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Feature Review

CRISPR/Cas-mediated plant genome editing: outstanding challenges a decade after implementation

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The discovery of the CRISPR/Cas genome-editing system has revolutionized our understanding of the plant genome. CRISPR/Cas has been used for over a decade to modify plant genomes for the study of specific genes and biosynthetic pathways as well as to speed up breeding in many plant species, including both model and non-model crops. Although the CRISPR/Cas system is very efficient for genome editing, many bottlenecks and challenges slow down further improvement and applications. In this review we discuss the challenges that can occur during tissue culture, transformation, regeneration, and mutant detection. We also review the opportunities provided by new CRISPR platforms and specific applications related to gene regulation, abiotic and biotic stress response improvement, and *de novo* domestication of plants.

Highlights

Climate change and the diversity of consumer needs require innovative methods to continuously and rapidly modify existing crops for the development of new varieties.

In the past decade genome editing by CRISPR/Cas and derivatives has emerged as a novel and effective technology for functional studies and gene discovery as well as for breeding new traits and genotypes.

The development of novel CRISPR/Cas platforms, methods for the delivery of editing reagents, and methods for controlling gene regulation and detection of mutants have all expanded the scope of genome editing and other CRISPR/Cas-based approaches.

A decade of CRISPR/Cas plant genome editing

Ensuring food security for a growing global population in a changing climate presents a great challenge for agriculture. Conventional breeding can only tackle this challenge to some extent; the classically obtained genetic gain will not suffice in the long term. Recent advances in genome editing such as gene engineering using **CRISPR/Cas** (see Glossary) have opened many opportunities to accelerate plant breeding and to bridge the gap between conventional breeding and the knowledge acquired through plant molecular biology to study and improve (complex) traits [1]. CRISPR/Cas-mediated genome editing enables very precise and efficient targeted modification in most crops, and thus largely increases the speed of crop improvement compared to conventional breeding [2]. Since the first description of CRISPR/Cas as a plant genome-editing technique, the technology has been successfully applied in close to 120 crops and model plants, with reports of wide applications for as many as half of them (Table S1 in the supplemental information online; www.eu-sage.eu/genome-search). CRISPR/Cas-edited plants are obtained by mutagenesis techniques using site-directed nucleases (SDNs) which can introduce targeted changes into specific DNA sequences of the genome to improve desired traits [3,4]. A distinction can be made between **site-directed nuclease type I (SDN-1)**, **site-directed nuclease type II (SDN-2)**, and **site-directed nuclease type III (SDN-3)** techniques because they result in different editing outcomes [5]. The CRISPR/Cas field is advancing very quickly (Figure 1, Key figure), and CRISPR systems are undergoing continual modification to increase specificity. **Base editing (BE)** and **prime editing (PE)** approaches do not rely on the repair of a double-strand break (DSB) in DNA. The former induces C to T or A to G transitions through a deaminase fused to either

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a **Cas nickase** or a **dead Cas (dCas)**, whereas the latter mediates targeted insertions, deletions, and all base-to-base conversions by exploiting an engineered reverse transcriptase enzyme and a PE guide RNA (pegRNA) fused to a Cas nickase (reviewed in [6]). Furthermore, new CRISPR/Cas delivery methods are being applied in addition to the classical transformation system using *Rhizobium radiobacter* or *Rhizobium rhizogenes* (formerly *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively). Some of these new technologies make genome alteration possible without the use of recombinant DNA [3]. Despite these advantages, many challenges remain regarding tissue culture and regeneration, mutant screening, polyploidy, and multiplexing. This review lists the known challenges and presents the methods and new platforms that are being developed to resolve some of these bottlenecks (Figure 1).

Tissue culture, transformation, and regeneration

Starting with the first attempts to deliver editing reagents through conventional transformation procedures, novel delivery systems have been developed for different species to facilitate *in vitro* tissue culture response, avoid stable transgene integration, test the functionality and efficiency of editing constructs, and develop genotype-independent transformation/regeneration protocols (Figure 2).

In vitro transformation and regeneration responses

Improvements in delivery methods and in fertile plant regeneration by *Rhizobium*-mediated transformation have been recently achieved to overcome genotype-dependency. A ternary pVir system, based on improved accessory plasmids characterized by small size, enhanced vector stability, improved bacterial selectable marker, and amended *vir* genes, has resulted in more efficient T-DNA delivery and stable plant transformation in recalcitrant maize and sorghum varieties [7–9]. Using this system, Che *et al.* [9] showed, however, that the main bottleneck in achieving genotype-independent transformation of immature sorghum embryos is not the delivery step but the tissue culture response for producing embryogenic callus.

The tissue culture response has recently been improved, for example, in *Brassica napus* [10], by using epicotyl and higher stem (internodal) segments as explants, as well as in barley, where an anther culture-based system was implemented [11]. These systems enable the development of genotype-independent transformation and editing protocols.

Developmental regulators operate in concert with plant hormones in tissue culture to induce embryogenesis or organogenesis from somatic cells [12]. The application of such regulators represents a recent improvement in tissue culture and transformation methods in various crops. In several instances the transfer of genes encoding such regulators, including *WUSCHEL* (*WUS2*), *BABY BOOM* (*BBM*), and *SHOOT MERISTEMLESS* (*STM*), has facilitated totipotency [13]. However, the expression of these genes can cause genotype-specific pleiotropic effects which prevent the recovery of normal, fertile plants. It is therefore important to modulate their expression, for instance by implementing inducible promoters, applying recombinase-mediated excision, or using cotransformation with two *Rhizobium* strains – one of which provides a T-DNA for *WUS2* expression and the other carries a T-DNA containing the gene of interest and a selectable marker in a neighboring cell (a phenomenon designated 'altruistic transformation') [9,14].

The overexpression of a chimeric protein combining transcription factor *GROWTH REGULATING FACTOR 4* (*GRF4*) and its cofactor *GRF-INTERACTING FACTOR 1* (*GIF1*), or *Arabidopsis GRF5* and/or its homologs, also enhanced regeneration and transformation in monocots and dicots, including woody species. Importantly, the GRF4–GIF1/GRF5 technology results in fertile and normal transgenic plants without the need for specialized promoters or transgene excision. This

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overcomes some of the limitations of transformation technologies with other morphogenic genes [15,16]. The GRF4–GIF1 complex could be also expressed transiently by biolistic delivery in wheat, and has promoted regeneration and editing efficiency in a range of cultivars [17].

Relying on the ability of short protospacer sequences (14–16 nt) to recruit transcriptional activators, a novel approach to promote plant regeneration (termed CRISPR-Combo) has recently been proposed [18]. The combination of Cas9 nuclease with regular and shorter protospacers allowed the editing of target genes and the simultaneous activation of genes encoding developmental regulators (WUS, WOX11, and BBM1) in poplar or rice, resulting in improved regeneration of edited plants.

Stable versus transient transformation

Genetic engineering by *Rhizobium*-mediated transformation mostly results in stable integration and expression of genes. The backcrossing procedure necessary to eliminate the transgene may present challenges, especially in heterozygous, polyploid, dioecious, or self-incompatible crops, as well as in species with an extended juvenile period. Transformation techniques leading to genetically modified plants (GMPs) reduce public acceptance of targeted mutagenized (edited) plants. This makes transient expression of Cas nucleases a desirable alternative because this creates transgene-free genome-edited plant lines with reduced off-target effects. An especially effective technique in this regard is to use ribonucleoprotein complexes (RNPs) that degrade rapidly once in the cell [19]. Polyethylene glycol (PEG)-mediated delivery of RNPs into cell wall-free protoplasts has been accomplished in various important plant species [20–22]. To avoid the potential toxicity of PEG in some protoplast systems, alternative transformation procedures based on electroporation, lipofection, and biolistics can be devised [23,24]. Nevertheless, protoplast transformation is confronted by a series of pitfalls that limit its potential use for editing plant genomes. Transformation success is limited by explant type and protoplast quality [25,26]. Protoplasts are also very sensitive and need gentle handling. Alternative isolation systems may need to be established, such as the 'Tape-*Arabidopsis* Sandwich' protoplast isolation protocol [20,27]. Regeneration relies on various parameters, including genotype, explant type, culture type, light conditions, media composition, and oxidative stress induced during isolation and culture [28]. Furthermore, **somaclonal variation** may complicate the evaluation of genome-editing events. Protoplasts are prone to these modifications, and show numerous chromosome rearrangements in regenerated plants [29,30]. Markers linked to regeneration and/or protoclonal variation could be designed based on chromocenter (re)assembly, reactive oxygen species (ROS) activity, DNA methylation or hydroxymethylation, histone methylation, phytohormone ratios, or gene expression, potentially resulting in the creation of custom-made and genotype-specific regeneration protocols that ensure the universal applicability of protoplast-based techniques.

