nuclease protein-gRNA ribonucleoprotein complexes (Kim *et al.* 2017, Toda *et al.* 2019, Woo *et al.* 2015). Toda *et al.* (2019) introduced Cas9-gRNA ribonucleoproteins into totipotent rice zygotes to generate plants carrying mutant alleles at *GRAIN WIDTH 7* (*GW7*), *DROOPING LEAF* (*DL*), and *PSEUDO-RESPONSE REGULATOR 37* (*PRR37*). Likewise, Zhang *et al.* (2016) introduced *in vitro*-transcribed transcripts for *Cas9* and the gRNA into common wheat by accelerated microparticles and obtained primary mutants. Subsequently, Liang *et al.* (2017) successfully performed targeted genome modification of *GW2* in common wheat using Cas9-gRNA ribonucleoproteins.

Targeted genome modification for agronomic trait improvement in rice

Rice is one of the most important cereal crops worldwide, particularly in Asian countries, where it is consumed as a staple food. The rice genome is smaller than those of other cereal crops and was the first cereal genome to be sequenced. Rice plants are also easier to transform than other members of the Poaceae family, explaining why it has long been used as a model system for monocot plants. In recent years, rice has also been used as an experimental platform to test and implement novel targeted genome modification tools. Such tools have been used to modify genes related to high yield and production stability in ways that would not have been possible via conventional breeding. In this section, we describe how genome modification tools have been used to modify genes of high agronomic importance in rice.

Table 1 lists several examples of targeted genome modification for agronomically important traits in cereal crops. In two pioneering studies, Li *et al.* (2012) and Zhou *et al.* (2015) used TALENs and Cas9 endonuclease to modify the promoter regions of the bacterial leaf blight susceptibility genes *SWEET14* and *SWEET13*, respectively. The resulting mutant plants showed increased resistance to *Xanthomonas oryzae* pv. *oryzae*, the pathogen that causes bacterial leaf blight in rice. Following these reports, Oliva *et al.* (2019), Xu *et al.* (2019), and Blanvillain-Baufumé *et al.* (2017) also created mutants that are highly resistant to the same

Table 1. Site-directed modification of agronomically relevant genes in cereal crops

Plant	Modified gene	Purpose	Nuclease	DNA repair by	Reference
rice	*ACC*	Herbicide resistance	Cas9 nuclease	NHEJ	Liu *et al.* (2020)
	ALS	Herbicide resistance	TALENs, Cas9 nuclease	HDR/TSI	Li *et al.* (2016c), Sun *et al.* (2016)
	BADH2	Fragrant rice	TALENs, Cas9 nuclease	NHEJ	Shan *et al.* (2015), Ashokkumar *et al.* (2020)
	CRTI and *PSY*	Carotenoid accumulation	Cas9 nuclease	HDR/TSI	Dong *et al.* (2020)
	CSA	Male sterility	Cas9 nuclease	NHEJ	Li *et al.* (2016b)
	DEP1	Grain yield	Cas9 nuclease	NHEJ	Huang *et al.* (2018)
	ELF4G	Virus resistance	Cas9 nuclease	NHEJ	Macovei *et al.* (2018)
	EPSPS	Herbicide resistance	Cas9 nuclease	NHEJ/TSI	Li *et al.* (2016a)
	ERF922	Blast disease resistance	Cas9 nuclease	NHEJ	Wang *et al.* (2016)
	GA20OX2	Plant height	Cas9 nuclease	NHEJ	Nawaz *et al.* (2020b)
	GN1a	Grain yield	Cas9 nuclease	NHEJ	Huang *et al.* (2018)
	GS3	Grain yield	Cas9 nuclease	NHEJ	Zeng *et al.* (2020b)
	GW2	Grain yield	Cas9 nuclease	NHEJ	Xu *et al.* (2016)
	GW5	Grain yield	Cas9 nuclease	NHEJ	Xu *et al.* (2016)
	LCT1	Cadmium accumulation	Cas9 nuclease	NHEJ	Liu *et al.* (2019)
	LOX3	Storage tolerance	TALENs	NHEJ	Ma *et al.* (2015)
	MTL (*PLA1*)	Haploid production	Cas9 nuclease	NHEJ	Yao *et al.* (2018)
	MYB30	Cold tolerance	Cas9 nuclease	NHEJ	Zeng *et al.* (2020b)
	NRAMP5	Cadmium accumulation	Cas9 nuclease	NHEJ	Tang *et al.* (2017a), Liu *et al.* (2019), Yang *et al.* (2019)
	PI21	Blast disease resistance	Cas9 nuclease	NHEJ	Nawaz *et al.* (2020a)
	PIN5b	Grain yield	Cas9 nuclease	NHEJ	Zeng *et al.* (2020b)
	RC	Proanthocyanidin and anthocyanin accumulation	Cas9 nuclease	NHEJ	Zhu *et al.* (2019)
	RR22	Salinity tolerance	Cas9 nuclease	NHEJ	Zhang *et al.* (2019)
	SBEIIb	Starch composition	Cas9 nuclease	NHEJ	Sun *et al.* (2017)
	SD1	Plant height	Cas9 nuclease	NHEJ	Hu *et al.* (2019), Biswas *et al.* (2020)
	SE5	Plant height	Cas9 nuclease	NHEJ	Hu *et al.* (2019)
	SWEET11	Bacterial leaf blight resistance	Cas9 nuclease	NHEJ	Oliva *et al.* (2019), Xu *et al.* (2019)
	SWEET13	Bacterial leaf blight resistance	Cas9 nuclease	NHEJ	Zhou *et al.* (2015), Oliva *et al.* (2019), Xu *et al.* (2019)
	SWEET14	Bacterial leaf blight resistance	TALENs, Cas9 nuclease	NHEJ	Li *et al.* (2012), Blanvillain-Baufumé *et al.* (2017), Oliva *et al.* (2019), Xu *et al.* (2019)
	TGW6	Grain size	Cas9 nuclease	NHEJ	Xu *et al.* (2016), Han *et al.* (2018)
	TMS5	Male sterility	Cas9 nuclease	NHEJ	Zhou *et al.* (2016)
	VP1	Germination speed	Cas9 nuclease	NHEJ	Jung *et al.* (2019)
	WAXY	Starch composition	Cas9 nuclease	NHEJ	Han *et al.* (2018), Zhang *et al.* (2018a), Huang *et al.* (2020), Zeng *et al.* (2020a)
	XA13	Bacterial blight resistance	Cas9 nuclease	NHEJ	Kim *et al.* (2019), Li *et al.* (2020a)
wheat	*α-GLIADINS*	Low gluten	Cas9 nuclease	NHEJ	Sánchez-León *et al.* (2018)
	CM3	Low α-amylase/trypsin inhibitor (allergenic proteins)	Cas9 nuclease	NHEJ	Camerlengo *et al.* (2020)
	CM16	Low α-amylase/trypsin inhibitor (allergenic proteins)	Cas9 nuclease	NHEJ	Camerlengo *et al.* (2020)
	EDR1	Powdery mildew disease resistance	Cas9 nuclease	NHEJ	Zhang *et al.* (2017)
	GW2	Grain size	Cas9 nuclease	NHEJ	Zhang *et al.* (2018b), Wang *et al.* (2018a, 2018b)
	HRC	Fusarium head blight disease resistance	Cas9 nuclease	NHEJ	Su *et al.* (2019)
	MLO	Powdery mildew disease resistance	TALENs	NHEJ	Wang *et al.* (2014)
	MS1	Male sterility	Cas9 nuclease	NHEJ	Okada *et al.* (2019)
	MS45	Male sterility	Cas9 nuclease	NHEJ	Singh *et al.* (2018)
	NP1	Male sterility	Cas9 nuclease	NHEJ	Li *et al.* (2020b)
	NFXL1	Fusarium head blight disease resistance	Cas9 nuclease	NHEJ	Brauer *et al.* (2020)
	QSD1	Grain dormancy	Cas9 nuclease	NHEJ	Abe *et al.* (2019), Liu *et al.* (2021)
	SBEIIb	Starch composition	Cas9 nuclease	NHEJ	Li *et al.* (2021)
	SD1	Plant height	Cas9 nuclease	NHEJ	Budhagatapalli *et al.* (2020)
barley	*β-1,3-GLUCANASE*	Increased callose formation	Cas9 nuclease	NHEJ	Kim *et al.* (2020)
	MORC	Fungal resistance	Cas9 nuclease	NHEJ	Kumar *et al.* (2018)
	NUD	Non-adherent hull	Cas9 nuclease	NHEJ	Gasparis *et al.* (2018), Gerasimova *et al.* (2020)
maize	*AAD1*	Herbicide resistance	ZFNs	HDR/TSI	Ainley *et al.* (2013)
	ARGOS8	Drought stress tolerance	Cas9 nuclease	HDR/TSI	Shi *et al.* (2017)
	GA20OX3	Plant height	Cas9 nuclease	NHEJ	Zhang *et al.* (2020)
	IPK1	Phytate accumulation in grain	ZFNs	NHEJ	Shukla *et al.* (2009)
	LOX3	Resistance to fungal infection	Cas9 nuclease	NHEJ	Pathi *et al.* (2020)
	MS45	Male fertility	Cas9 nuclease	NHEJ	Svitashev *et al.* (2016)
	MTL (*PLA1*)	Haploid production	Cas9 nuclease	NHEJ	Kelliher *et al.* (2017)
	PAT	Herbicide resistance	ZFNs	HDR/TSI	Shukla *et al.* (2009), Ainley *et al.* (2013)

