Applications of CRISPR–Cas in agriculture and plant biotechnology

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Abstract | The prokaryote-derived CRISPR–Cas genome editing technology has altered plant molecular biology beyond all expectations. Characterized by robustness and high target specificity and programmability, CRISPR–Cas allows precise genetic manipulation of crop species, which provides the opportunity to create germplasms with beneficial traits and to develop novel, more sustainable agricultural systems. Furthermore, the numerous emerging biotechnologies based on CRISPR–Cas platforms have expanded the toolbox of fundamental research and plant synthetic biology. In this Review, we first briefly describe gene editing by CRISPR–Cas, focusing on the newest, precise gene editing technologies such as base editing and prime editing. We then discuss the most important applications of CRISPR–Cas in increasing plant yield, quality, disease resistance and herbicide resistance, breeding and accelerated domestication. We also highlight the most recent breakthroughs in CRISPR–Cas-related plant biotechnologies, including CRISPR–Cas reagent delivery, gene regulation, multiplexed gene editing and mutagenesis and directed evolution technologies. Finally, we discuss prospective applications of this game-changing technology.

Green revolution
Refers to a great increase in crop production in the second half of the twentieth century through the use of fertilizers, the sue of agrochemicals, cultivation of high-yield crop varieties and mechanization.

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Global agricultural production is facing unprecedented challenges. By 2050, the world's population will reach 9.6 billion, and the demand for staple crops will have increased by 60% (REF.[1]). Since the rate of increase of yields brought about by the green revolution has been steadily declining, and detrimental climate change is expected to further limit plant production, cultivars with enhanced resilience to adverse environments and with increased yields and improved quality need to be generated. However, the conventional strategies used for crop breeding are laborious, time-consuming and complicated, and more-effective and time-saving breeding methods are required[2].

With the rapid progress in sequencing technologies, genomic information on an ever-increasing number of plant species is becoming available, and genome editing systems are offering the opportunity to edit genes with precision and creating new opportunities for crop improvement. The basic strategy of genome editing is to use a sequence-specific nuclease to induce a DNA double-strand break (DSB) at a target site. Thereafter, either the donor-dependent homology-directed repair (HDR) pathway or the error-prone non-homologous end joining pathway repairs the DSB and introduces some kind of genetic change. Early sequence-specific nucleases, including meganuclease[3], zinc-finger nucleases[4] and transcription activator-like effector nucleases[5], have been shown to be effective for plant genome editing, but their construction requires complex protein engineering, which limits their applicability.

CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated protein) is an adaptive phage immunity system in archaea and bacteria. As they rely on DNA–RNA recognition and binding for sequence-specific nucleic acid cleavage, CRISPR–Cas9 and other CRISPR–Cas systems can be easily programmed to introduce DSBs at any desired target site at minimal cost[6]. Since its first applications in plants in 2013 (REFS[7–9]), CRISPR–Cas has been used for genome editing in a variety of crop species, introducing into many of them agricultural traits of great value[10]. The newly developed precise CRISPR–Cas technologies, in particular, promise to have a major impact on agriculture owing to their capacity to induce precise nucleotide changes. However, CRISPR–Cas is capable of doing much more than just editing specific loci for crop improvement. A number of novel plant biotechnologies based on this versatile game-changing platform have emerged that are capable of promoting gene regulation and protein engineering. These technologies have already had an impact on fundamental biological research and have raised the prospect of widespread applications.

In this Review, we first describe the CRISPR–Cas-based molecular platforms used for precise genome editing. We then discuss the latest applications of

Donor
A nucleic acid (single-stranded DNA, double-stranded DNA or RNA) that has some homology with the region flanking a CRISPR–Cas-generated DNA break that can serve as a template during homology-directed repair.

Gene targeting
A genome editing technology that creates genome modifications, such as gene substitutions, insertions and deletions, through homology-directed repair.

Cas9 nickase
(nCas9). A term for catalytically defective Cas9 variants that cut only one strand of the target DNA; they include Cas9 bearing the mutations D10A or H840A, which cut the target strand and non-target strand, respectively.

Base transitions
Single-nucleotide changes that substitute one pyrimidine for another or one purine for another.

CRISPR–Cas in crop improvement, agricultural breeding and domestication of wild species. We also discuss several CRISPR–Cas-related plant biotechnologies, including novel delivery methods, gene-expression modulation, multiplexed and high-throughput gene editing, and in situ directed evolution. Our goal is to provide a comprehensive summary of the developments in CRISPR–Cas techniques in plants and to consider the prospects of future applications.

Precise genome editing in plants

Gene targeting technology in plants relies on HDR, which enables precise genome editing through the introduction of insertions, sequence replacements and nucleotide substitutions[11]. However, the low editing efficiency achieved with HDR has limited its application in plants[11,12]. Deaminase-mediated base editing and reverse transcriptase-mediated prime editing technologies are alternative genome editing technologies; as they do not involve DSB formation and do not require donor DNA, these CRISPR–Cas-based tools induce precise sequence editing and are more efficient than HDR in plants. Following the development of cytosine base editor (CBE) and adenine base editor (ABE) in human cells, dual base editor and base-editing-derived precise DNA deletion strategies were first developed in plants. In this section, we briefly describe the newly developed CRISPR–Cas technologies that are used to precisely edit plant genomes (TABLE 1).

Cytosine base editing

CBE is composed of a Cas9 nickase (nCas9) bearing the D10A mutation, which deactivates RuvC (one of the two Cas9 nuclease domains), fused with two proteins: a cytidine deaminase and an uracil DNA glycosylase (UDG) inhibitor (UGI). CBE introduces C:G>T:A base transitions directly into DNA sites targeted by single guide RNA (sgRNA)[13]. The deaminase deaminates cytidines to uridines in the non-target strand, which is the single-strand DNA (ssDNA) part of the R-loop generated by the nCas9 (D10A)–sgRNA complex, while the UGI prevents UDG from deaminating cytidines to apyrimidinic (AP) sites. When nCas9 (D10A) induces a nick on the target strand, the DNA mismatch repair pathway (or other DNA repair pathways) is activated and preferentially resolves the U:G mismatch into the desired U:A, and following DNA replication a T:A product, thereby generating a C:G>T:A base transition (FIG. 1a).