Delivery through biolistics, leading to either stable or transient expression of editing reagents, with different cargos and target tissues as well as editing pathways, has been reported in a range of species [31]. Among others, transient expression of CRISPR/Cas has been achieved by delivering DNA, RNA, or RNPs into wheat and maize [19,32,33]. In the latter species, biolistic delivery enabled targeted mutagenesis, precise gene editing or insertion, and promoter insertion/exchange [34,35]. Although the biolistics approach can help to overcome the limitations of protoplast isolation and regeneration, it requires an expensive apparatus and can favor random integration of multiple transgene copies in nuclear or organellar genomes.

Testing the functionality and efficiency of editing constructs

The efficiency of editing constructs can vary extensively. Furthermore, when several genes are simultaneously targeted by using more than one guide RNA (gRNA), the editing efficiency of each gRNA in the polycistronic transcripts is not the same; those located at the end of the coding

Glossary

Base editing (BE): CRISPR-based editing technology relying on either a Cas nickase or a dead Cas fused to a deaminase enzyme to achieve single-base C to T or A to G transitions.
Cas nickase (nCas9): a mutant version of Cas9 that generates single-strand breaks in the DNA.
CRISPR/Cas: clustered regularly interspaced short palindromic repeats (CRISPR) are spacer sequences that have been identified in the genomes of prokaryotic organisms, and are derived from DNA fragments of infecting bacteriophages to serve as elements of acquired immunity to future infections. CRISPR-associated protein (Cas) is an enzyme that uses CRISPR-encoded sequences as a guide to recognize and cleave specific strands of DNA complementary to the CRISPR sequence.
Dead Cas (dCas): also known as endonuclease-deficient Cas9, a mutant form of Cas9 that has no endonuclease activity but can bind to the DNA strand that is targeted.
Indel: insertion and/or deletion of nucleotide base(s) at a locus in the genome.
Non-homologous end-joining (NHEJ): an error-prone DNA repair mechanism.
Prime editing (PE): an editing technology based on a Cas nickase fused to an engineered reverse transcriptase enzyme and a PE guide RNA (pegRNA) that specifies the target site and encodes the desired edits.
Protospacer adjacent motif (PAM): a short specific sequence at the 5′ or 3′ end of the target DNA sequence that is essential for cleavage by Cas nuclease.
Site-directed nuclease type I (SDN-1): induces Indel mutations in a predefined region in the genome as a result of NHEJ repair of a double-strand break (DSB).
Site-directed nuclease type II (SDN-2): generates specific small Indel or single-nucleotide variant (SNV) modifications as a result of the introduction into the cell of a repair DNA template (donor DNA) homologous to the targeted area. This enables precise repair of a DSB by homologous recombination (HR).

sequence can have lower editing capacity [36]. Several systems have been developed to validate editing constructs before their stable/transient transformation to produce edited tissues, including protoplasts [19,37], hairy roots [38,39], plant cell suspension cultures (PCSCs), or a biolistics-based leaf epidermis transient expression assay [40]. PCSCs, which reflect many physiological and biological characteristics of a whole plant while being a stable system, allow controlled testing of the effects of SDNs in plants. They can be utilized as a stable and uniform source of protoplasts for transformation [20], and can be used directly for *Rhizobium*-mediated transformation [41] or biolistic and electroporation-based introduction of elements for genetic engineering. A system based on *Rhizobium*-mediated transformation of wheat cell suspension cultures was used to evaluate the editing efficiency of gRNA/Cas9 constructs designed for the *ABA 8′-HYDROXYLASE 1* gene [42]. Both hairy root and PCSC systems can also be used to regenerate edited plants, particularly when other transformation methods are ineffective [43,44].

Site-directed nuclease type III (SDN-3): induces the insertion of DNA sequences into a desired locus in the genome enabled by the delivery of a large (up to several kilobases) stretch of a recombinant DNA molecule. The insertion can take place either by HR or NHEJ.
Somaclonal variation: genetic variation in plants regenerated through tissue culture.

Transformation and regeneration in woody plants and polyploids

Conventional breeding of perennial woody plants presents specific challenges that include long generation time, seasonal dormancy, huge genomes, dioecy and polyploidy, and a prolonged evaluation period for mature traits. All of these can be overcome by genome editing. CRISPR/Cas systems have been successfully applied in several trees such as *Malus*, *Coffea*, *Citrus*, and *Populus*, among others [45,46]. In woody species, however, the low transformation and *in vitro* regeneration ability (except for *Populus*), as well as their naturally slow growth rate, represent bottlenecks for wider implementation of genome-editing technologies [47]. In addition, chimeric regenerants, in which only a part of the plant descends from an edited cell, may occur (e.g., in apple and pear) [48]. *Rhizobium*-mediated low transformation efficiencies were increased by using other *Rhizobium* species and strains [49–51], or preinfection with other bacteria such as *Xanthomonas citri* [52]. To shorten the long juvenile period and promote early flowering of woody plants, thus ensuring their early maturation, overexpression of the *BpMADS4* gene may be employed [53]. Flachowsky *et al.* [54] reported that overexpression of *BpMADS4* in apples significantly shortened the juvenile period and enabled early flowering. Ectopic expression of *FLOWERING LOCUS T* (*FT*) from various donor species reduced the generation time of European plum [55], *Eucalyptus* [56], *Populus* [57], and sweet orange [58]. In addition to *FT* genes, arabidopsis *APETALA1* (*AP1*) overexpression was effective in sweet orange and citrange [59].

Polyploid plants, especially when vegetatively propagated, can present difficulties to obtain homozygous individuals and can have a long reproductive cycle. These also encounter specific difficulties for both classical breeding and genome-editing techniques [60]. Duplicated genes, heterozygosity, repetitive DNA, and genome irregularities contribute to their complex genetic background. In addition, genetic redundancy prevents functional genomic studies and breeding approaches owing to the difficulty of simultaneously mutating multiple alleles [61]. However, despite these limitations, mutations in all alleles in hexaploid *Camelina* [62], tetraploid potato [63], tetraploid/hexaploid wheat [19], and other polyploids have been obtained by using CRISPR/Cas and various delivery methods. Genome editing in polyploid plants can also be affected by poor or ineffective transfer of CRISPR/Cas reagents into tissues with high regeneration capacity. Fluorescence-activated cell sorting (FACS) can be used to select the transformed cells. This technique, which is mostly used in mammalian and human cells, was used for the first time in *Arabidopsis* [64], and was also applied successfully in some polyploid plants such as *Nicotiana benthamiana* [65].

Genotype-independent *in planta* transformation and regeneration procedures

To overcome the bottlenecks related to *de novo* regeneration *in vitro*, genotype-independent genome-editing protocols that do not rely on tissue culture procedures are highly desirable in many species. However, these methods are at an advanced stage of development

Key figure

Challenges and opportunities related to various Cas-based tools, their delivery into plant cells, and the outcomes and applications