NHEJ; non-homologous end-joining, HDR; homology-directed repair, TSI; targeted sequence insertion.

pathogen by targeting the promoter regions of *SWEET11*, in addition to *SWEET13* and *SWEET14*. In another study, Wang *et al.* (2016) used Cas9 nuclease to modify *ETHYLENE RESPONSIVE TRANSCRIPTION FACTOR 922* (*OsERF922*), thereby increasing resistance to rice blast caused by *Magnaporthe oryzae*, one of the most destructive plant diseases worldwide. The authors further showed that the induced mutations were inherited by the progeny. Compared to wild-type plants, mutant lines exhibited much smaller blast lesions at both the seedling and tillering stages.

Nawaz *et al.* (2020a) also successfully improved resistance to rice blast by modifying another gene, *PYRICULARIA ORYZAE RESISTANCE 21* (*PI21*). Other examples of mutants generated via site-directed genome modification for higher tolerance to biotic stress were reported by Kim *et al.* (2019) and Li *et al.* (2020a) for bacterial blight resistance and by Macovei *et al.* (2018) for virus resistance.

In concerted efforts to improve grain yield, quality, and composition in rice, Tang *et al.* (2017a), Liu *et al.* (2019), and Yang *et al.* (2019) modified *NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 5* (*NRAMP5*) and *LOW-AFFINITY CATION TRANSPORTER 1* (*LCT1*) using Cas9 nuclease to reduce cadmium accumulation, which causes Itai-itai disease. The resulting mutant grains accumulated less cadmium compared to the wild-type, thereby contributing to a safer food supply. Sun *et al.* (2017) modified *STARCH BRANCHING ENZYME IIb* (*SBEIIb*) using Cas9, which produced genome-edited plants whose grains had high resistant starch content. Likewise, Han *et al.* (2018), Zhang *et al.* (2018a), Huang *et al.* (2020), and Zeng *et al.* (2020a) targeted the *WAXY* gene, resulting in edited grains with modified starch composition and altered amylose/amylopectin contents. Shan *et al.* (2015) and Ashokkumar *et al.* (2020) used TALENs and Cas9 nuclease, respectively, to generate fragrant mutants producing 2-acetyl-1-pyrroline, the aroma compound that gives jasmine and basmati rice their flavor, via site-directed mutagenesis of *BETAINE ALDEHYDE DEHYDROGENASE 2* (*BADH2*). Xu *et al.* (2016) obtained mutants with larger grains by simultaneously targeting *GW2*, *GW5*, and *THOUSAND-GRAIN WEIGHT 6* (*TGW6*) by Cas9 nuclease-mediated multiplex targeted mutagenesis. Ma *et al.* (2015) improved seed longevity via TALEN-mediated inactivation of *LIPOXYGENASE 3* (*LOX3*). Zhang *et al.* (2019) and Zeng *et al.* (2020b) focused on improving tolerance to high-salinity and low-temperature stress, which are of particular relevance in rice, and obtained tolerant mutants by Cas9-mediated genome modification of *RESPONSE REGULATOR 22* (*RR22*) and *MYB30*, respectively.

The precise modification of *ACETOLACTATE SYNTHASE* (*ALS*) and *5-ENOL-PYRUVYL SHIKIMATE-3-PHOSPHATE SYNTHASE* (*EPSP*) using TALENs or Cas9 nuclease resulted in herbicide-resistant rice plants (Li *et al.* 2016a, 2016c, Sun *et al.* 2016). In another Cas9-based approach, Dong *et al.* (2020) inserted an expression cassette of two carotenoid biosynthetic genes, *CAROTENE DESATURASE I* (*CTRI*) and *PHYTOENE SYNTHASE* (*PSY*), at a predefined target site without the use of a selectable marker. The resulting plants produced carotenoid-enriched rice that accumulates β-carotene in the grains, which may help overcome vitamin A deficiency in the developing world.

Studies involving targeted genome modification have also contributed to a revolution in crop breeding by focusing on genes that do not directly affect agronomic traits. For instance, Zhou *et al.* (2016) targeted *THERMOSENSITIVE 5* (*TMS5*), a gene involved in thermosensitive genic male sterility, by Cas9 nuclease-mediated mutagenesis and successfully produced male-sterile mutants. Li *et al.* (2016b) also modified *CARBON STARVED ANTHER* (*CSA*), which is involved in photosensitive genic male sterility. These studies have accelerated the expansion of genetic resources for male sterility and the development of a hybrid breeding system in rice. To produce haploid rice, Yao *et al.* (2018) performed Cas9-mediated modification of the rice ortholog of maize *PHOSPHOLIPASE A1* (*PLA1*, also called *MATRILINEAL* [*MTL*] and *NOT LIKE DAD* [*NLD*]), which encodes a pollen-specific phospholipase whose inactivation stimulates the development of the unfertilized egg into a haploid embryo. The authors succeeded in producing haploid mutants, which represents another essential tool for modern plant breeding.