As this base editing technology provides high efficiency of precise editing, CBE systems have been optimized and developed in various plant species[14–16]. Several cytidine deaminase orthologues with different base editing features have been incorporated into plant CBEs (TABLE 1). Rat APOBEC1-based CBEs edit cytosines in editing windows of approximately seven nucleotides from position 3 to position 9 within the protospacer, and, depending on the sequence motif, prefer TC not GC. By contrast, *Petromyzon marinus* (lamprey eel) cytidine deaminase 1 (CDA1)-based CBEs and human

Table 1 | **Features of precise editing technologies in plants**

Editing technology	Main effectors	Cas protein	With UGI	Sequence changes	Product purity[a]	Motif preference	Editing window	Editing efficiency relative to prime editor
CBE	Rat APOBEC1 (REF.[16])	nCas9 (D10A)	Yes	C:G>T:A	High	No preference for GC motif	3–9	3
	Petromyzon marinus CDA1 (REF.[14])	nCas9 (D10A)			High	No	1–9	4
	Human AID*Δ[17]	nCas9 (D10A)			High	No	3–12	3
	Human APOBEC3A[15]	nCas9 (D10A)			High	No	1–17	5
	Human APOBEC3Bctd-VHM[18]	nCas9 (D10A)			High	No preference for GC motif	4–8	4
	Human APOBEC3Bctd-KKR[18]	nCas9 (D10A)			High	No	4–7	4
ABE	ecTadA-ecTadA*[22,23] or ecTadA*[24]	nCas9 (D10A)	No	A:T>G:C	Very high	No	4–8	2
STEME	APOBEC3A–ecTadA–ecTadA*[19]	nCas9 (D10A)	Yes	C:G>T:A and A:T>G:C	High	No	C_1-C_{17}, A_4-A_8	2
AFID	APOBEC3A[28]	Cas9	Yes	Designed multinucleotide deletion	Moderate	No	NA	5
	APOBEC3Bctd[28]	Cas9			Moderate	Preference for TC motif	NA	5
Prime editor	M-MLV reverse transcriptase or CaMV reverse transcriptase[30–32]	dCas9 or nCas9 (H840A)	No	12 kinds of base substitutions, multiple base substitutions, and precise insertions (<15 bp) and deletions (<40 bp)	Moderate	No	1–50	1

ABE, adenine base editor; AFID, APOBEC–Cas9 fusion-induced deletion system; APOBEC3Bctd, carboxy-terminal catalytic domain of APOBEC3B; CBE, cytosine base editor; CaMV, cauliflower mosaic virus; dCas9, catalytically inactive Cas9; ecTadA, *Escherichi coli* tRNA-specific adenosine deaminase; ecTadA*, *Escherichi coli* tRNA-specific adenosine deaminase variant; M-MLV, Moloney murine leukaemia virus; NA, not available; nCas9, Cas9 nickase; STEME, saturated targeted endogenous mutagenesis editor; UGI, uracil DNA glycosylase inhibitor. [a]Product purity is evaluated by the proportion of desired editing products.