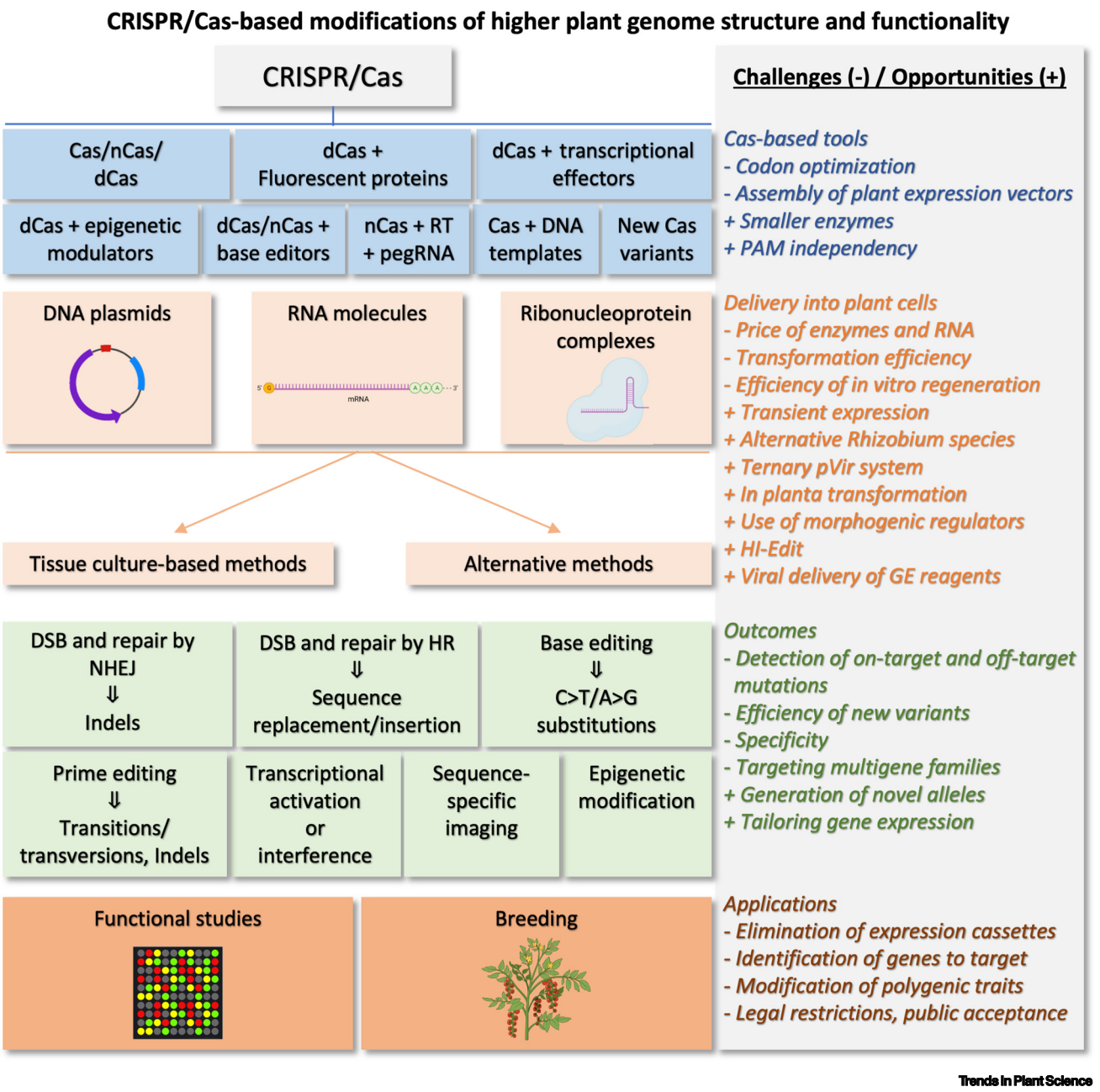

Figure 1. Abbreviations: dCas, dead (endonuclease-deficient) Cas; DSB, double-strand break; GE, genome editing; HR, homologous recombination; Indel, insertion/deletion; nCas, Cas nickase; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; pegRNA, prime editing guide RNA; RT, reverse transcriptase.

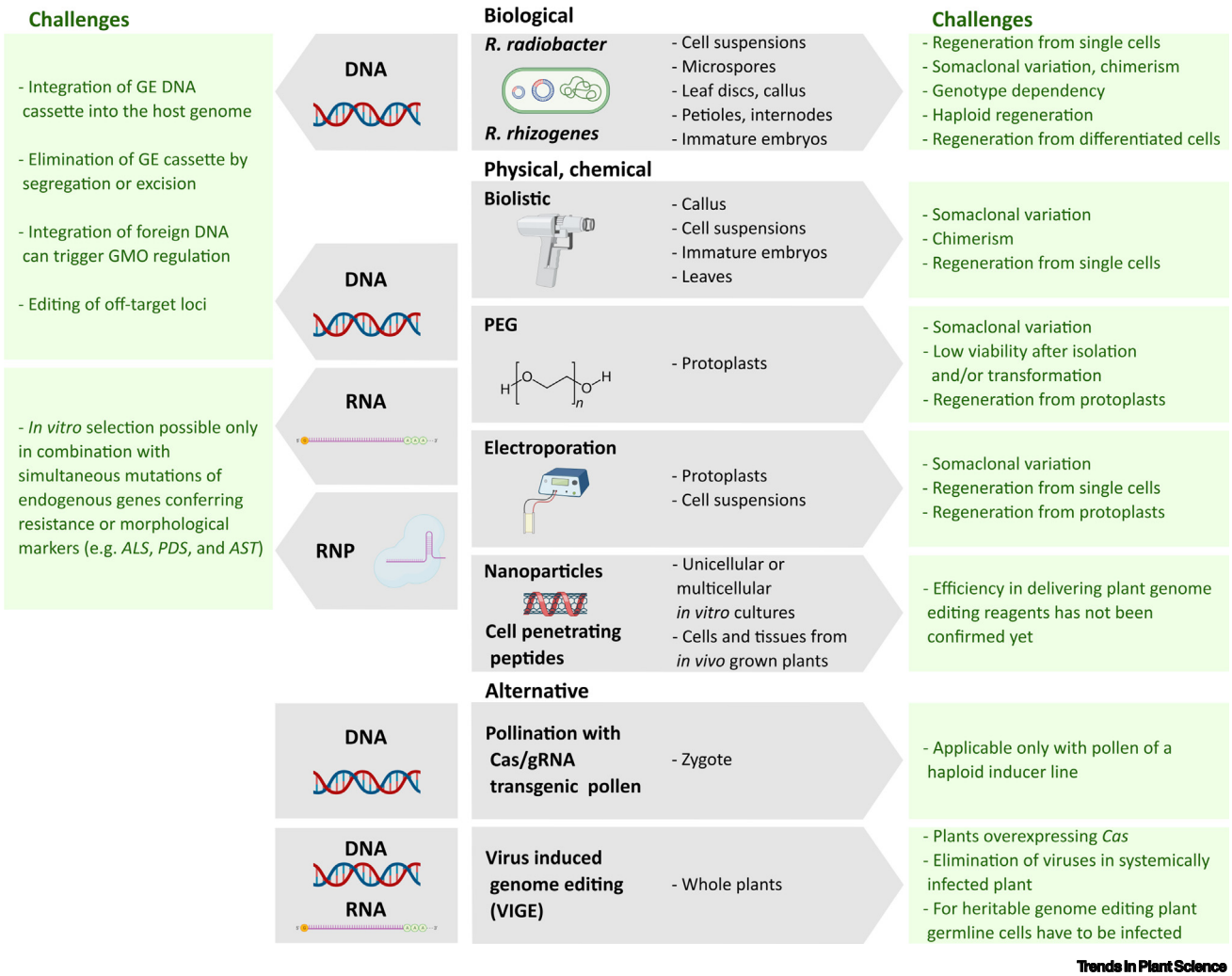

Figure 2. CRISPR/Cas plant genome-editing (GE) methods to deliver various macromolecules into plant cells and associated challenges.
Abbreviations: GMO, genetically modified organism; gRNA, guide RNA; PEG, polyethylene glycol.

in only a few species, and in most cases require further work to advance from the proof-of-concept stage (Table 1). The floral dip method has been recently improved to reduce chimerism in transformed tissues, and to enhance *in planta* gene targeting and frequency of mutagenesis, but so far it has only been successfully employed to deliver editing reagents in *A. thaliana* and a few close relatives [66–71]. Several studies [72–74] showed the possibility to recover CRISPR/Cas-induced mutations (edits) in non-transgenic maternal or paternal haploids of *Zea mays*, *A. thaliana*, *Triticum aestivum*, and *Triticum durum*. However, the use of such an approach requires a system for haploid induction based on chromosome elimination after fertilization and the possibility to transform the haploid-inducer genotype to express editing reagents.