Targeted genome modification for agronomic trait improvements in wheat

Wheat is a major staple food crop in nearly every region of the world. Durum wheat (*Triticum durum*) and common wheat (*T. aestivum*) are allopolyploid crops with two and three subgenomes (AB and ABD), respectively. Since these wheats have multiple copies of each gene, conventional mutagenesis affecting any one gene in one subgenome may not result in a phenotypic change due to functional redundancy with the remaining homoeologs from the other subgenome(s). Thus, the most promising method for targeted genome modification of durum and common wheat may be the introduction of mutations in all homoeologs simultaneously or successively. In temperate areas with a rainy season around wheat harvest time, such as Japan, pre-harvest sprouting and *Fusarium* head blight disease are major problems. However, genetic resources for tolerance or resistance are limited, making it imperative to develop and deploy breeding techniques that allow for the generation of novel useful genetic variation. In this section, we review agronomically relevant examples of targeted genome modification in wheat.

In common wheat, the cultivars ‘Bobwhite’ and ‘Fielder’ have mainly been used for targeted mutagenesis due to their outstanding transformation efficiency. However, the method of Budhagatapalli *et al.* (2020) holds great promise to expand the scope of wheat genome editing to other more recalcitrant cultivars.

In an early study of targeted mutagenesis in common wheat, Wang *et al.* (2014) successfully addressed all three homoeoalleles of *POWDERY-MILDEW-RESISTANCE LOCUS O* (*MLO*), a susceptibility factor for powdery mildew disease. Using TALENs, the authors generated single, double, and triple homozygous mutants. Only the triple mutant (*aabbdd*) displayed resistance to infection by *Blumeria graminis* f. sp. *tritici*, whereas all single and double knockout lines remained susceptible. This study demonstrates the power of customizable endonucleases

when knockouts in all homoeoalleles of a given target gene must be generated in polyploid species. Zhang *et al.* (2017) focused on the modification of *ENHANCED DISEASE RESISTANCE 1* (*EDR1*) using Cas9 nuclease to increase resistance to powdery mildew in triple homozygous wheat mutants as well. Moreover, Zhang *et al.* (2018b) and Wang *et al.* (2018a, 2018b) performed targeted genome modification of wheat *GW2*, whose rice ortholog is negatively associated with grain width and thousand-grain weight. In these studies, the wheat cultivars 'Kenong 199' and 'Bobwhite' were used as starting germplasm, and the inactivation of genes from the B and D subgenomes had more significant phenotypic effects on grain size than their respective single knockout mutants. Sánchez-León *et al.* (2018) and Camerlengo *et al.* (2020) produced common wheat and durum wheat with grains that accumulated substantially reduced levels of allergenic constituents by Cas9 endonuclease-mediated targeting of the gene families encoding α-GLIADINs and subunits of α-AMYLASE/TRYPSIN INHIBITOR (CHLOROFORM/METHANOL 3 [CM3] and CM16), respectively. The immunoreactivity of the resulting wheat mutants was significantly reduced, as demonstrated by enzyme-linked immunosorbent assay (ELISA) in both studies. These approaches may therefore greatly contribute to the mitigation of celiac disease in the human population. Abe *et al.* (2019) succeeded in the site-directed mutagenesis of the wheat gene *QTL FOR SEED DORMANCY 1* (*QSD1*), affecting grain dormancy and resistance to pre-harvest sprouting. The triple mutant (*aabbdd*) germinated much later than the wild-type counterpart and thus exhibited higher dormancy levels, whereas single and double mutants germinated at similar times to the wild-type. Not all successful attempts at targeted genome modification require the inactivation of all homoeologs, however. Su *et al.* (2019) identified *HISTIDINE-RICH CALCIUM-BINDING PROTEIN* (*TaHRC*) as a quantitative trait locus (QTL) responsible for resistance to *Fusarium* head blight. The cloning of the causal gene identified a single deletion in the B subgenome copy that was sufficient to cause resistance to *Fusarium* head blight. This result was validated by independent site-directed mutagenesis of the *TaHRC* homoeolog from the B subgenome. In an alternative approach using the cultivar 'Fielder', Brauer *et al.* (2020) generated knockout mutants of *NUCLEAR TRANSCRIPTION FACTOR, X-BOX BINDING 1-LIKE* (*NFXL1*) and obtained resistance to *Fusarium* head blight. Okada *et al.* (2019), Singh *et al.* (2018) and Li *et al.* (2020b) generated male-sterile wheat mutants by targeting *MALE STERILITY 1* (*MS1*), *MS45*, and *NO POLLEN 1* (*NP1*). These studies lead to the production of male-sterile mutants, which are needed for a hybrid seed production system in wheat.

Budhagatapalli *et al.* (2020) generated doubled haploid wheat with mutations targeting *SEMI-DWARF 1* (*SD1*). Such mutations were obtained in various genotypes, including common and durum wheat, using Cas9/gRNA-transgenic maize as the pollen donor to induce the formation of haploids. Mutant progenies had reduced plant height compared to the wild-type.

Targeted genome modification for agronomic trait improvements in barley

Barley is a self-pollinating diploid crop that has long been used as a research model representative of the Triticeae species. Since barley is used as a food for animals and humans and for malting, breeding efforts have focused on yield- and quality-related traits, including resistance to fungal and viral pathogens, tolerance to preharvest sprouting, starch grain content, beta-glucan content, protein content, and brewing suitability. Like wheat, only a limited number of barley genotypes are amenable to efficient genetic transformation, with the cultivars 'Golden Promise' and 'Igri' being particularly suitable (Hoffie *et al.* 2021, Kumlehn *et al.* 2006).

Targeted genome modification via Cas9 endonuclease to target genes of interest for basic research and agronomically relevant traits was recently reported in barley. For example, Kumar *et al.* (2018) explored the function of the *MICRORCHIDIA PROTEIN* (*MORC*) gene in barley and demonstrated its effect on enhancing resistance to viral diseases. Gasparis *et al.* (2018) and Gerasimova *et al.* (2020) used the Cas9 endonuclease to perform site-directed mutagenesis of the *ERF* family gene known as naked caryopsis gene, *NUDUM* (*NUD*), which is responsible for the particularly strong adherence of hulls to the pericarp of barley grains. Knockout of this gene resulted in the conversion of hulled barley into barley with naked grains, the form used for human consumption.

Targeted genome modification for agronomic trait improvements in maize

Maize, the cereal crop with the greatest production worldwide, is primarily grown in North and South America and is commonly used for livestock feed and as an ingredient in processed foods such as oil and cornstarch. Genetically engineered maize, represented by herbicide-resistant and *Bt* (*Bacillus thuringiensis*) corn, has long been cultivated in several countries. It is hoped that new varieties exhibiting both herbicide resistance and a waxy grain phenotype generated by targeted genome modification will be released in the near future (Waltz 2016). However, like wheat and barley, only a few maize germplasms are suitable for genetic transformation. In particular, high type II (Hi-II) hybrids have mainly been used for genetic engineering in the past due to their very high transformation efficiency.