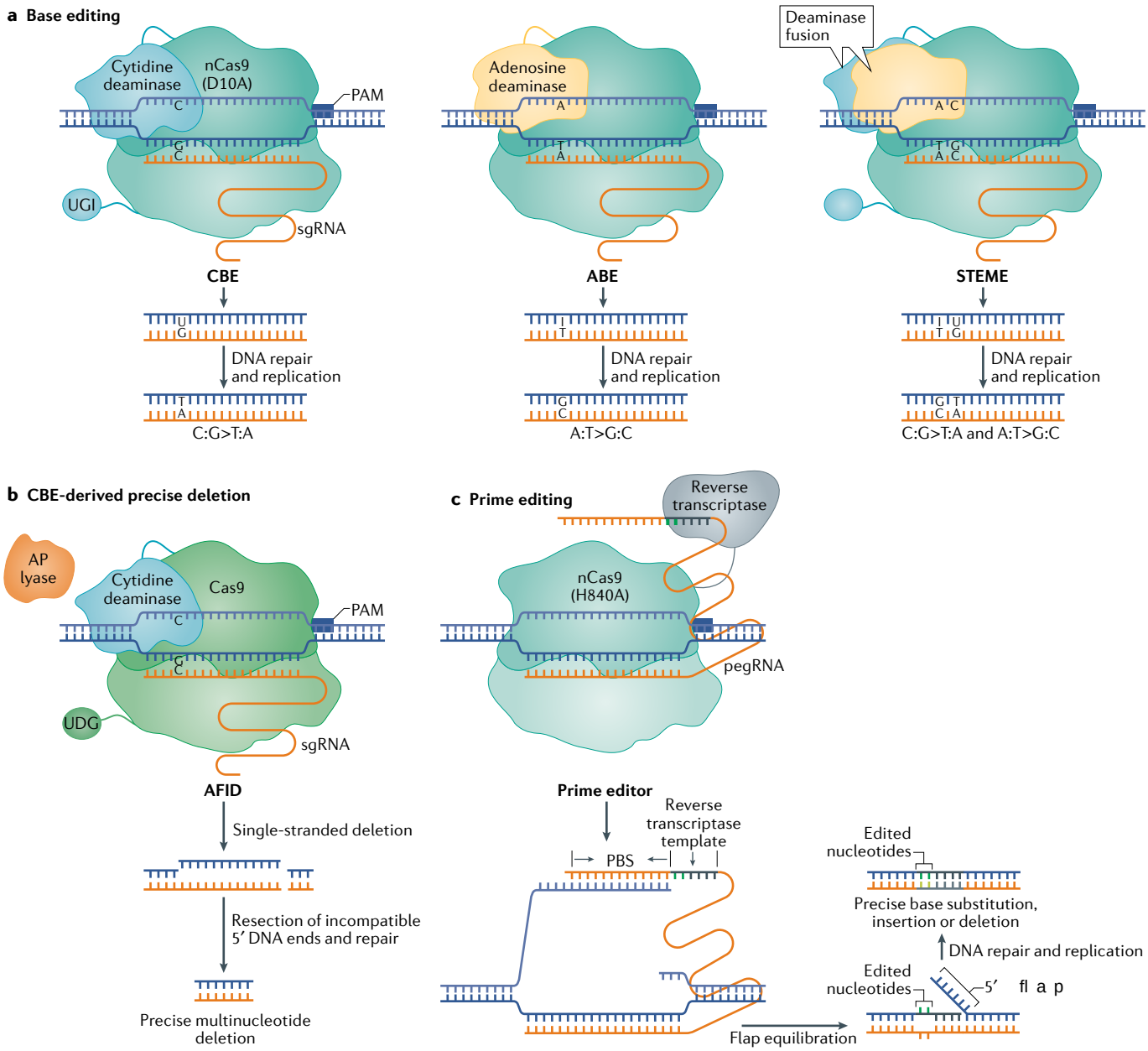

Fig. 1 | **Deaminase-mediated and reverse transcriptase-mediated precise genome editing technologies in plants. a** | Base editing technologies in plants. Base editors comprise Cas9 nickase (nCas9; bearing a D10A mutation) fused with two proteins: a deaminase and an uracil DNA glycosylase inhibitor (UGI). The cytosine base editor (CBE) generates C:G>T:A base substitutions, whereas the adenine base editor (ABE) generates A:T>G:C base substitutions. A fusion of nCas9 with these two base editors, referred to as a 'saturated targeted endogenous mutagenesis editor' (STEME), generates simultaneous C:G>T:A and A:T>G:C dual base substitutions using one single guide RNA (sgRNA). **b** | The APOBEC–Cas9 fusion-induced deletion system (AFID). Cytidine deaminase converts cytidine to uridine, and then a uracil DNA glycosylase (UDG), which is fused to the cytidine deaminase–Cas9 complex, excises the uridine and generates an apyrimidinic (AP) site, which is nicked by the separate yet co-expressed AP lyase. Following base excision repair, a precise deletion is generated between the site of the Cas9-induced DNA double-strand break and the deaminated cytidine. **c** | Prime editing technology. The prime editor tool is composed of a fusion of nCas9 with reverse transcriptase and a prime editing guide RNA (pegRNA). The pegRNA carries the desired mutations (green pegs) at the 3′ end of the reverse transcriptase template. The primer-binding site (PBS) binds to the nicked DNA strand, thereby priming reverse transcription of the template into the desired DNA sequence. The edited nucleotides are then inserted into the target site in a precise way. PAM, protospacer adjacent motif.

Single guide RNA (sgRNA). An artificial fusion of CRISPR RNA and *trans*-activating CRISPR RNA, which guides Cas9 to the target site through DNA–RNA recognition.

activation-induced cytidine deaminase (AID)-based CBEs are much more efficient in GC motifs in rice and seem to have no strong motif preference[14,17]. Similarly to *P. marinus* CDA1-based and human AID-based CDEs, human APOBEC3A (hAPOBEC3A)-based CBEs also have high base editing efficiencies without motif preference, with base editing windows from position 1 to position 17 in the protospacer[15]. Very recently, two newly developed CBEs, based on rationally designed truncated human APOBEC3B (hAPOBEC3B) showed high specificity and precision in rice plants[18]. Finally, Cas9 dependent on variants of the protospacer adjacent motif (PAM) and Cas9 orthologues have been developed to avoid targeting restriction by the canonical PAM

R-loop
A nucleic acid structure formed when the Cas9–single guide RNA (sgRNA) complex invades the target DNA and the sgRNA forms a DNA–RNA hybrid with the target strand while displacing the non-target strand.

Editing windows
Regions of the target DNA in which base substitutions are induced by base editors; the window is usually numbered in ascending order from the distal end of the protospacer adjacent motif.

Protospacer
A two- to six-nucleotide sequence within the guide RNA that determines the target site of CRISPR–Cas. It is located at the 5′ terminus of the single guide RNA in Cas9 and the 3′ terminus of the CRISPR RNA in Cas12a.

Protospacer adjacent motif
(PAM). The DNA motif flanking the target sequence, which is indispensable for target recognition and cleavage by CRISPR–Cas. For *Streptococcus pyogenes* Cas9, the PAM is 5′-NGG-3′.

Editing scope
The length of the genomic sequence that can be targeted for editing given the requirements of the particular protospacer adjacent motif.

sgRNA scaffold
(scRNA). A single guide RNA harbouring RNA aptamer hairpins in its tetraloop, stem loop 2 or 3′ end.

Directed evolution
A protein engineering method that generates user-defined proteins or DNA by mimicking the process of natural selection.

(NGG; where N is any nucleotide) and expand the editing scope of CBE systems in plants[19,20].

Adenine base editing

ABEs expand base editing to include A:T>G:C substitutions using adenosine deaminase as an effector, fused with nCas9 (D10A)[21]. Adenosine deaminase deaminates adenosines to inosines, which are recognized as guanosines by DNA polymerase during DNA repair and replication (FIG. 1a). Although there is no natural adenosine deaminase for deaminating ssDNA, such an enzyme has been evolved from *Escherichia coli* tRNA-specific adenosine deaminase (ecTadA)[21].

ABEs based on evolved ecTadA variants (ecTadA*) have been developed in rice, wheat, *Arabidopsis thaliana* and rapeseed[22,23] (TABLE 1). However, they are inefficient at some targets, and several strategies have been used to increase their editing efficiency in monocots, such as adding three SV40 nuclear localization sequences to the C terminus of nCas9, generating enhanced sgRNAs by modifying the sgRNA scaffold (scRNA) and using a simplified ecTadA* monomer version[22,24]. In *A. thaliana* and rapeseed, the *RPS5A* gene promoter driving the expression of plant ABEs is more efficient than the constitutive 35S promoter or the egg-cell specific *YAO* promoter used for *Agrobacterium*-mediated transformation[23]. The editing scope of the ABE system has also been expanded with use of PAM variants, but these are less efficient than the original *Streptococcus pyogenes* Cas9 (SpCas9) or *Staphylococcus aureus* Cas9 (SaCas9) versions[19,20], and several ABE8 versions recently developed for human cells may be useful for increasing the efficiency of A>G base editing in plants[25,26].