In vivo delivery of editing reagents in meristems, which results in edits that are transmittable to progeny, has been performed in only a few species [75–78]. Efficient *in planta* transformation

Table 1. Recent examples of protocols for the straightforward generation of GE plants bypassing *de novo* regeneration in tissue culture[a]

Procedure	Species	Organ/tissue	Editing reagents delivered	Transfer technology	Expression of delivered reagents	Target genes	Edits transmittable to progeny	Refs
Floral dip	*Arabidopsis thaliana*, *Camelina sativa*, *Thlaspi arvense*, *Arabis alpina*	Egg cells	Cas9-sgRNA Cas12a-sgRNA plasmid DNA	*Rhizobium radiobacter*	Stable	*AtGLABRA2*, *AtALS*, *AtAP3*, *AtLFY*, *AtAG*, *CsFAE1*, *TaFAE1*, *AaSPL15*	Yes	[66–71]
Haploid inducer	*Zea mays*, *A. thaliana*, *Triticum aestivum*	Sexual zygote, developing embryo	Cas9/gRNA transgenes in pollen	None	Stable[b]	*ZmVLHP1*, *ZmVLHP2*, *ZmGW2-1*, *ZmGW2-2*, *AtGL1*, *TaGT1*	Yes	[72]
	Z. mays	Sexual zygote, developing embryo	Cas9/gRNA transgenes in pollen	None	Stable[b]	*ZmLG1*, *ZmUB2*	Yes	[73]
	T. aestivum, *T. durum*	Sexual zygote, developing embryo	Cas9/gRNA transgenes in pollen	None	Stable[b]	*TaBRI1*[c], *TaSD1*[c]	Yes	[74]
Zygotes	*Oryza sativa*	*In vitro* produced zygotes	Cas9-sgRNA plasmid DNA, Cas9-sgRNA RNP complex	PEG-Ca^{2+}	Stable, transient	*DsRed2*, *DL*, *GW7*, *GCS1*, *PRR37*	Yes	[156]
Meristem targeting	*Gossypium hirsutum*	Shoot apex	Cas9-sgRNA plasmid DNA	*R. radiobacter*	Stable	*GhCLA1*, *GhVP*	Yes	[75]
	T. aestivum	Seed shoot apical meristem	Cas9-sgRNA plasmid DNA	Biolistics	Transient	*TaGASR7*, *TaQsd1*	Yes	[76,78]
	Solanum tuberosum	Apical and lateral meristems	Cas9-sgRNA RNP complex	Biolistics, vacuum infiltration	Transient	*StCoilin*	Nr[d]	[77]
De novo induction of meristems	*Nicotiana benthamiana* (*Cas9*$^+$)	Cotyledons (seedlings), cut shoot apices (adult plants)	Plasmid DNA [sgRNA + developmental regulators (DRs)[e]]	*R. radiobacter*	Stable, transient	*NbPDS1*, *NbPDS2*	Yes	[79]
Virus-induced genome editing (VIGE)	*N. benthamiana* (*Cas9*$^+$)	Leaves	sgRNA::*FT*mRNA sgRNA:: $tRNA^{Me}$/$tRNA^{Gly}$/$tRNA^{Ile}$ [tobacco rattle virus (TRV)-based vectors]	*R. radiobacter*	Transient	*NbPDS*, *NbAG*	Yes	[84]
	N. benthamiana (*Cas9*$^+$)	Leaves	sgRNA::*tFT*mRNA [potato virus X (PVX) vector]	*R. radiobacter*	Transient	*NbFT*, *NbPDS3*, *NbXT2B*	Yes	[157]
	A. thaliana (*Cas9*$^+$)	Leaves	sgRNA::t*FTm*RNA [cotton leaf crumple virus (CLCrV) vector]	*R. radiobacter*	Transient	*AtGL2*, *AtBRI1*	Yes	[158]
	T. aestivum (*Cas9*$^+$)	Leaves	sgRNA with/without fusion to *FT* or tRNA mobile elements [barley stripe mosaic virus (BSMV) vectors]	None	Transient	*TaPDS*, *TaGW2*, *TaGASR7*	Yes	[159]
	N. benthamiana	Leaves	Cas9-sgRNA [*Sonchus* yellow net rhabdovirus (SYNV) vector]	*R. radiobacter*	Transient	*GFP* transgene, *NbPDS*, *NbRDR6*, *NbSGS3*	No[f]	[81]

Table 1. (continued)

Procedure	Species	Organ/tissue	Editing reagents delivered	Transfer technology	Expression of delivered reagents	Target genes	Edits transmittable to progeny	Refs
	N. benthamiana	Leaves	Cas9-sgRNA [foxtail mosaic virus (FoMV) vector]	*R. radiobacter*	Transient	*NbPDS*	No	[82]
Nanoparticles	*T. aestivum*	Leaves	Cas9-sgRNA plasmid DNA (carbon dots)	Foliar spraying	Transient	*SPO11*	No	[160]
Grafting	*A. thaliana*	Egg cells (rootstock)	Cas9-TLS[g], sgRNA-TLS plasmid DNA	*R. radiobacter*	Stable (in rootstock)	*AtNIA1*	Yes	[89]

[a]In some cases, only *in vitro* culture of pre-formed meristems was carried out.
[b]In haploid-inducer lines.
[c]Conserved across all two (AABB) or three (AABBDD) homeologs of the target genes in durum and bread wheat, respectively.
[d]Not relevant.
[e]Developmental regulators (various combinations of *BBM*, *Wus2*, *ipt*, *STM*).
[f]Edits were recovered only in the progenies of plants regenerated *in vitro*.
[g]TLS, tRNA-like sequence motifs.

protocols require further development. Gene-edited *N. benthamiana* plants have been recently obtained by *in situ* delivery of single guide RNA (sgRNA) and several combinations of developmental regulators which induced the formation of new meristems on somatic tissues *in vivo* [79]. The latter study made use of transgenic plants expressing Cas9, and the feasibility of codelivering Cas nuclease, sgRNA, and developmental regulators remains to be demonstrated. In addition, to avoid negative pleiotropic effects, the expression of developmental regulators should be regulated.

Several viruses have been considered as vectors for delivering editing reagents *in planta*, but their limited ability to accommodate large molecules (e.g., Cas9) and/or infect the germline prevents the efficient induction of edits and their transmission to progeny [80]. Recently, the exploitation of new viruses made it possible to overcome the first limitation [81–83], and fusion of sgRNA to meristem-specific regulatory elements increased the efficiency of mutation transmission through generations [84]. A combination of the two approaches and/or the use of smaller nucleases [85] might open new perspectives for alternative genome-editing methods. Nanoparticles have been also suggested as novel delivery carriers for tissue culture-free genome editing [86] and pollen engineering [87,88], but even in this case the size of Cas9 and respective nucleases presently limits their delivery through the cell wall and subsequent editing of the germline.

Transgene-free heritable editing was recently accomplished in *Arabidopsis* by transforming rootstocks with Cas9 and gRNA sequences to which tRNA-like motifs had been added [89]. As a result, mobile editing reagent transcripts could move from the rootstock to the scion, thus allowing germline editing. Alternative ways to deliver and express mobile editing reagents in the rootstock may allow this novel approach to be exploited in various combinations of compatible grafts.

Mutant detection

To detect edits after CRISPR/Cas-induced genomic modifications, several detection methods can be applied. Because different methods have distinct outcomes, it is not reliable to compare studies regarding the efficiency of mutation induction. To make studies comparable, standardization will be essential to determine/quantify the mutation efficiency and identify which standard

data should be obtained as an outcome. Regardless of the detection method, specificity regarding the exclusive response of the target of interest is a key property. Which technique is suitable for a specific application largely depends on the data required; for example, whether it is necessary to determine the exact alteration, and what knowledge is available about the target sequence, gene, and gene family (Figure 3). Mutational outcomes are not random because they depend on the DNA sequence at the targeted location [90]. Data from more than 10^9 mutational outcomes revealed that most mutations are insertions of a single base pair, short deletions, or longer micro-homology-mediated deletions [90]. This presents a challenge in developing analytical methodologies that can identify unique markers of such small genetic alterations.

PCR-based methods

Real-time PCR methods are often used to detect genome-edited plants, but they have limitations related to the detection of single-nucleotide variants (SNVs). A crucial aspect here is the conjunction of real-time PCR with locked nucleic acids (LNAs) in primer design because this increases assay specificity in genome-edited plants such as in canola [91] and rice [92].

DNA change	Analyzed plant material	Detection methods**
Indel and SNV*, SDN-1 or SDN-2 Short insertion Short deletion Short substitution	Pooled single cell cultures: protoplasts, cells, microspores Undifferentiated multicellular structure: callus	Targeted NGS T7E1, Cell, Surveyor PCR-RFLP Sanger sequencing in combination with DSDecode, TIDE, ICE PCR, agarose electrophoresis for long deletions
Indel and SNV*, SDN-1 or SDN-2 Short insertion Short deletion Short substitution	Regenerants M0 Progeny M1	Real-time PCR (qPCR), HRM ddPCR Sanger sequencing Southern blot Targeted NGS, WGS PCR, agarose electrophoresis for long deletions
Gene integration, SDN-3 Gene integration	Regenerants T0 Progeny T1	PCR, agarose electrophoresis qPCR, ddPCR Sanger sequencing Southern blot Targeted NGS, WGS

M0 - first generation of plants with induced mutations; M1 - progeny of M0;
T0 - first generation of transgenic plants; T1 - progeny of T0
** SNV induced with base or prime editing; ** definitions in Glossary*

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Figure 3. Detection methods used in the development of protocols for CRISPR/Cas genome editing of plants. Abbreviations: ddPCR, droplet digital PCR; DSDecode, degenerate sequence decode; ICE, inference of CRISPR edits; HRM, high-resolution melting; Indel, insertion/deletion; NGS, next-generation sequencing; qPCR, quantitative PCR; RFLP, restriction fragment length polymorphism; SDN, site-directed nuclease; SNV, single-nucleotide variant; TIDE, tracking of indels by decomposition; WGS, whole-genome sequencing.