As mentioned above, Shukla *et al.* (2009) succeeded in performing site-directed mutagenesis of the *IPK1* gene as well as site-specific DNA insertion into the *PAT* gene in maize using ZFNs. A few years later, Ainley *et al.* (2013) performed homology-mediated DNA insertion of two herbicide-

resistance genes in maize encoding ARYLOXYALKANOATE DIOXYGENASE 1 (AAD1) and ALS using ZFNs and Cas9 endonuclease, respectively. Shi *et al.* (2017) improved grain yield in maize plants exposed to drought stress via Cas9-mediated insertion of the *GOS2* promoter onto the upstream region of *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE 8* (*ARGOS8*), thereby upregulating its expression. Pathi *et al.* (2020) performed site-directed genome modification using the Cas9 nuclease at *LOX3*, a susceptibility factor for smut disease in maize, resulting in moderately resistant plants to *Ustilago maydis* infection.

To develop haploid technology in maize, Kelliher *et al.* (2017) used Cas9 nuclease to introduce knockout mutations at *PLA1*/*MTL*/*NLD*, which encodes a pollen-specific phospholipase required for regular fertilization. These mutants generated haploid plants at efficiencies suitable for the development of maize inbred lines. Using preassembled Cas9/gRNA ribonucleoproteins for site-directed mutagenesis, Svitashev *et al.* (2016) successfully generated male-sterile alleles at *MS2* and *MS45*, holding great promise for the development of a reliable method for hybrid breeding.

Future prospects of targeted genome modification in cereal breeding

Crop breeding has traditionally involved the identification of desirable genetic variants, followed by their incorporation into other germplasm by repeated crosses. To increase the frequency of genetic diversity, plant materials have been exposed to mutagenic chemicals or ionizing radiation. Using these methods, mutations are randomly introduced into the genome. Consequently, very large plant populations had to be phenotyped, processed, and analyzed to identify and validate mutations with potential agronomic value. The major advantages of targeted genome modification are that 1) virtually any target gene may be modified and 2) downstream analyses can be substantially accelerated. Another feature of targeted genome modification using customizable endonucleases is that multiple target genes of interest can be modified simultaneously, which is particularly important for polyploid species or to improve traits that require complex genetic modifications.

However, to fully realize the potential of targeted genome modification, elite germplasms must be amenable to modification, as backcrossing procedures unnecessarily prolong the production of breeding lines until a suitable genetic background is ready for approval and release. Various attempts have been made to overcome the challenge of genotype dependence for genetic engineering. For example, Yeo *et al.* (2014), Hisano and Sato (2016), and Hisano *et al.* (2017) attempted to identify genes involved in the transformation amenability of barley. Once these genes are identified, any elite background may become transformable. Using another approach to reduce genotype dependence, Hamada *et al.* (2017, 2018) and Liu *et al.* (2021) developed a new method of *in planta* transformation and targeted genome modification in wheat including commercial varieties, which relies on ballistic DNA transfer to the shoot apex of mature embryos and may allow for the genetic engineering of a wide range of germplasm.

In another approach, Kelliher *et al.* (2019) and Budhagatapalli *et al.* (2020) used Cas9/gRNA-transgenic haploidy-inducing lines as pollen donors to induce targeted mutagenesis during early embryogenesis in maize and wheat, respectively. In the case of wheat, this method was effective in accessions typically recalcitrant to transformation. A similar technique for barley using pollen of wild barley (*Hordeum bulbosum*), the so-called *bulbosum* method may be employed to create doubled haploid barley carrying targeted genome modifications using any desired elite background (Satpathy *et al.* 2021).

Conclusion

Mutagenesis has advanced from the era of large-scale screening of random mutant populations to the production of desirable mutants by precisely targeting known genomic sequences. The use of customizable endonucleases has accelerated the generation of knock-in/out gene variants and thus is poised to revolutionize the breeding of field crops. In this review, we provided comprehensive information on generating targeted genome modifications with agronomic relevance for cereal crops. This technology has been steadily progressing and will increasingly contribute to crop breeding in the future. It remains necessary, however, to further increase the efficiency and precision of genome modifications to help usher in the new age of customizable crops.

Author Contribution Statement

HH and JK wrote the draft of the manuscript. All authors revised the manuscript and approved of its submission to Breeding Science.

Acknowledgments

HH and JK were supported by the Joint Usage/Research Center, Institute of Plant Science and Resources, Okayama University (FY2017). HH and FA were supported by the Cabinet Office, Government of Japan, Cross-ministerial Strategic Innovation Promotion Program (SIP), “Technologies for Creating Next-generation Agriculture, Forestry and Fisheries” (funding agency: Bio-oriented Technology Research Advancement Institution, NARO). RH is grateful to the German Ministry of Research and Education (BMBF) for financial support in the context of the IDEMODERESBAR project (FKZ 031B0199C and 031B0887C). HH is supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (B) (Grant Number JP19H02930).

Literature Cited

Abe, A., S. Kosugi, K. Yoshida, S. Natsume, H. Takagi, H. Kanzaki, H. Matsumura, K. Yoshida, C. Mitsuoka, M. Tamiru *et al.* (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat. Biotechnol. 30: 174–178.

Abe, F., E. Haque, H. Hisano, T. Tanaka, Y. Kamiya, M. Mikami, K. Kawaura, M. Endo, K. Onishi, T. Hayashi *et al.* (2019) Genome-edited triple-recessive mutation alters seed dormancy in wheat. Cell Rep. 28: 1362–1369.e4.

Ahloowalia, B.S. and M. Maluszynski (2001) Induced mutations—A new paradigm in plant breeding. Euphytica 118: 167–173.

Ahloowalia, B.S., M. Maluszynski and K. Nichterlein (2004) Global impact of mutation-derived varieties. Euphytica 135: 187–204.

Ainley, W.M., L. Sastry-Dent, M.E. Welter, M.G. Murray, B. Zeitler, R. Amora, D.R. Corbin, R.R. Miles, N.L. Arnold, T.L. Strange *et al.* (2013) Trait stacking via targeted genome editing. Plant Biotechnol. J. 11: 1126–1134.

Anzalone, A.V., P.B. Randolph, J.R. Davis, A.A. Sousa, L.W. Koblan, J.M. Levy, P.J. Chen, C. Wilson, G.A. Newby, A. Raguram *et al.* (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576: 149–157.

Arora, S., B. Steuernagel, K. Gaurav, S. Chandramohan, Y. Long, O. Matny, R. Johnson, J. Enk, S. Periyannan, N. Singh *et al.* (2019) Resistance gene cloning from a wild crop relative by sequence capture and association genetics. Nat. Biotechnol. 37: 139–143.