Dual base editing

A cytosine and adenine dual base editor has been created to simultaneously perform C:G>T:A and A:T>G:C editing in plants using a single sgRNA[19] (TABLE 1). It uses a cytidine deaminase (APOBEC3A), an adenosine deaminase (ecTadA–ecTadA*), nCas9 (D10A) and a UGI fusion, and is referred to as 'saturated targeted endogenous mutagenesis editor' (STEME) (FIG. 1a). The STEME system deaminates cytidines to uridine and adenosines to inosines in the editing window of the protospacer, and these are then copied by DNA repair and replication, generating dual C:G>T:A and A:T>G:C substitutions. An SpCas9–NG PAM variant[27], which recognizes NG PAMs, has been used to expand the editing scope and to increase the ability to edit as many targets as possible. These dual base editors facilitate directed evolution of endogenous plant genes in situ. STEME might also be used to change *cis* elements in regulatory regions and genome-wide screening in a high-throughput manner in plants.

CBE-based precise DNA deletion

In CBEs, uridine generated by deaminating cytidines are preserved by the UGI, which inhibits the activity of the cellular UDG. The opposite situation, in which UDG is overexpressed, should trigger base excision repair and lead to excision of the uridines and generation of AP sites, which can be nicked by AP lyases[28]. The combination of such a nick with the formation nearby of a DSB by Cas9 should produce a specified and precise deletion between the deaminated cytidine and the Cas9 cleavage site (FIG. 1b). Following this rationale, tools for generating precise multinucleotide deletions, which comprise a cytidine deaminase, Cas9, UDG and AP lyase — referred to as 'APOBEC–Cas9 fusion-induced deletion systems' (AFIDs) — have been developed to induce specific deletions within the protospacer (FIG. 1; TABLE 1). Two cytidine deaminases, hAPOBEC3A and the C-terminal catalytic domain of hAPOBEC3B (hAPOBEC3Bctd) have been used in AFIDs: hAPOBEC3A yields a predictable DNA deletion ranging from the targeted cytidine to the Cas9-induced DSB, and hAPOBEC3Bctd yields precise DNA deletions ranging from the TC-preference motif to the Cas9-induced DSB; these deletions ensure more uniform products. AFIDs add to the precise editing systems that facilitate the formation of in-frame deletions, interfere with DNA regulatory elements and allow editing of microRNAs.

Prime editing

Although CBE and ABE can induce precise base transitions, tools for generating base transversions are limited. A revolutionarily genome editing technology that solves this problem was developed in 2019. Termed 'prime editor', this technology can produce in human cells all 12 kinds of base substitutions, precise insertions of up to 44 bp, deletions of up to 80 bp and combinations of these edits[29]. Prime editor uses a fusion of nCas9 (H840A) and reverse transcriptase, which is complexed with a prime editing guide RNA (pegRNA). The latter is composed of a reverse transcriptase template and a primer-binding site at the 3′ end of the sgRNA. The reverse transcriptase template contains the genetic information for the desired mutations, and the primer-binding site pairs with the nCas9 (H840A)-nicked ssDNA strand, thereby priming reverse transcription and incorporating the genetic information from the reverse transcriptase template into the genome (FIG. 1c). This is then followed by equilibration between the edited 3′ flap and the unedited 5′ flap, ligation and repair, which generate the desired edit. As prime editor generates base substitutions and short insertions and deletions at a relatively wide range of positions (+1 to +33, counting from the first base 3′ of the pegRNA-induced nick), it is not substantially constrained by its PAM.

This powerful and elegant technology was shown to create and correct mutations that cause genetic diseases in human cells. Subsequently, prime editor systems were developed and tested in rice and wheat, and were shown to generate all 12 base substitutions, multiple base substitutions simultaneously, and insertions and deletions in rice and wheat[30–32]. However, the editing efficiency of prime editor in plants remains limited despite the use of orthogonal strategies, such as use of reverse transcriptase orthologues with different catalytic activities, use of ribozymes to produce precise pegRNAs, raising of the culture temperature to increase catalytic activities, incorporation of enhanced sgRNA scaffold modifications into pegRNA to increase the binding activity of Cas9 and manipulation of selective markers for enrichment of edited cells[30–32]. Of note, the ability of prime editor to induce precise edits has been shown only in

rice and wheat; its activity in other plants still needs to be tested. Moreover, the ability of prime editor to produce larger genetic modifications (hundreds of bases) and its specificity have not been demonstrated in either mammalian cells or plants. Thus, more work is needed to improve and expand plant prime editing technology.

CRISPR–Cas in crop upgrade and breeding

Owing to its unparalleled ability to precisely manipulate plant genomes, CRISPR–Cas has emerged as a powerful tool in agriculture. It has not only helped to develop novel varieties with desirable traits but has also revolutionized current breeding systems. In addition, CRISPR–Cas has offered the possibility to domesticate novel species in a short time. Most studies discussed in this section used SpCas9 for genome editing, except where otherwise specified.

Applications in crop improvement

Unlike conventional breeding approaches, CRISPR–Cas technology provides a rapid way to generate ideal germplasms by deleting negative genetic elements responsible for undesired traits or introducing gain-of-function mutations through precise genome editing. As shown in Supplementary Table 1, in the past 2 years, the use of CRISPR–Cas has improved several crop characteristics, including yield, quality, disease resistance and herbicide resistance.

Increasing yield. Among the numerous factors affecting yield, manipulating cytokinin homeostasis is a practical way to increase cereal yield. Editing the C terminus of *Oryza sativa LOGL5*, which encodes a cytokinin-activation enzyme in rice, enhanced grain yield in a variety of environmental conditions[33]. Similarly, knocking out the gene that encodes cytokinin oxidase/dehydrogenase (CKX), an enzyme that catalyses cytokinin degradation, generated high-yield phenotypes in wheat[34]. Through knocking out the gene that encodes amino acid permease 3, which is involved in nutrient partitioning, rice varieties were bred with elevated tiller numbers and increased yields in combination with maintaining grain quality[35]. CRISPR–Cas-mediated editing of other genes, including *O. sativa PIN5b* (regulating panicle size), *O. sativa GS3* (regulating grain size) and *Triticum aestivum GW2*, *O. sativa GW2* and *O. sativa GW5* (regulating grain weight), has also given rise to crop plants with increased yields[36–39]. In addition to cereals, researchers have also increased the yield of fruit crops by editing *CLV*[40] and *ENO*[41], which control meristem size.