Droplet digital PCR (ddPCR) [5,93] is a promising tool for genome-editing detection, as has been shown for detecting SNVs in oncology research [94], in edited cells [95,96], and recently in edited plants to identify wild-type, homozygous, and heterozygous mutations induced by CRISPR/Cas9 in rice [92] and rapeseed [93]. Similarly, a duplex ddPCR approach distinguished CRISPR/Cas-induced small **Indels** in *ALPHA-GLIADIN* from large deletions in polyploid bread wheat [97]. Furthermore, high-resolution melting (HRM) analysis is a post-PCR approach based on monitoring the gradual denaturation of amplicons that can detect small sequence differences [98] such as small Indels or even SNVs in gene-edited rice [99]. However, HRM analysis also has limitations associated with the use of small target amplicons (100–200 bp) which might hamper the detection of large Indels. All these PCR-based techniques are relatively cheap, easy to implement, and allow rapid routine screening of gene-edited mutant collections. However, they only indirectly show the presence of a mutation, and do not identify the exact alteration at the nucleotide level.

Sequencing-based methods

Amplification and sequencing of the target loci allow identification of the specific nucleotides that are deleted, inserted, or substituted. In addition, when a large number of regenerated polyploid plants need to be analyzed for the presence of the multi-allelic structure, sequencing-based detection methods are valuable. Furthermore, many traits are determined by multiple small-effect genes operating in gene families and/or complex interactive networks, and thus require high-throughput, multiplex CRISPR approaches. Off-targets also need to be determined. All of these represent additional challenges and increase the demand for efficient detection techniques for proper screening and data analysis based on new sequencing-based and bioinformatic tools. Sanger sequencing of (cloned) amplicons enables the identification of induced mutations at on-target sites and makes it possible to determine the frequency and the type of mutations at a specific locus. However, Sanger sequencing can lead to confusing results when applied to polyploid organisms with heterozygous or multiallelic mutations [100]. Bioinformatic tools such as TIDE (tracking of indels by decomposition; http://tide.nki.nl) and ICE (inference of CRISPR edits; https://ice.synthego.com/#/) can be used for automatic decoding of overlapping electropherograms derived from PCR amplicons that hold different types of mutations. The sensitivity of Sanger sequencing is (only) about 15%, meaning that if a pooled strategy is used, 15% of the pool must contain the mutation before it can be detected. Low-efficiency editing is thus likely to be overlooked by Sanger sequencing [101].

Recent advances in sequencing technologies have led to the development of high-throughput next-generation sequencing (NGS) technologies using second- (short-read) and third-generation (long-read) sequencing technologies. These enable massively parallel sequencing and analysis of heterogeneous samples. NGS sequencing has a sensitivity of 0.1% to 1%, allowing efficient pooling and screening of protoplast samples. To analyze the resulting huge datasets, bioinformatic tools are available such as CRISPResso2 [102] and SMAP (stacked mapping anchor points) [103]. In the study of Lorenzo *et al.* [1], multiplex genome editing of whole gene families was combined with crossing schemes to improve complex traits (yield, drought stress). NGS sequencing and SMAP data analysis were used to screen for knockouts of 48 growth-related genes in maize, and a collection of >1000 gene-edited plants were regenerated. Recent progress is also reported related to gRNA library-based CRISPR screens for high-throughput loss-of-function screens in plants, even at a genome-wide scale [104,105]. gRNA libraries can be created and introduced in bulk in plant cells in a way that individual cells receive different gRNAs. Unique gRNAs can serve as barcodes and then be identified in a pool of cells by high-throughput sequencing. Such CRISPR screens enable rapid connection between the genotype and the phenotype.

In addition to targeted sequencing, NGS also enables whole-exome and whole-genome sequencing (WGS) which is an untargeted detection approach for unknown alterations (small Indels and SNPs) as well as a way to detect structural variants such as inversions, rearrangements, duplications, and major deletions [106,107]. WGS therefore makes it possible to detect on-target mutations induced by Cas endonucleases including off-target mutations induced by endonucleases as well as naturally occurring mutations. WGS has demonstrated its efficacy for revealing rare off-target mutations in rice [108], cotton [109], and grapevine [110].

In general, NGS is currently considered to be the most comprehensive and reliable technique for analysis of genome-editing events [111], even in polyploid or chimeric plants, as well as for tracing large insertions/deletions and mutations in multi-edited plants. Knowing the exact mutation at the nucleotide level allows the mutated allele to be placed in its gene context and thus enables the effect of the mutation on the structure and function of the encoded protein to be predicted. On the other hand, NGS produces large datasets. However, because high-throughput multiplex mutation screening is possible, the per sample per locus costs are substantially reduced. In addition, although datasets can be huge, especially for plants with large genomes, they are systematically structured such that high-throughput automated analysis becomes possible. Skilled investigators and suitable bioinformatic pipelines are able to process and detect all haplotypes, at all loci, in all samples, and in parallel. However, even using NGS, it is impossible to distinguish an artificially edited genome from one containing natural mutations.

New CRISPR/Cas actions and platforms

Gene regulation

CRISPR/Cas can be employed in several ways to regulate gene expression. miRNAs are endogenous small RNA molecules that control the abundance of target mRNA in diverse pathways [112]. Fine-tuning of their abundance by CRISPR/Cas to regulate miRNA-regulated plant processes is feasible; however, the research is in its early phase and still represents a challenge, especially in polyploid species [112]. One drawback is that miRNA editing with a sgRNA is not necessarily efficient because frameshifts do not always affect miRNA function [113]. For this reason, dual sgRNA design is recommended to target both ends of a mature miRNA coding sequence. In an optimal situation, this would result in the removal of the whole miRNA sequence, or at least sufficiently perturb the structure of pre-miRNA to impair the processing of miRNA [114]. miRNA modulation by a sgRNA is also feasible, although less efficient [115,116].

Different approaches have been implemented to target miRNA genes in several plant species (Table S2 in the supplemental information online). miRNA control of gene expression can also be altered by inducing mutations in miRNA binding sites of regulated genes.

In addition, *cis*-regulatory elements (CREs) in the genes to be modified can be disrupted or replaced by CRISPR/Cas-based genome editing [34,117]. In tomato, the use of CRISPR/Cas to dissect CREs allowed the generation of novel variation underlying quantitative trait variation and pleiotropy that both control the phenotypic variation of useful traits [118–120]. Finally, gene expression can also be modulated, without stable genome modification, by fusing dCas9 to transcriptional effectors or epigenetic modulators, or by exploiting the cited ability of short protospacers, combined with a standard nuclease, to recruit transcriptional activators [18,121,122]. Challenges for future research on dCas-based technologies include improvement of the activation/repression efficiency and of the epigenetic manipulation specificity, together with the possibility of controlling gene expression simultaneously in multiple targets and/or in an inducible or tissue-specific manner [121].

Advanced CRISPR/Cas variants

Despite many successful examples of first-generation CRISPR methods in plants, introduced changes were predominantly knockout mutations of the targeted loci. Advanced plant breeding programs need precise changes in plant genomes, which cannot be accomplished by **non-homologous end-joining (NHEJ)** following the generation of a DSB. Moreover, many loci cannot be targeted by the first generation of Cas9 nucleases from *Streptococcus pyogenes* (*Sp*Cas9) owing to the lack of suitable **protospacer adjacent motif (PAM)** sequences.

Advanced CRISPR/Cas variants are therefore being developed [123] for precise targeting using newly discovered SDN or modified Cas proteins combined with the catalytic activity of DNA-modifying proteins. Acquired fusion proteins for BE and PE can generate single-nucleotide substitutions, in-frame insertions, and large deletions. In addition, these variants allow DSB-free and template-free editing with improved efficiency and increased specificity. Some of the new variants have already been successfully used in plants (Table 2). Although these new techniques will need more experimental data to be widely and routinely applied for crop improvement, they provide a more efficient induction of intended edits, thus increasing the chance of obtaining the desired mutants even from plant genotypes with low regeneration capacity. Wider implementation of these new CRISPR variants in different plant species and their optimization for plant expression (e.g., Golden Gate Domestication, codon optimization) will foster plant genome editing. The new CRISPR toolboxes are already overcoming bottlenecks related to PAM restriction of traditional *Sp*Cas9 and target specificity. The large size of *Sp*Cas9 is addressed by the adoption of smaller SDN enzymes [124–126] that, combined with split-Cas systems [127,128], will also enable *in planta* virus-mediated expression of SDN.