Ashokkumar, S., D. Jaganathan, V. Ramanathan, H. Rahman, R. Palaniswamy, R. Kambale and R. Muthurajan (2020) Creation of novel alleles of fragrance gene *OsBADH2* in rice through CRISPR/Cas9 mediated gene editing. PLoS ONE 15: e0237018.

Bayer, P.E., A.A. Golicz, A. Scheben, J. Batley and D. Edwards (2020) Plant pan-genomes are the new reference. Nat. Plants 6: 914–920.

Begemann, M.B., B.N. Gray, E. January, G.C. Gordon, Y. He, H. Liu, X. Wu, T.P. Brutnell, T.C. Mockler and M. Oufattole (2017) Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases. Sci. Rep. 7: 11606.

Bettgenhaeuser, J. and S.G. Krattinger (2019) Rapid gene cloning in cereals. Theor. Appl. Genet. 132: 699–711.

Biswas, S., J. Tian, R. Li, X. Chen, Z. Luo, M. Chen, X. Zhao, D. Zhang, S. Persson, Z. Yuan *et al.* (2020) Investigation of CRISPR/Cas9-induced *SD1* rice mutants highlights the importance of molecular characterization in plant molecular breeding. J. Genet. Genomics 47: 273–280.

Blanvillain-Baufumé, S., M. Reschke, M. Solé, F. Auguy, H. Doucoure, B. Szurek, D. Meynard, M. Portefaix, S. Cunnac, E. Guiderdoni *et al.* (2017) Targeted promoter editing for rice resistance to *Xanthomonas oryzae* pv. *oryzae* reveals differential activities for *SWEET14*-inducing TAL effectors. Plant Biotechnol. J. 15: 306–317.

Brauer, E.K., M. Balcerzak, H. Rocheleau, W. Leung, J. Schernthaner, R. Subramaniam and T. Ouellet (2020) Genome editing of a deoxynivalenol-induced transcription factor confers resistance to *Fusarium graminearum* in wheat. Mol. Plant Microbe Interact. 33: 553–560.

Budhagatapalli, N., T. Rutten, M. Gurushidze, J. Kumlehn and G. Hensel (2015) Targeted modification of gene function exploiting homology-directed repair of TALEN-mediated double-strand breaks in barley. G3 (Bethesda) 5: 1857–1863.

Budhagatapalli, N., S. Schedel, M. Gurushidze, S. Pencs, S. Hiekel, T. Rutten, S. Kusch, R. Morbitzer, T. Lahaye, R. Panstruga *et al.* (2016) A simple test for the cleavage activity of customized endonucleases in plants. Plant Methods 12: 18.

Budhagatapalli, N., T. Halbach, S. Hiekel, H. Büchner, A.E. Müller and J. Kumlehn (2020) Site-directed mutagenesis in bread and durum wheat via pollination by *cas9*/guide RNA-transgenic maize used as haploidy inducer. Plant Biotechnol. J. 18: 2376–2378.

Camerlengo, F., A. Frittelli, C. Sparks, A. Doherty, D. Martignago, C. Larré, R. Lupi, F. Sestili and S. Masci (2020) CRISPR-Cas9 multiplex editing of the α-amylase/trypsin inhibitor genes to reduce allergen proteins in durum wheat. Front. Sustain. Food Syst. 4: 104.

D'Halluin, K., C. Vanderstraeten, E. Stals, M. Cornelissen and R. Ruiter (2008) Homologous recombination: a basis for targeted genome optimization in crop species such as maize. Plant Biotechnol. J. 6: 93–102.

Dong, O.X., S. Yu, R. Jain, N. Zhang, P.Q. Duong, C. Butler, Y. Li, A. Lipzen, J.A. Martin, K.W. Barry *et al.* (2020) Marker-free carotenoid-enriched rice generated through targeted gene insertion using CRISPR-Cas9. Nat. Commun. 11: 1178.

Endo, A., M. Mikami, H. Kaya and S. Toki (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. Sci. Rep. 6: 38169.

FAO, IFAD, UNICEF, WFP and WHO (2018) The state of food security and nutrition in the world. Building climate resilience for food security and nutrition. Rome, FAO.

Feng, Z., B. Zhang, W. Ding, X. Liu, D.-L. Yang, P. Wei, F. Cao, S. Zhu, F. Zhang, Y. Mao *et al.* (2013) Efficient genome editing in plants using a CRISPR/Cas system. Cell Res. 23: 1229–1232.

Gasparis, S., M. Kała, M. Przyborowski, L.A. Łyżnik, W. Orczyk and A. Nadolska-Orczyk (2018) A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heritable mutations in barley (*Hordeum vulgare* L.). Plant Methods 14: 111.

Gerasimova, S.V., C. Hertig, A.M. Korotkova, E.V. Kolosovskaya, I. Otto, S. Hiekel, A.V. Kochetov, E.K. Khlestkina and J. Kumlehn (2020) Conversion of hulled into naked barley by Cas endonuclease-mediated knockout of the *NUD* gene. BMC Plant Biol. 20: 255.

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

Gurushidze, M., G. Hensel, S. Hiekel, S. Schedel, V. Valkov and J. Kumlehn (2014) True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. PLoS ONE 9: e92046.

Hamada, H., Q. Linghu, Y. Nagira, R. Miki, N. Taoka and R. Imai (2017) An *in planta* biolistic method for stable wheat transformation. Sci. Rep. 7: 11443.

Hamada, H., Y. Liu, Y. Nagira, R. Miki, N. Taoka and R. Imai (2018) Biolistic-delivery-based transient CRISPR/Cas9 expression enables *in planta* genome editing in wheat. Sci. Rep. 8: 14422.

Han, Y., D. Luo, B. Usman, G. Nawaz, N. Zhao, F. Liu and R. Li (2018) Development of high yielding glutinous cytoplasmic male sterile rice (*Oryza sativa* L.) lines through CRISPR/Cas9 based mutagenesis of *Wx* and *TGW6* and proteomic analysis of anther. Agronomy 8: 290.

Hensel, G. and J. Kumlehn (2019) Genome engineering using TALENs. *In*: Harwood, W.A. (ed.) Barley—Methods and Protocols. Methods in Molecular Biology, Springer Nature: Berlin Heidelberg, Chapter 13, pp. 195–215.

Hisano, H. and K. Sato (2016) Genomic regions responsible for amenability to *Agrobacterium*-mediated transformation in barley. Sci. Rep. 6: 37505.

Hisano, H., B. Meints, M.J. Moscou, L. Cistue, B. Echávarri, K. Sato and P.M. Hayes (2017) Selection of transformation-efficient barley genotypes based on *TFA* (*transformation amenability*) haplotype and higher resolution mapping of the *TFA* loci. Plant Cell Rep. 36: 611–620.

Hoffie, R.E., I. Otto, H. Hisano and J. Kumlehn (2021) Site-directed mutagenesis in barley using RNA-guided Cas endonucleases during microspore-derived generation of doubled haploids. *In*: Segui-Simarro, J.M. (ed.) Doubled Haploid Technology, Methods in Molecular Biology, Vol. 2287, Chapter 9, Springer, pp. 199–214.