Improving quality. Crop characteristics other than yield are also critical for agricultural production. Grain with low amylose content has better eating and cooking quality, and widespread applications in the textile and adhesives industries. As granule-bound starch synthase 1 (GBSS1) is crucial for amylose biosynthesis, waxy maize varieties were generated in 12 elite inbred lines by disruption of *GBSS1* with CRISPR–Cas9 (REF.[42]), and rice lines with a continuum of low amylose contents were created by alteration of the amino acid sequence of GBSS1 with a CBE[43]. These strategies can be easily applied in other crops (Supplementary Table 1). However, low amylose content is not always desirable, as cereal grains high in amylose are beneficial to human health. By targeting of the starch branching enzyme that forms the amylopectin biosynthesis pathway, rice lines with a higher content of amylose were created[44]. Gluten proteins in wheat grains can cause coeliac disease in susceptible individuals. As gluten proteins are encoded by ~100 loci in the wheat genome, traditional breeding methods cannot substantially decrease gluten content. By use of CRISPR–Cas to target the conserved region of gluten genes, low-gluten wheat lines with up to 85% loss of immunoreactivity have been created[45]. Moreover, CRISPR–Cas has also facilitated the breeding of high-quality crops with enriched carotenoid[46,47] and γ-aminobutyric acid[48], reduced phytic acid[49] and high oleic acid contents[50].

Disease resistance. Compared with introducing dominant resistance genes, which tends to promote the reciprocal evolution of resistance in pathogens, disrupting host susceptibility factors using CRISPR–Cas is a more promising approach for protecting plants against biotic stress. Global rice production is strongly threatened by bacterial blight, which is a devastating disease caused by *Xanthomonas oryzae* pv. *oryzae*. During infections, a group of bacterial factors can activate transcription of the SWEET genes, whose products are needed for disease susceptibility. By mutating the promoter region of *O. sativa SWEET11*, *O. sativa SWEET13* and *O. sativa SWEET14* using CRISPR–Cas, researchers have generated rice lines with broad-spectrum resistance to *X. oryzae* pv. *oryzae*[51,52]. Similarly, targeting the promoter region of *Citrus* × *sinensis LOB1* can confer resistance to *Xanthomonas citri* subsp. *citri* in citrus[53].

As a biotrophic fungus, *Blumeria graminis* f. sp. *tritici* can cause powdery mildew in wheat. *Enhanced disease resistance 1* (*EDR1*) is a gene encoding an MAPK kinase kinase that inhibits defence responses to powdery mildew; simultaneous mutation of the three wheat homologues of *EDR1* by CRISPR–Cas gave rise to plants with enhanced resistance to *B. graminis* f. sp. *tritici*[54]. Likewise, simultaneous mutation of all three *mildew-resistance locus O* (*MLO*) homologues created a wheat variety with broad-spectrum resistance to powdery mildew[55], and targeting *Solanum lycopersicum MLO1* by CRISPR–Cas in tomato conferred tolerance to *Oidiumneo lycopersici*, which causes powdery mildew in tomato[56].

Owing to its ability to induce DSBs, CRISPR–Cas9 can be programmed to cleave the genomes of plant DNA viruses and confer virus resistance. However, careful assessment of the CRISPR–Cas9 approach should still be considered, as the technology has been reported to generate plants with no or limited levels of virus resistance, thereby driving rapid generation of viral escapes[167]. Using this tactic, researchers have established plant immune systems against geminivirus[57] and caulimovirus[58]. Similarly, by harnessing of the RNA-targeting Cas13a, Cas13b, Cas13d and *Francisella novicida* Cas9, defence against a number of RNA viruses has been created[59,60]. Knocking out plant susceptibility genes is also an effective way of generating broad-spectrum virus resistance. Potyviruses are a group of plant RNA viruses that hijack

Hybrid vigour
The phenomenon of heterozygotes formed from homozygous parents often exhibiting better agronomic performance than either parent.

the host factor eukaryotic translation initiation factor 4E (eIF4E) and its isoforms to initiate their translation. As eIF4E is not essential for plant growth, disrupting the *Cucumis sativus* eIF4E gene conferred broad-spectrum resistance to potyviruses in cucumber without causing fitness penalty[61].

Herbicide resistance. As weed problems are globally increasing, developing herbicide-resistant germplasms becomes a cost-effective way of maintaining high crop productivity and preventing soil degradation. Compared with conventional transgenic methods that introduce foreign herbicide-resistant genes such *bar*, which encodes phosphinothricin *N*-acetyltransferase into crops, editing herbicide-targeted genes to confer endogenous resistance using CRISPR–Cas is attractive on account of its speed, flexibility and transgene-free nature. Acetolactate synthase (ALS) is a key enzyme in branched-chain amino acid biosynthesis and the target of herbicides such as sulfonylurea and imidazolinone. Studies of naturally occurring point mutations in the ALS gene have revealed that specific amino acid substitutions in ALS can bring about herbicide tolerance[62]. Therefore, introduction of particular base transitions into *O. sativa ALS* with use of CBEs conferred herbicide resistance to rice while retaining ALS activity[14,155]. Similar procedures were also used in other species, where certain mutations in *ALS* were introduced through HDR to confer herbicide tolerance[63]. Acetyl coenzyme A carboxylase (ACCase) is a crucial enzyme in lipid biosynthesis and another valuable herbicide target. Introduction of a C2186R substitution by an ABE into the *O. sativa* ACCase gene generated rice strains tolerant of haloxyfop-R-methyl[22]. Similarly, quizalofop-resistant wheat was produced by inducing an A1992V substitution in *T. aestivum* ACCase[64]. Other amino acid substitutions in ACCase, such as W2125C and P1927F, which were discovered by CRISPR-based screening, also confer haloxyfop resistance in rice[19,156]. Furthermore, editing *EPSPS*[65], *PPO*[66], *TubA2* (REF.[67]) and *SF3B1* (REF.[68]) has been reported to confer resistance to glyphosate, butafenacil, trifluralin and herboxidiene (GEX1A), respectively. In addition to their agricultural applications, these herbicide resistance alleles can also be used as selective markers to enrich for gene editing events[14,64].