Opportunities and challenges related to specific applications

Biotic and abiotic stresses

CRISPR/Cas genome editing has been applied to enhance disease resistance and abiotic stress tolerance. Despite the great potential and some outstanding results [129], several issues still limit its efficiency as a tool for mitigation of plant stresses.

To induce disease resistance via genome editing, the identification and functional annotation of plant susceptibility (*S*) and/or resistance (*R*) genes is needed. This is labor-intensive and demands access to whole-genome sequences. However, limited or no data are available on the molecular functions of *S*/*R* genes in most non-model plants [130]. Furthermore, many *S* genes have dual roles in plant physiology and susceptibility to pathogens, which often makes them essential for the survival of the host. Inactivating these genes often leads to disease resistance but is also accompanied by pleiotropic effects including plant growth inhibition, phenotypic abnormalities, and increased susceptibility to abiotic stress and/or other pathogenic agents [131–134].

Similarly to other methods for inducing disease resistance, the widespread and long-term planting of gene edited plants, especially in monocultures, might lead to the appearance of new or adapted pathogen strains. Simultaneous protection against different races or strains of the same species and/or several taxonomically unrelated pathogens can be an additional challenge [130,135,136]. The possibility of using CRISPR/Cas for the fast induction of new mutations that lead to plant resistance can help to manage resistance genes and edited plants such as by producing multi-lines or stacked lines with multiple resistance mechanisms.

The frequency and intensity of climate extremes are increasing, which means that disease resistance should be combined with tolerance to abiotic stress. Resilience in the face of abiotic stress usually depends on complex morphophysiological mechanisms controlled by multiple genes,

Table 2. New CRISPR/Cas variants for enhanced and more precise plant genome editing[a,b]

CRISPR variant	Component	Novelty or mode of action	Expression platform and editing efficiency	Refs
CBE (cytosine base editor)	n*Sp*Cas9[a] + rAPOBEC1 (rat cytidine deaminase) + UGI (uracil DNA glycosylase inhibitor)	Introduction of nucleotide changes without inducing a DSB: C to T base transition. Higher efficiency compared to HDR-mediated base pair substitution, lower occurrence of undesirable mutations.	*Rhizobium radiobacter*-mediated transformation of watermelon and cauliflower. Efficiency: 23% in watermelon T0 generation and 22% or 87% in cauliflower, depending on the targeted locus.	[161,162]
	n*Sp*Cas9 or *Sp*RY (PAM-less) + UNG (uracil DNA glycosylase) + rAPOBEC1	Introduction of nucleotide changes without inducing a DSB: C to G base transversion. Higher efficiency than HDR-mediated base pair substitution, lower frequency of undesirable mutations.	Rice and tomato protoplasts; *R. radiobacter*-mediated transformation of rice and poplar. Efficiency: C to G substitution in 38% of transgenic rice lines and 6.3% in poplar; C to T edits and Indels among byproducts.	[163]
	n*Sp*Cas9(D10A) + rationally designed human A3Bctd-BE3	Reduced sgRNA-independent genomic off-target activity of CBEs producing mainly single and double C to T edits, with marginally reduced on-target activity in rice.	Rice protoplasts transfection and *R. radiobacter*-mediated transformation of rice calli. Efficiency: up to 30% on-target efficiency across the four target sites and reduced sgRNA-independent off-target activity.	[164]
	n*Sp*Cas9-NG + evoFERNY + rice UNG or human UNG	Introduction of nucleotide changes without inducing a DSB. Recognition of NG PAM sequence. C to T base transition, C to G or C to A transversion. Efficient monoallelic base editing with the significant number of insertion/deletion byproducts.	*R. radiobacter*-mediated transformation of rice calli. Efficiency: Up to 21% of C to T, up to 8.2% of C to G and up to 3.7% C to A on-target efficiency in T0 transformants.	[165]
	n*Sp*Cas9-pBE and VQRn-pBE	*Petromyzon marinus* cytidine deaminase 1 (PmCDA1) and uracil DNA glycosylase inhibitor (UGI) fused to *Sp*Cas9. VQRn variant created by D1135V, R1335Q, and T1337R amino acid substitutions in the *Sp*Cas9 sequence. Efficiency additionally increased by modified gRNA. C to T base substitutions. Deamination window within 1–7 nt upstream the PAM.	*R. radiobacter*-mediated transformation of rice. Efficiency: up to 90% in rice T0 plants.	[166]
ABE (adenine base editor)	n*Sp*Cas9 (D10A) + engineered *E. coli* tRNA adenine deaminase (TadA)	Introduction of nucleotide changes without inducing a DSB. A to G base transition. Higher efficiency than HDR-mediated base pair substitution, lower occurrence of undesirable mutations.	*R. radiobacter*-mediated transformation of rice. Efficiency: A to G transition in 26% of transgenic lines.	[167]
	*Sp*Gn + adenine deaminase TadA8e with *E. coli* endonuclease V (EndoV) or human alkyladenine DNA glycosylase (hAAG)	Introduction of nucleotide changes without inducing a DSB. A to G base transition. High efficiency and broad target range owing to the variable PAM sequence.	*R. radiobacter*-mediated transformation of rice calli. Efficiency: average 56% of A to G on-target efficiency.	[165]
	PhieABE variants: n*Sp*Cas9-NG, n*Sp*G or n*Sp*RY (PAM-less) + TadA8e adenine deaminase + single stranded DNA-binding domain (DBD)	Introduction of nucleotide changes without inducing a DSB. A to G base transition at the NGN PAM target site. DBD enhanced on-target activity and decreased off-target and self-editing.	Rice T0 transformation with *R. radiobacter*. Efficiency: up to 78.5% average on-target editing across the 17 target sites.	[168]

Table 2. (continued)

CRISPR variant	Component	Novelty or mode of action	Expression platform and editing efficiency	Refs
STEME (saturated targeted endogenous mutagenesis editors)	n*Sp*Cas9 (D10A) + cytidine deaminase + adenosine deaminase + UGI	Saturating mutagenesis of a single locus with a single protein that uses a single sgRNA to achieve C:G > T:A and A:T > G:C substitutions.	PEG-mediated transfection of rice protoplasts. Efficiency: STEME-1 edited cytosine and adenine at the same target site with C > T efficiency up to 61.6% and simultaneous C > T and A > G efficiency up to 15.1%. STEME-NG produced 73.2% mutagenesis.	[169]
SWISS (CRISPR simultaneous and wide-editing induced by a single system)	n*Sp*Cas9 (D10A) + sgRNA scaffolds with different RNA aptamers + binding proteins fused with cytidine or adenosine deaminase	Multiplex base editing, insertions and deletions. Editing adenosines and cytidines at separate sites.	*R. radiobacter*-mediated transformation of rice callus cells. Efficiency: cytosine conversion 25.5%, adenine conversion 16.4%, Indels 52.7%, and simultaneous triple mutations 7.3% in rice mutants.	[170]
Prime editing – PE, PE2, PE3	PE: n*Sp*Cas9 (H840A) + RT + pegRNA PE3: PE, pegRNA and a second gRNA nicking the non-edited DNA strand to trigger repair of the edited strand PE3b: PE, pegRNA and a second gRNA nicking the non-edited DNA strand with a spacer matching the edited sequence	Reverse transcription of an editing template from pegRNA (a modified gRNA) directly into the target locus which is nicked and extended by the prime editor. Introduction of single or multiple base substitutions, Indels, long deletions.	Maize inbred lines transformation with *R. radiobacter* LBA4404/pVIS1–VIR2. Rice protoplasts are transfected by the PEG-mediated method. Efficiency: increased efficiency of PE in maize from 6.5% up to 71.7% by two strategies: the Csy4 RNA processing system and the tRNA and HDV ribozyme RNA-processing system integrated with two drivers, polymerase II (35S enhancer-CmYLCV) and III (shortened U6-26) promoters. From 1% to 7% efficiency in rice protoplasts depending on the vector used.	[171]
	ePPE	Based on original n*Sp*Cas9 + M-MLV RT. The RNAse H domain was removed from fused M-MLV RT and additionally fused to a viral NC peptide with nucleic acid chaperone activity.	Rice protoplasts and transformed plants. Efficiency: 5.8-fold higher than the original plant PE.	[172]
	enpPE2	n*Sp*Cas9 with sequence modifications (R221K/N394K; PEmax) fused to M-MLV RT plus modified pegRNA expressed under a composite CaMV 35S enhancer-CmYLCV-U6 promoter.	Rice protoplasts and transformed plants. Efficiency: 64.58–77.08% in rice T0 plants.	[173]
AFIDs (APOBEC–Cas9 fusion-induced deletion systems)	*Sp*Cas9 + human APOBEC3A (A3A) + uracil DNA-glucosidase + apurinic or apyrimidinic site lyase	Introduction of larger deletions.	PEG-mediated transfection of rice and wheat protoplasts, *R. radiobacter*-mediated transformation of rice callus cells, and biolistic transformation of immature wheat embryo cells. Efficiency: AFID-3 generated deletions from 5′-deaminated C bases to the Cas9-cleavage site in rice and wheat protoplasts. One-third of these deletions in protoplasts (30.2%) and regenerated plants (34.8%) were predictable.	[174]
Cas9-VirD2	*Sp*Cas9 + VirD2 relaxes protein from *R. radiobacter* to tether end-protected single-stranded DNA repair template for HDR to the targeted DSB	More efficient gene targeting through HDR.	Bombardment of rice calli with plasmids. Efficiency: fivefold increase over the non-tethered control in rice. Up to 9.9% efficiency of gene editing through HDR in the absence of selection.	[175]