Hu, X., Y. Cui, G. Dong, A. Feng, D. Wang, C. Zhao, Y. Zhang, J. Hu, D. Zeng, L. Guo *et al.* (2019) Using CRISPR-Cas9 to generate semi-dwarf rice lines in elite landraces. Sci. Rep. 9: 19096.

Hua, K., Y. Jiang, X. Tao and J.-K. Zhu (2020) Precision genome engineering in rice using prime editing system. Plant Biotechnol. J. 18: 2167–2169.

Huang, L., R. Zhang, G. Huang, Y. Li, G. Melaku, S. Zhang, H. Chen, Y. Zhao, J. Zhang, Y. Zhang *et al.* (2018) Developing superior alleles of yield genes in rice by artificial mutagenesis using the CRISPR/Cas9 system. Crop J. 6: 475–481.

Huang, L., Q. Li, C. Zhang, R. Chu, Z. Gu, H. Tan, D. Zhao, X. Fan and Q. Liu (2020) Creating novel *Wx* alleles with fine-tuned amylose levels and improved grain quality in rice by promoter editing using CRISPR/Cas9 system. Plant Biotechnol. J. 18: 2164–2166.

Jung, Y.J., H.J. Lee, S. Bae, J.H. Kim, D.H. Kim, H.K. Kim, K.H. Nam, F.M. Nogoy, Y.-G. Cho and K.K. Kang (2019) Acquisition of seed dormancy breaking in rice (*Oryza sativa* L.) via CRISPR/Cas9-targeted mutagenesis of *OsVP1* gene. Plant Biotechnol. Rep. 13: 511–520.

Kelliher, T., D. Starr, L. Richbourg, S. Chintamanani, B. Delzer, M.L. Nuccio, J. Green, Z. Chen, J. McCuiston, W. Wang *et al.* (2017) MATRILINEAL, a sperm-specific phospholipase, triggers maize haploid induction. Nature 542: 105–109.

Kelliher, T., D. Starr, X. Su, G. Tang, Z. Chen, J. Carter, P.E. Wittich, S. Dong, J. Green, E. Burch *et al.* (2019) One-step genome editing of elite crop germplasm during haploid induction. Nat. Biotechnol. 37: 287–292.

Kim, H., S.-T. Kim, J. Ryu, B.-C. Kang, J.-S. Kim and S.-G. Kim (2017) CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat. Commun. 8: 14406.

Kim, S.-Y., T. Bengtsson, N. Olsson, V. Hot, L.-H. Zhu and I. Åhman (2020) Mutations in two aphid-regulated β-1,3-glucanase genes by CRISPR/Cas9 do not increase barley resistance to *Rhopalosiphum padi* L. Front. Plant Sci. 11: 1043.

Kim, Y.-A., H. Moon and C.-J. Park (2019) CRISPR/Cas9-targeted mutagenesis of *Os8N3* in rice to confer resistance to *Xanthomonas oryzae* pv. *oryzae*. Rice (N Y) 12: 67.

Koeppel, I., C. Hertig, R. Hoffie and J. Kumlehn (2019) Cas endonuclease technology—a quantum leap in the advancement of barley and wheat genetic engineering. Int. J. Mol. Sci. 20: 2647.

Kumar, N., M. Galli, J. Ordon, J. Stuttmann, K.-H. Kogel and J. Imani (2018) Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. Plant Biotechnol. J. 16: 1892–1903.

Kumlehn, J., L. Serazetdinova, G. Hensel, D. Becker and H. Lörz (2006) Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Plant Biotechnol. J. 4: 251–261.

Kumlehn, J. and G. Hensel (2009) Genetic transformation technology in the Triticeae. Breed. Sci. 59: 553–560.

Kumlehn, J., J. Pietralla, G. Hensel, M. Pacher and H. Puchta (2018) The CRISPR/Cas revolution continues: From efficient gene editing for crop breeding to plant synthetic biology. J. Integr. Plant Biol. 60: 1127–1153.

Kumlehn, J., S. Hiekel and N. Budhagatapalli (2019) Site-directed genome modification in barley and wheat. *In*: Ordon, F. and W. Friedt (eds.) Advances in breeding techniques for cereal crops, Burleigh Dodds Science Publishing, Cambridge, UK, Chapter 20, pp. 559–576.

Li, C., Y. Zong, Y. Wang, S. Jin, D. Zhang, Q. Song, R. Zhang and C. Gao (2018) Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. Genome Biol. 19: 59.

Li, C., W. Li, Z. Zhou, H. Chen, C. Xie and Y. Lin (2020a) A new rice breeding method: CRISPR/Cas9 system editing of the *Xa13* promoter to cultivate transgene-free bacterial blight-resistant rice. Plant Biotechnol. J. 18: 313–315.

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

Li, J., G. Jiao, Y. Sun, J. Chen, Y. Zhong, L. Yan, D. Jiang, Y. Ma and L. Xia (2021) Modification of starch composition, structure and properties through editing of *TaSBEIIa* in both winter and spring wheat varieties by CRISPR/Cas9. Plant Biotechnol. J. 19: 937–951.

Li, J., Z. Wang, G. He, L. Ma and X.W. Deng (2020b) CRISPR/Cas9-mediated disruption of *TaNP1* genes results in complete male sterility in bread wheat. J. Genet. Genomics 47: 263–272.

Li, Q., D. Zhang, M. Chen, W. Liang, J. Wei, Y. Qi and Z. Yuan (2016b) Development of *japonica* photo-sensitive genic male sterile rice lines by editing *carbon starved anther* using CRISPR/Cas9. J. Genet. Genomics 43: 415–419.

Li, T., B. Liu, M.H. Spalding, D.P. Weeks and B. Yang (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat. Biotechnol. 30: 390–392.

Li, T., B. Liu, C.Y. Chen and B. Yang (2016c) TALEN-mediated homologous recombination produces site-directed DNA base change and herbicide-resistant rice. J. Genet. Genomics 43: 297–305.

Liang, Z., K. Chen, T. Li, Y. Zhang, Y. Wang, Q. Zhao, J. Liu, H. Zhang, C. Liu, Y. Ran *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat. Commun. 8: 14261.

Lin, Q., Y. Zong, C. Xue, S. Wang, S. Jin, Z. Zhu, Y. Wang, A.V. Anzalone, A. Raguram, J.L. Doman *et al.* (2020) Prime genome editing in rice and wheat. Nat. Biotechnol. 38: 582–585.

Liu, S., J. Jiang, Y. Liu, J. Meng, S. Xu, Y. Tan, Y. Li, Q. Shu and J. Huang (2019) Characterization and evaluation of *OsLCT1* and *OsNramp5* mutants generated through CRISPR/Cas9-mediated mutagenesis for breeding low Cd rice. Rice Sci. 26: 88–97.

Liu, X., R. Qin, J. Li, S. Liao, T. Shan, R. Xu, D. Wu and P. Wei (2020) A CRISPR-Cas9-mediated domain-specific base-editing screen enables functional assessment of ACCase variants in rice. Plant Biotechnol. J. 18: 1845–1847.