Although most plant varieties created by genome editing are still at the experimental stage, so far more than 100 plant varieties created by genome editing technologies have been designated as not regulated by the US Department of Agriculture, allowing commercial cultivation in the USA, including oleic acid-enriched soybean varieties produced by disruption of *Glycine max FAD2* (REF.[50]), powdery mildew resistant wheat[55] and high-oil content camelina[69].

Applications in breeding technologies

Although CRISPR–Cas has shown great ability to improve crops, combining it with conventional breeding methods would further benefit agricultural production. A number of novel breeding approaches that target reproduction-related genes using CRISPR–Cas have recently emerged.

Haploid induction. Doubled haploid technology can stabilize the genetic background of hybrid lines within two generations, compared with the six to eight generations of selfing that traditional approaches require to produce homozygosity. Frameshift mutations in *MATRILINEAL* (*MTL*), which encodes the pollen-specific phospholipase A1, can trigger elimination of the paternal chromosomes in the zygote, leading to the formation of haploid maize embryos[70] (FIG. 2a). Haploid induction lines of wheat and rice have also been created by CRISPR-mediated mutagenesis of *MTL*[71,72]. Editing of other genes, such as *CENH3* (REF.[73]) and *DMP*[74,75], by CRISPR–Cas has efficiently induced haploidization.

Generating male sterile lines. Hybrid vigour has been exploited extensively in agricultural breeding to increase yields and improve quality. However, to commercially produce hybrid seeds, self-pollination of the female parent has to be avoided to eliminate homozygous seeds. Among several ways to solve this problem, establishing male sterility in maternal lines has been the most effective and practical approach. Although a number of male-sterile lines have been documented in various crops, transbreeding male sterility into other genetic backgrounds is generally time-consuming and laborious. Gene editing by CRISPR–Cas provides a rapid way to establish male sterility in transformable lines. By targeting *male sterile 1* (*Ms1*) and *Ms45*, which encode a glycosylphophatidylinositol-anchored lipid transfer protein and a strictosidine synthase-like enzyme, respectively, researchers introduced male sterility into hexaploid wheat cultivars[76,77]. A male-sterile tomato line was also created by mutation of a putative strictosidine synthase gene[78]. These strategies have also been generalized to other species. Moreover, by disruption of *thermosensitive genic male-sterile 5* (REF.[79]) and *carbon starved anther*[80], thermo-sensitive and photoperiod-sensitive genic male sterile lines, which are more flexible and easy to use, have been established in rice and maize, respectively.

Fixation of hybrid vigour. Although systems for production of hybrid seeds based on male-sterile lines are well established, they remain costly and laborious in some crops. Inducing apomixis, which is a naturally occurring asexual reproduction pathway, would be an alternative solution for fixing elite hybrid backgrounds. Studies have shown that rice and *A. thaliana* with mitosis instead of meiosis (*MiMe*) genotypes comprising the *PAIR1*, *REC8* and *OSD1* triple mutation induced by CRISPR–Cas (genes that cause the abolishment of meiotic recombination, the separation of sister chromatids in the first meiotic division and the skipping of the second meiotic division, respectively), produce clonal diploid gametes and tetraploid seeds. By ectopic expression of *BABY BOOM 1*, which promotes embryogenesis in egg cells of *MiMe* rice, parthenogenesis can be triggered and results in progeny genetically identical to the female parents[81] (FIG. 2b). In a similar way, clonal diploid embryos in rice were generated by disruption of *MTL* (causing paternal genome elimination after fertilization) and *MiMe* genes[82] (FIG. 2b). Although these synthetic-apomictic germplasms cannot yet be used to mass-produce hybrid