(continued on next page)

Table 2. (continued)

CRISPR variant	Component	Novelty or mode of action	Expression platform and editing efficiency	Refs
CRISPR PLUS: C9R and C9G	C9R: *Sp*Cas9 + 5′-to-3′ exonuclease RecJ C9G: *Sp*Cas9 + *gfp*	Increase in both mutagenesis and knock-in efficiency; off-target effects were not significantly increased relative to the Cas9 (C9) structure alone.	PEG transfection of *Nicotiana benthamiana* protoplasts with RNPs. Efficiency: 2.6-fold increased editing efficiency of C9R compared to control C9 in protoplasts of *N. benthamiana*.	[176]
CRISPR-Combo	Cas9-Act3.0 system: catalytically active Cas9 nuclease, MS2 bacteriophage coat protein (MCP)-SunTag-activator complex, and a 15 or 20 nt sgRNA	Gene editing or gene activation optimization.	*Arabidopsis* plants, poplar and rice calli, and rice and tomato protoplasts.	[18]
CasΦ	CasΦ variants: wtCasΦ, vCasΦ, and nCasΦ	Small, hypercompact enzyme, requiring a T-rich minimal PAM, efficient over a wide range of working temperatures, sensitive to chromatin environment, and with higher editing specificity.	Arabidopsis and maize protoplasts, and transgenic *Arabidopsis* plants.	[125,177]
	Cas12j2 variants: wtCas12j2, vCas12j2 and nCas12j2	Small enzyme, highly specific nuclease activity, T-rich PAM site (preferably 5′-NTTV-3′). Efficient editing in non-dividing cells. Gene activation and epigenome editing for fine-tuning target gene expression in plants.	Rice protoplasts and stable transformed lines, tomato protoplasts, and poplar transgenic plants.	[126]

[a]Abbreviations: CaMV, cauliflower mosaic virus; CBE, cytosine base editor; CmYLCV, *Cestrum* yellow leaf curling virus; DSB, double-strand break; evoFERNY, evolved cytosine deaminase; HDR, homology-directed repair; HDV, hepatitis delta virus; M-MLV, Moloney murine leukemia virus; NC, nucleocapsid; PAM, protospacer adjacent motif; PEG, polyethylene glycol; pegRNA, prime editing guide RNA; RNP, ribonucleoprotein; RT, reverse transcriptase.
[b]n*Sp*Cas9, *Sp*Cas9 nickase from *Streptococcus pyogenes* in which one endonuclease domain is mutated (D10A or H840A) and therefore only introduces single-strand nicks.

and thus implies the need to develop multiplex CRISPR-based approaches [130,135]. The role of tolerance (*T*) and sensitivity (*S*) genes, which positively or negatively regulate stress tolerance and adaptation, has been proposed [137]. Genes having a structural or a regulatory role, as well as *cis*-regulatory sequences, can be edited by CRISPR/Cas, but the possible occurrence of detrimental pleiotropic effects after such manipulations is an additional challenge to consider. In addition, modification of *R*/*T* genes requires the development of alternative, and often more complex, approaches based on genome editing/replacement by homologous recombination (HR), BE, or PE. The strategy of Lorenzo *et al.* [1] is based on the combination of simultaneous editing of gene families using up to 12 sgRNAs followed by subsequent crossings. This resulted in multiple mutant maize plants with improved response to water-deficient conditions which could be utilized in breeding programs.

Field trials with genome-edited plants are of paramount importance to confirm the efficacy of induced changes under normal production conditions, to demonstrate the durability of resistance or tolerance, and to check for the absence of off-target effects.

De novo domestication and rewilding

In the current scenario of global population increase and global climate changes, CRISPR/Cas approaches can facilitate the development of novel plant varieties that combine good agronomic performance with adaptability to stress-inducing environments and low-input management practices. This has the potential to enhance plant genetic resources for food and agriculture (PGRFA) and their utilization in agricultural systems based on 'sustainable intensification'. Although plant

domestication and genetic improvement have contributed to the present level of productivity and quality traits in elite cultivars when grown under favorable environments, these also have reduced genetic diversity and less resilience to stress conditions in comparison to their wild relatives. This has been confirmed at the genomic level by *de novo* assembly of the pan-genome in several crops, which highlighted the evolution of copy number and the presence/absence of variants. In some cases these were also related to response to stress conditions [138–140].

To capture this 'lost diversity' in elite cultivars, the 'rewilding' concept has been advanced by Palmgren *et al.* [141]. It aims to reintroduce mutations that are no longer present in the cultivated gene pool but are still available in wild progenitors (Figure 4). Rewilding can be achieved by introgression breeding, specific insertion of candidate genes, and precision mutagenesis. CRISPR/Cas-based approaches, including more advanced technological developments (e.g., multiplex methods, BE, and PE), can help to achieve these goals by favoring not only gene knockout but also gene knock-in and recombination at specified genomic locations [142].

To combine agronomically desirable traits with useful traits from wild species (Figure 4), *de novo* domestication approaches, including redomestication of crop progenitors as well as domestication of wild species, have been demonstrated in *Solanum pimpinellifolium* (a stress-tolerant wild tomato relative), *Physalis pruinosa* (groundcherry, an orphan crop distantly related to tomato), and *Oryza alta* (a wild tetraploid rice) by modifying 'domestication genes' via CRISPR/Cas-based technologies [138,143–145]. Because regulatory variants have been linked to domestication traits in various crops [146,147], novel *de novo* domestication approaches will soon involve CRISPR/Cas approaches in the modification of *cis*-regulatory sequences [142,148]. Other wild species, in some cases relatives of important crops such as potato, kiwi, and pepper, are in the pipeline for further applications [149,150].

Landraces derived from domestication and participatory breeding show adaptation to regional growing conditions and are appreciated by local consumers. Nevertheless, they often present defects that are incompatible with modern agriculture. CRISPR/Cas methods can be used to correct such defects without altering the genetic background and the general phenotype (Figure 4).

Nevertheless, to fully exploit these new breeding approaches, it will not only be important to apply available editing tools but also to select suitable genetic materials to start with, as well as to develop high-quality genomic information and efficient methods for the delivery of editing components and regeneration of edited cells.

Concluding remarks and future perspectives

In the first decade of use, CRISPR/Cas technology exceeded initial expectations by providing a precise, easy to implement, and high-throughput tool for targeted genetic modifications to plant research and breeding activities [151]. It was quickly adopted in most life-science laboratories, even those with no previous expertise of genetic modifications. Owing to its broad range of applications and adaptability to various molecular biology protocols, CRISPR/Cas is now the method of choice in fundamental research. It is used predominantly for gene targeting and knockouts, regulation of gene expression, introduction of heterologous DNA sequences at predefined genomic loci, and chromosome tagging. The future of plant genome editing relies on an expansion of the use of CRISPR/Cas based on more efficient multiplexing, high-throughput editing strategies, and applications towards chromosomal rearrangements and epigenomic changes. Furthermore, current difficulties with delivering Cas nucleases and gRNAs into plant organelles hinder their use for editing plastomes and chondriomes.

Outstanding questions

Is CRISPR/Cas technology readily adoptable by applied plant breeding programs? The tissue culture response of crop genotypes is one of the main bottlenecks for implementing CRISPR/Cas-based genome editing in applied breeding programs. More research will be necessary to develop novel transient methods for delivering editing reagents into plant cells. Such methods are unlikely to be based on conventional tissue culture regeneration protocols.