Liu, Y., W. Luo, Q. Linghu, F. Abe, H. Hisano, K. Sato, Y. Kamiya, K. Kawaura, K. Onishi, M. Endo *et al.* (2021) *In planta* genome editing in commercial wheat varieties. Front. Plant Sci. 12: 648841.

Ma, L., F. Zhu, Z. Li, J. Zhang, X. Li, J. Dong and T. Wang (2015) TALEN-based mutagenesis of lipoxygenase LOX3 enhances the storage tolerance of rice (*Oryza sativa*) seeds. PLoS ONE 10:

e0143877.

Macovei, A., N.R. Sevilla, C. Cantos, G.B. Jonson, I. Slamet-Loedin, T. Čermák, D.F. Voytas, I.-R. Choi and P. Chadha-Mohanty (2018) Novel alleles of rice *eIF4G* generated by CRISPR/Cas9-targeted mutagenesis confer resistance to *Rice tungro spherical virus*. Plant Biotechnol. J. 16: 1918–1927.

McCallum, C.M., L. Comai, E.A. Greene and S. Henikoff (2000) Targeting induced local lesions in genomes (TILLING) for plant functional genomics. Plant Physiol. 123: 439–442.

Miao, J., D. Guo, J. Zhang, Q. Huang, G. Qin, X. Zhang, J. Wan, H. Gu and L.-J. Qu (2013) Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res. 23: 1233–1236.

Nawaz, G., B. Usman, H. Peng, N. Zhao, R. Yuan, Y. Liu and R. Li (2020a) Knockout of *Pi21* by CRISPR/Cas9 and iTRAQ-based proteomic analysis of mutants revealed new insights into *M. oryzae* resistance in elite rice line. Genes 11: 735.

Nawaz, G., B. Usman, N. Zhao, Y. Han, Z. Li, X. Wang, Y. Liu and R. Li (2020b) CRISPR/Cas9 directed mutagenesis of *OsGA20ox2* in high yielding basmati rice (*Oryza sativa* L.) line and comparative proteome profiling of unveiled changes triggered by mutations. Int. J. Mol. Sci. 21: 6170.

Okada, A., T. Arndell, N. Borisjuk, N. Sharma, N.S. Watson-Haigh, E.J. Tucker, U. Baumann, P. Langridge and R. Whitford (2019) CRISPR/Cas9-mediated knockout of *Ms1* enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. Plant Biotechnol. J. 17: 1905–1913.

Oliva, R., C. Ji, G. Atienza-Grande, J.C. Huguet-Tapia, A. Perez-Quintero, T. Li, J.-S. Eom, C. Li, H. Nguyen, B. Liu *et al.* (2019) Broad-spectrum resistance to bacterial blight in rice using genome editing. Nat. Biotechnol. 37: 1344–1350.

Pathi, K.M., P. Rink, N. Budhagatapalli, R. Betz, I. Saado, S. Hiekel, M. Becker, A. Djamei and J. Kumlehn (2020) Engineering smut resistance in maize by site-directed mutagenesis of *LIPOXYGENASE 3*. Front. Plant Sci. 11: 543895.

Rasheed, A., Y. Hao, X. Xia, A. Khan, Y. Xu, R.K. Varshney and Z. He (2017) Crop breeding chips and genotyping platforms: progress, challenges, and perspectives. Mol. Plant 10: 1047–1064.

Sánchez-León, S., J. Gil-Humanes, C.V. Ozuna, M.J. Giménez, C. Sousa, D.F. Voytas and F. Barro (2018) Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol. J. 16: 902–910.

Sánchez-Martín, J., B. Steuernagel, S. Ghosh, G. Herren, S. Hurni, N. Adamski, J. Vrána, M. Kubaláková, S.G. Krattinger, T. Wicker *et al.* (2016) Rapid gene isolation in barley and wheat by mutant chromosome sequencing. Genome Biol. 17: 221.

Satpathy, P., S. Audije de la Fuente, V. Ott, A. Müller, H. Büchner, D.S. Daghma and J. Kumlehn (2021) Generation of doubled-haploid barley by interspecific pollination with *Hordeum bulbosum*. *In*: Segui-Simarro, J.M. (ed.) Doubled Haploid Technology, Methods in Molecular Biology, Vol. 2287, Chapter 10, Springer, pp. 215–226.

Shan, Q., Y. Wang, J. Li, Y. Zhang, K. Chen, Z. Liang, K. Zhang, J. Liu, J.J. Xi, J.-L. Qiu *et al.* (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31: 686–688.

Shan, Q., Y. Zhang, K. Chen, K. Zhang and C. Gao (2015) Creation of fragrant rice by targeted knockout of the *OsBADH2* gene using TALEN technology. Plant Biotechnol. J. 13: 791–800.

Shi, J., H. Gao, H. Wang, H.R. Lafitte, R.L. Archibald, M. Yang, S.M. Hakimi, H. Mo and J.E. Habben (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol. J. 15: 207–216.

Shukla, V.K., Y. Doyon, J.C. Miller, R.C. DeKelver, E.A. Moehle, S.E. Worden, J.C. Mitchell, N.L. Arnold, S. Gopalan, X. Meng *et al.* (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature 459: 437–441.

Singh, M., M. Kumar, M.C. Albertsen, J.K. Young and A.M. Cigan (2018) Concurrent modifications in the three homeologs of *Ms45* gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). Plant Mol. Biol. 97: 371–383.

Steuernagel, B., S. Periyannan, I. Hernández-Pinzón, K. Witek, M.N. Rouse, G. Yu, A. Hatta, M. Ayliffe, H. Bariana, J.D.G. Jones *et al.* (2016) Rapid cloning of disease-resistance genes in plants using mutagenesis and sequence capture. Nat. Biotechnol. 34: 652–655.

Su, Z., A. Bernardo, B. Tian, H. Chen, S. Wang, H. Ma, S. Cai, D. Liu, D. Zhang, T. Li *et al.* (2019) A deletion mutation in *TaHRC* confers *Fhb1* resistance to Fusarium head blight in wheat. Nat. Genet. 51: 1099–1105.

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

Sun, Y., G. Jiao, Z. Liu, X. Zhang, J. Li, X. Guo, W. Du, J. Du, F. Francis, Y. Zhao *et al.* (2017) Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. Front. Plant Sci. 8: 298.

Svitashev, S., C. Schwartz, B. Lenderts, J.K. Young and A. Mark Cigan (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. Nat. Commun. 7: 13274.

Tang, L., B. Mao, Y. Li, Q. Lv, L. Zhang, C. Chen, H. He, W. Wang, X. Zeng, Y. Shao *et al.* (2017b) Knockout of *OsNramp5* using the CRISPR/Cas9 system produces low Cd-accumulating *indica* rice without compromising yield. Sci. Rep. 7: 14438.

Tang, X., L.G. Lowder, T. Zhang, A.A. Malzahn, X. Zheng, D.F. Voytas, Z. Zhong, Y. Chen, Q. Ren, Q. Li *et al.* (2017a) A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat. Plants 3: 17018.