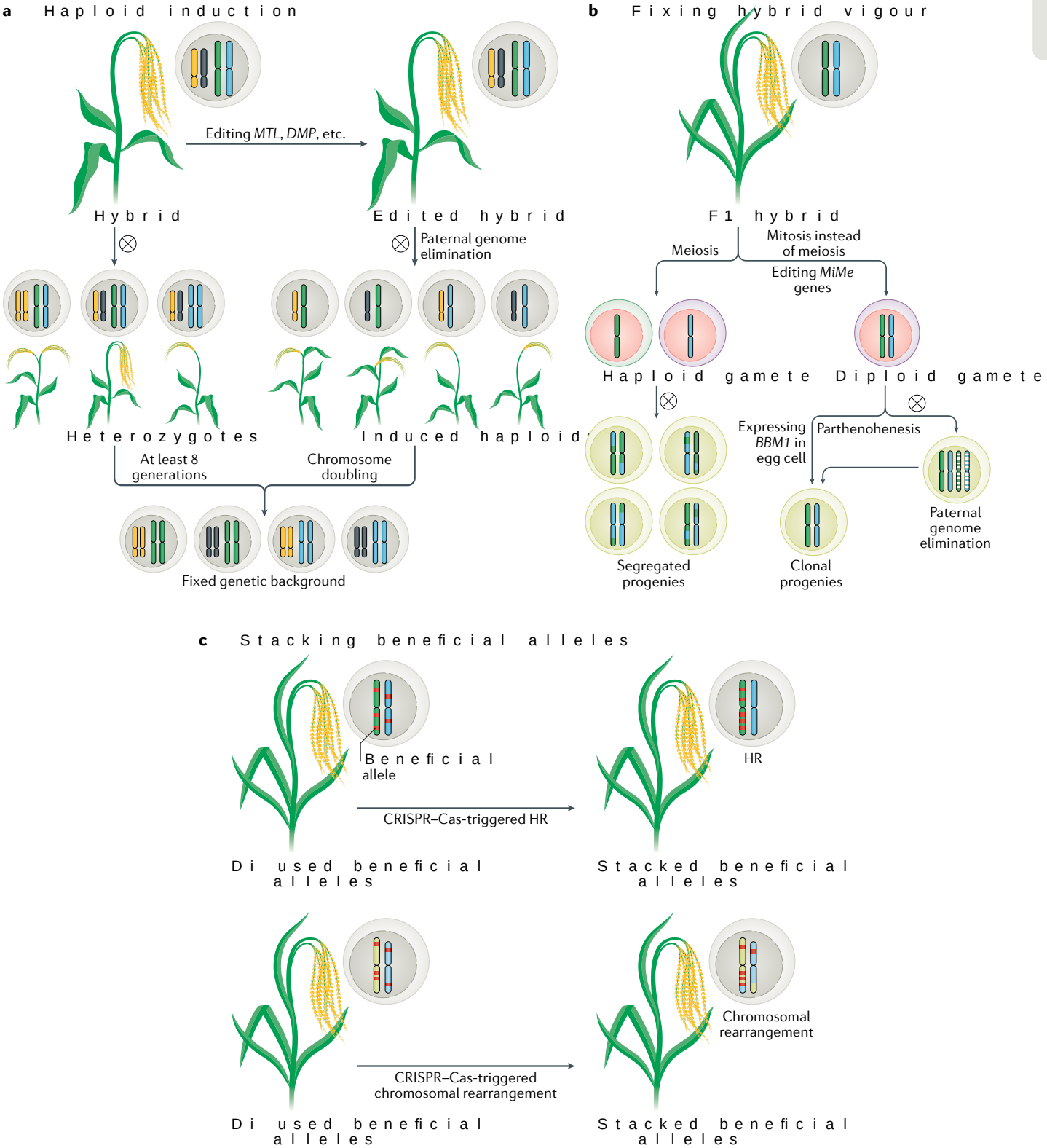

Fig. 2 | **Applications of CRISPR–Cas9 in breeding technologies. a** | Haploid induction. CRISPR–Cas9 editing of genes such as *MATRILINEAL* (*MTL*) and *DMP* creates haploid induction lines, which shortens the time required to stabilize genetic backgrounds. **b** | Inducing apomixis to fix hybrid vigour. Plants with the mitosis instead of meiosis (*MiMe*) genotype can generate diploid gametes, either by ectopically expressing *BABY BOOM 1* (*BBM1*) in egg cells and triggering parthenogenesis or by disrupting *MTL* and thus causing elimination of the paternal genome. **c** | Triggering homology-directed repair or a chromosomal translocation at intended sites by CRISPR–Cas to consolidate beneficial alleles or break undesirable genetic linkages. HR, homologous recombination.

Self-incompatibility
Situations in which female and male gametes are both fertile but the pollen cannot germinate on stigmas with the same or a similar genotype.

Orphan crops
Crops cultivated and consumed regionally, which are generally not fully domesticated and are especially essential in developing countries.

seed owing to their poor fertility and low apomixis induction rate, these approaches can be applied directly to crops such as vegetables and pastures, in which seed production is less valuable.

Manipulating self-incompatibility. Genetic improvement of some crops such as potato has been hampered by the lack of inbred lines due to their intrinsic self-incompatibility. Through mutation of *S-RNase* by CRISPR–Cas, which is a co-dominant gene responsible for gametophytic self-incompatibility in the Solanaceae, self-compatible potato lines have been created[83]. Furthermore, sporophytic self-incompatibility has been overcome by disruption of *M-locus protein kinase* and *S-receptor kinase* in oilseed rape[84] and cabbage[85], respectively. In addition to reducing heterozygosity, this approach also promises to overcome interspecific reproductive barriers and eliminate the need for pollinizers in fruit trees. In addition, through mutation of genes such as *farnesyl pyrophosphate synthase 2*, CRISPR–Cas can also have a role in restoring self-incompatibility[83], and can be used to develop more-effective hybrid breeding systems and achieve the production of seedless fruit in citrus crops by triggering parthenocarpy.

Other breeding technologies. Crossing between distant lines usually results in severe hybrid sterility, which is caused by deleterious genetic interactions between divergent alleles and hinders the exploitation of hybrid vigour. By knocking out partial copies of the *Sc-i* allele, which suppress the expression of the pollen-essential *Sc-j* allele, the male-fertility of *O. sativa japonica-indica* hybrids can be restored[86]. Hybrid-compatible African–Asian rice hybrid lines have also been bred by disruption of *Oryza glaberrima TPR1* (REF.[87]). Homologous recombination during meiosis rarely occurs at desired sites, but specifically targeting one parental allele by CRISPR–Cas[88] can trigger meiotic homologous recombination at specific sites (FIG. 2c). In addition, introducing a DSB on each of two heterologous chromosomes can trigger reciprocal chromosomal translocations[89] (FIG. 2c). Such approaches could be used to stack beneficial alleles, break undesirable genetic linkages and rapidly create near-isogenic lines.

CRISPR–Cas-accelerated domestication

Since the inception of agriculture, more than 10,000 years ago, cultivation has involved artificially selecting for desirable traits such as high yield, nutrient richness and ease of harvest. However, this productivity-directed breeding process generally results in loss of genetic diversity and vulnerability to biotic and abiotic stresses[90]. It is estimated that 70% of the calories humans need come from only 15 of a total of 30,000 edible plants[91]. In comparison with established crops, nature has provided us with a huge reservoir of genetic variation that we do not yet exploit: wild species and orphan crops often have favourable nutritional attributes or stress resilience and are better adapted to local climates. Therefore, domesticating wild species or use of semidomesticated crops is an attractive way to meet the ever-increasing demand for food. Traditional domestication is a lengthy process involving changes to many loci, only a few of which have key roles in driving the desired outcome[92]. CRISPR–Cas, with its capacity for accurate genome manipulation, could undoubtedly accelerate the process of domesticating crops.