Can complex agronomic traits, such as productivity or tolerance to stress, be efficiently modified by CRISPR/Cas-based technologies? Functional studies and breeding of multigenic traits will require the development of efficient high-throughput and multiplexed CRISPR methods.

Are techniques available to detect products derived from CRISPR/Cas-based editing? Several advances have been proposed to trace gene-edited plants based on ddPCR and NGS. However, these techniques do not distinguish edited genomes from naturally occurring mutations. This is a major bottleneck for the EU approval of gene-edited plants.

Are available CRISPR/Cas-based editing tools sufficient to deal with all intended applications? Several new platforms have been developed in the past decade. Nevertheless, the discovery and development of new Cas enzymes characterized by small size, high fidelity, and different sequence recognition signals will be necessary to develop novel delivery methods, reduce off-target mutations, and broaden the sequence types that can be modified.

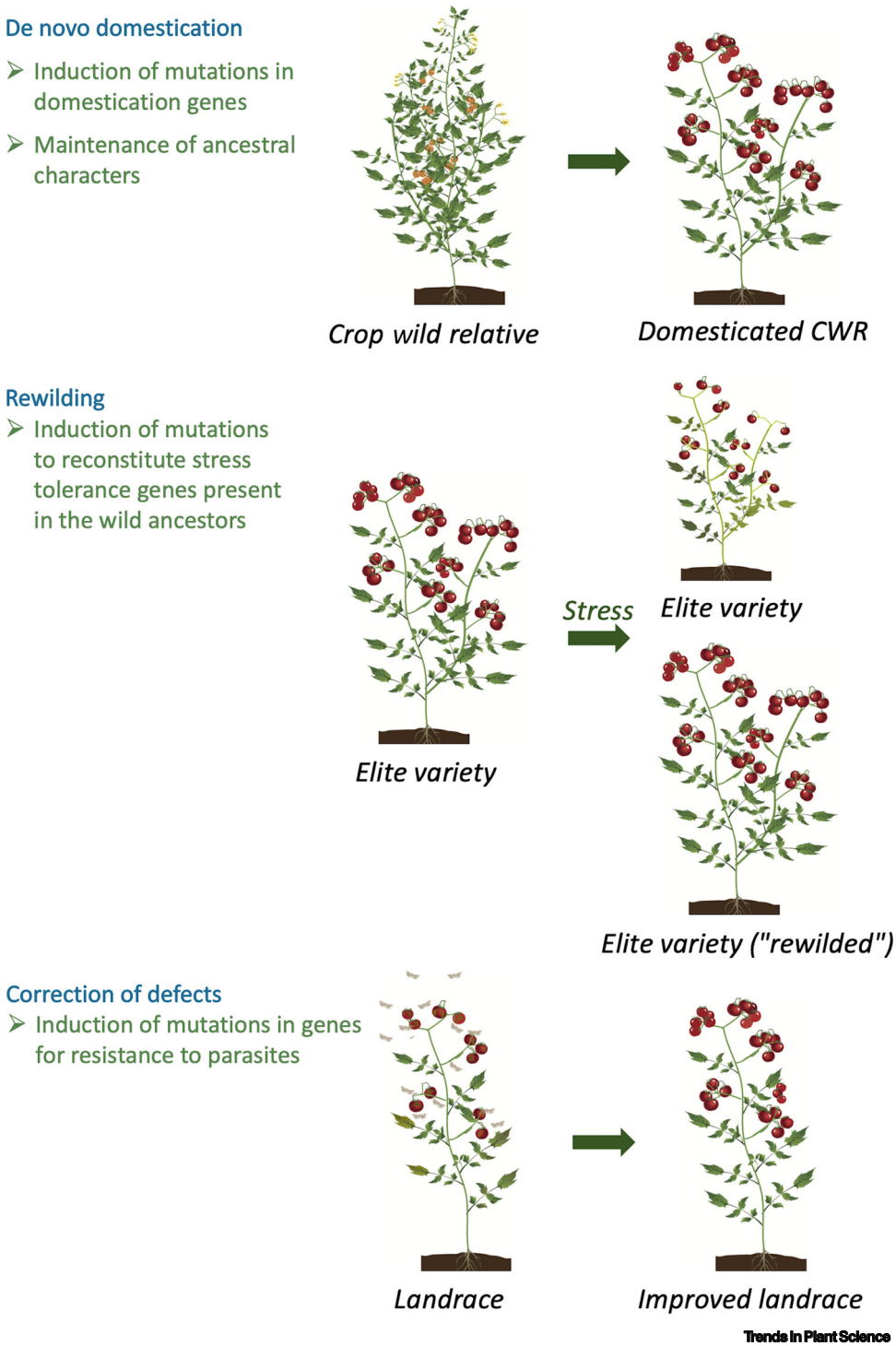

Figure 4. Application of CRISPR/Cas to induce mutations aiming at *de novo* domestication of crop wild relatives, rewilding of elite varieties, or correction of defects in landraces. Abbreviation: CWR, crop wild relative.

The application of high-throughput screening in plants is still in its infancy compared to animal cell culture systems. Nevertheless, high-throughput CRISPR experiments will be expanded [152], enabling the generation of mutant collections and CRISPR libraries using multiplexed CRISPR vectors and combinatorial vector assembly. New techniques for pooled plant transformation and CRISPR knockout screens will be developed. In addition, editing efficiencies are and will be increased by changing promoters, using tissue-specific promoters, and Cas codon optimization. New Cas enzymes will be discovered or are being developed, thus minimizing the limits on choosing the right target.

When performing extensive CRISPR screens in plants, it is crucial to produce a large number of regenerated plants. When studying crops with low transformation efficiency (e.g., maize), only a few genotypes will have the capacity to generate large screening populations. Improving the efficiency of plant regeneration will make it possible to scale up CRISPR studies. Alternative methods [virus-induced genome editing (VIGE) and nanotechnology-based] have been developed to avoid the need for *de novo* regeneration from tissue culture. However, to increase the adoption of these technologies, it will be important to overcome the limitations set by the size of the Cas enzyme.

In the future it will be important to broaden the targets for crop improvement. Researchers and breeders need to have a better understanding of the biological processes and genes involved, as well as of the pathways and their interaction with environmental factors. CRISPR will be a very valuable tool for functional gene studies. Genome-scale CRISPR mutant libraries could be made if some limitations are overcome [152], namely by developing prediction tools for gRNA efficiency and the editing outcome, recording genome-editing events at the single-cell level, and setting up large-scale combinatorial CRISPR screens.

Regarding the implementation of CRISPR-based systems in plant breeding schemes, the dependence on genotype for *in vitro* regeneration capacity undoubtedly remains one of the main challenges. However, some strategies discussed here open new perspectives, such as the activation of endogenous genes encoding developmental regulators, (transient) coexpression of transgenes with the same function, and the development of *in planta* editing protocols. The use of CRISPR-Combo to activate flowering-promoting factors [18] can also play a game-changing role in reducing juvenility in woody plants and shortening the duration of breeding cycles. Finally, analyses of pan-genomes have revealed the widespread presence of natural structural variants, highlighting their role during the evolution and domestication of crop plants. The possibility of inducing targeted chromosomal rearrangements sets new horizons for the increase/decrease of meiotic recombination, resulting in wider access to genetic variation in crop relatives in the former case and preservation of positive gene assets in the latter [153–155].

The technical challenges of CRISPR/Cas have been either (partially) overcome or will be tackled in future research, but the uncertain regulatory status of genome-edited plant varieties in the EU remains a major limitation for the applicability of CRISPR/Cas and other genome-editing technologies in plant breeding. Currently, EU legislation hinders adapted crops from being planted in the field and/or being marketed. The current regulation of genome-edited organisms as genetically manipulated organisms (GMOs), regardless of the type of introduced changes, restricts the use of precise plant breeding by genome editing to major, broad-acre crops such as maize and soybean. Their cultivation is thus financially feasible only for large (multinational) companies. To address these challenges in the EU, various initiatives [Cooperation in Science and Technology (COST) Action PlantEd 2019–2023, https://plantgenomeediting.eu/; and EU-SAGE (Sustainable Agriculture through Genome Editing), https://www.eu-sage.eu/] have been

launched. These initiatives aim to disseminate science-based information about new breeding techniques, which will be necessary to foster innovation and support advanced plant breeding as part of the transition to a more sustainable food production system in a fast-changing and challenging environment. New data on plant genome editing should promote the development of European and EU Member State policies based on scientific and socioeconomic aspects, thus enabling responsible use of genome editing for sustainable agriculture and food production (see also Outstanding questions).

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Declaration of interests

The authors declare no conflicts of interest.

Supplemental information

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