Thind, A.K., T. Wicker, H. Šimková, D. Fossati, O. Moullet, C. Brabant, J. Vrána, J. Doležel and S.G. Krattinger (2017) Rapid cloning of genes in hexaploid wheat using cultivar-specific long-range chromosome assembly. Nat. Biotechnol. 35: 793–796.

Toda, E., N. Koiso, A. Takebayashi, M. Ichikawa, T. Kiba, K. Osakabe, Y. Osakabe, H. Sakakibara, N. Kato and T. Okamoto (2019) An efficient DNA- and selectable-marker-free genome-editing system using zygotes in rice. Nat. Plants 5: 363–368.

Waltz, E. (2016) CRISPR-edited crops free to enter market, skip regulation. Nat. Biotechnol. 34: 582.

Wang, F., C. Wang, P. Liu, C. Lei, W. Hao, Y. Gao, Y.-G. Liu and K. Zhao (2016) Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene *OsERF922*. PLoS ONE 11: e0154027.

Wang, W., Q. Pan, F. He, A. Akhunova, S. Chao, H. Trick and E. Akhunov (2018a) Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. CRISPR J. 1: 65–74.

Wang, W., J. Simmonds, Q. Pan, D. Davidson, F. He, A. Battal, A. Akhunova, H.N. Trick, C. Uauy and E. Akhunov (2018b) Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of *TaGW2* homoeologues to grain size and weight in wheat. Theor. Appl. Genet. 131: 2463–2475.

Wang, Y., X. Cheng, Q. Shan, Y. Zhang, J. Liu, C. Gao and J.-L. Qiu (2014) Simultaneous editing of three homoeoalleles in hexaploid

bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. 32: 947–951.

Watanabe, K., U. Breier, G. Hensel, J. Kumlehn, I. Schubert and B. Reiss (2015) Stable gene replacement in barley by targeted double-strand break induction. J. Exp. Bot. 67: 1433–1445.

Woo, J.W., J. Kim, S.I. Kwon, C. Corvalán, S.W. Cho, H. Kim, S.-G. Kim, S.-T. Kim, S. Choe and J.-S. Kim (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat. Biotechnol. 33: 1162–1164.

Xu, R., Y. Yang, R. Qin, H. Li, C. Qiu, L. Li, P. Wei and J. Yang (2016) Rapid improvement of grain weight *via* highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. J. Genet. Genomics 43: 529–532.

Xu, Z., X. Xu, Q. Gong, Z. Li, Y. Li, S. Wang, Y. Yang, W. Ma, L. Liu, B. Zhu *et al.* (2019) Engineering broad-spectrum bacterial blight resistance by simultaneously disrupting variable TALE-binding elements of multiple susceptibility genes in rice. Mol. Plant 12: 1434–1446.

Yang, C.-H., Y. Zhang and C.-F. Huang (2019) Reduction in cadmium accumulation in *japonica* rice grains by CRISPR/Cas9-mediated editing of *OsNRAMP5*. J. Integr. Agric. 18: 688–697.

Yao, L., Y. Zhang, C. Liu, Y. Liu, Y. Wang, D. Liang, J. Liu, G. Sahoo and T. Kelliher (2018) *OsMATL* mutation induces haploid seed formation in *indica* rice. Nat. Plants 4: 530–533.

Yeo, F.K.S., G. Hensel, T. Vozábová, A. Martin-Sanz, T.C. Marcel, J. Kumlehn and R.E. Niks (2014) Golden SusPtrit: a genetically well transformable barley line for studies on the resistance to rust fungi. Theor. Appl. Genet. 127: 325–337.

Yin, X., A.K. Biswal, J. Dionora, K.M. Perdigon, C.P. Balahadia, S. Mazumdar, C. Chater, H.-C. Lin, R.A. Coe, T. Kretzschmar *et al.* (2017) CRISPR-Cas9 and CRISPR-Cpf1 mediated targeting of a stomatal developmental gene *EPFL9* in rice. Plant Cell Rep. 36: 745–757.

Zeng, D., T. Liu, X. Ma, B. Wang, Z. Zheng, Y. Zhang, X. Xie, B. Yang, Z. Zhao, Q. Zhu *et al.* (2020a) Quantitative regulation of Waxy expression by CRISPR/Cas9-based promoter and 5′UTR-intron editing improves grain quality in rice. Plant Biotechnol. J. 18: 2385–2387.

Zeng, Y., J. Wen, W. Zhao, Q. Wang and W. Huang (2020b) Rational improvement of rice yield and cold tolerance by editing the three genes *OsPIN5b*, *GS3*, and *OsMYB30* with the CRISPR–Cas9 system. Front. Plant Sci. 10: 1663.

Zhang, A., Y. Liu, F. Wang, T. Li, Z. Chen, D. Kong, J. Bi, F. Zhang, X. Luo, J. Wang *et al.* (2019) Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the *OsRR22* gene. Mol. Breed. 39: 47.

Zhang, J., H. Zhang, J.R. Botella and J.-K. Zhu (2018a) Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the *Waxy* gene in elite rice varieties. J. Integr. Plant Biol. 60: 369–375.

Zhang, J., X. Zhang, R. Chen, L. Yang, K. Fan, Y. Liu, G. Wang, Z. Ren and Y. Liu (2020) Generation of transgene-free semidwarf maize plants by gene editing of *gibberellin-oxidase20-3* using CRISPR/Cas9. Front. Plant Sci. 11: 1048.

Zhang, Y., Z. Liang, Y. Zong, Y. Wang, J. Liu, K. Chen, J.-L. Qiu and C. Gao (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. 7: 12617.

Zhang, Y., Y. Bai, G. Wu, S. Zou, Y. Chen, C. Gao and D. Tang (2017) Simultaneous modification of three homoeologs of *TaEDR1* by genome editing enhances powdery mildew resistance in wheat. Plant J. 91: 714–724.

Zhang, Y., D. Li, D. Zhang, X. Zhao, X. Cao, L. Dong, J. Liu, K. Chen, H. Zhang, C. Gao *et al.* (2018b) Analysis of the functions of *TaGW2* homoeologs in wheat grain weight and protein content traits. Plant J. 94: 857–866.

Zhou, H., M. He, J. Li, L. Chen, Z. Huang, S. Zheng, L. Zhu, E. Ni, D. Jiang, B. Zhao *et al.* (2016) Development of commercial thermo-sensitive genic male sterile rice accelerates hybrid rice breeding using the CRISPR/Cas9-mediated *TMS5* editing system. Sci. Rep. 6: 37395.

Zhou, J., Z. Peng, J. Long, D. Sosso, B. Liu, J.-S. Eom, S. Huang, S. Liu, C. Vera Cruz, W.B. Frommer *et al.* (2015) Gene targeting by the TAL effector PthXo2 reveals cryptic resistance gene for bacterial blight of rice. Plant J. 82: 632–643.

Zhu, Y., Y. Lin, S. Chen, H. Liu, Z. Chen, M. Fan, T. Hu, F. Mei, J. Chen, L. Chen *et al.* (2019) CRISPR/Cas9-mediated functional recovery of the recessive *rc* allele to develop red rice. Plant Biotechnol. J. 17: 2096–2105.