Several pioneering studies of accelerated domestication have already been conducted. Modern tomatoes are easily affected by environmental stresses, whereas *Solanum pimpinellifolium*, a putative ancestor of tomato, is highly resilient to bacterial spot disease and salt. However, to develop *S. pimpinellifolium* into a commercial crop, a set of undesirable features, such as a sprawling growth pattern, small fruit, poor nutritional value and day-length sensitivity, have to be changed. Using accumulated knowledge of these phenotypes, researchers have used a multiplexed CRISPR–Cas system to simultaneously edit related genes, including *SP* (plant growth habit), *SP5G* (floral induction), *CLV3* and *WUS* (fruit size), *MULT* (fruit number), *OVATE* (fruit shape), *GGP1* (vitamin C content) and *CycB* (lycopene content), and brought *S. pimpinellifolium* a step closer to becoming an attractive tomato cultivar[93,94]. Importantly, these domesticated plants retained the excellent resistance of *S. pimpinellifolium* to pathogenic bacteria and salt. Similarly, additional domestication of ground cherry (*Physalis pruinosa*), which is an orphan Solanaceae crop, was achieved by disruption of three genes, *SP*, *SP5G* and *CLV1*, and the resulting plants were shorter and had more flowers and larger fruits[95]. Studies aimed at domesticating African rice (*O. glaberrima*)[96] have also been implemented. These studies laid the foundation for accelerated domestication.

Other species are also attractive candidates for future agricultural exploitation. Intermediate wheatgrass (*Thinopyrum intermedium*), a perennial relative of wheat, is of agricultural interest because it takes up water and nutrients more effectively than wheat and requires less labour. However, several characteristics, such as seed shattering and low yield, hinder its expanded cultivation[97]. Quinoa (*Chenopodium quinoa*), another orphan crop, is also an ideal domestication candidate due to its excellent tolerance to abiotic stress and high nutritional value, but its short-day requirement and heat sensitivity require modification. Unlike modern potatoes, wild potatoes (*Solanum* spp.) are highly resilient to late blight disease and have a healthier glycaemic index, but their high levels of glycoalkaloid content and small tuber size make them not suitable for large-area planting. Other crops, such as lupin (*Lupinus* spp.), alfalfa (*Medicago sativa*) and pennycress (*Thlaspi arvense*)[98] also have outstanding features. Through CRISPR–Cas-mediated editing of the corresponding genes, it should be possible to overcome their shortcomings and create novel strains with favourable traits.

Although CRISPR–Cas-accelerated domestication holds great promise, the process still includes several bottlenecks. Because precise knowledge of functional genomics is required for domestication, additional studies are needed to obtain basic genetic knowledge of wild species and mine domestication genes. Furthermore, as wild species are often recalcitrant to regeneration, robust transformation systems need to be developed to enable

their domestication. Finally, as producing an ideal cultivar requires alteration of several loci, more efficient multiplexed genome editing methods are required.

CRISPR–Cas-related plant biotechnology

In addition to greatly facilitating improvements in agriculture, numerous CRISPR–Cas-related plant biotechnologies have recently emerged. For example, when the lack of robust delivery systems became a bottleneck of gene editing in plants, several novel tools for generating edited plants were developed that allow genome manipulation without the use of exogenous DNA (BOX 1). Later, CRISPR–Cas was leveraged to study the regulation of gene expression in various contexts. Furthermore, because of its ease of use and robust orthogonal features, CRISPR–Cas was adapted to performing multiplexed and high-throughput genome editing, and to a versatile platform in plant synthetic biology.

CRISPR–Cas reagent delivery in plants

Effective application of CRISPR–Cas9 in plants requires a robust and universal way of delivering CRISPR–Cas reagents into plant cells (FIG. 3a). However, both of the widely used delivery methods on which plant transformation has relied for decades — biolistic bombardment and *Agrobacterium*-mediated delivery — have limitations. Biolistic bombardment can deliver genetic material through rigid cell walls by mechanical force, but it is restricted by its low efficiency and can damage genome sequences. *Agrobacterium* bacteria can infect a large range of plants, but the integration of foreign DNA is unavoidable, and transformation efficiency is greatly affected by the recipient genotype, especially in monocots[96]. Furthermore, none of these conventional methods can avoid lengthy tissue culture procedures. Consequently, novel delivery strategies are urgently needed.

De novo meristem induction. The regeneration-boosting activities of morphogenetic regulators are becoming powerful tools for enabling CRISPR–Cas-mediated gene editing in plants (BOX 2). In addition to assisting the transformation of recalcitrant cultivars or species[99], morphogenetic regulators can be engineered to induce de novo meristems on plants, thereby completely circumventing the need for tissue culture. Recently, by injection of *Agrobacterium tumefaciens* carrying the morphogenetic regulators WUS2, IPT and STM and sgRNA cassettes into pruned sites on Cas9-overexpressing *Nicotiana benthamiana* where meristems had been removed, gene-edited plants were obtained directly from the resulting shoots, and the induced mutations were inheritable[100] (FIG. 3b). In the same study, the method was also used in potato, tomato and grape[100]. This excellent work provides a generalizable in planta delivering method that halves the time required for generating gene-edited *N. benthamiana*, and should the approach be adapted to diverse species, it will greatly facilitate plant research.

Virus-assisted gene editing. Harnessing plant viruses is a promising approach to obtaining gene-edited plants without the need for tissue culture. Because viruses undergo replication and move around in planta, virus-assisted gene editing is quite efficient and supports systemic genome editing (FIG. 3a). In recent years, positive-sense single-stranded RNA viruses, including tobacco rattle virus[101,102], tobacco mosaic virus[103], pea early-browning virus[102], barley stripe mosaic virus[104], foxtail mosaic virus[105] and beet necrotic yellow vein virus[106], and the ssDNA cabbage leaf curl virus[107] have been developed for sgRNA delivery in plants, and editing efficiencies of up to 80% have been achieved. However, because of their limited cargo capacities, Cas9 cannot be co-encoded with the sgRNA by these viruses, and pre-existing Cas9-overexpressing plant lines are required instead. To solve this problem, two groups inserted Cas9 and sgRNA cassettes concurrently into the genome of barley yellow striate mosaic virus[108] and sonchus yellow net rhabdovirus[109], two negative-sense single-stranded RNA viruses with excellent genome stability and delivering capacity, and achieved systemic gene editing in wild-type *N. benthamiana*. Another factor obstructing virus-assisted gene editing is that virus-induced mutations cannot be passed on to the next generation, because intact viruses cannot enter the meristem or reproductive tissue. To bypass this limitation, researchers cleverly fused sgRNAs with RNA mobile elements and introduced them into tobacco rattle virus RNA2. Following *Agrobacterium*-mediated infiltration into plant somatic tissues, the mobile elements directed them into shoot apical meristem cells, thereby inducing heritable mutations with efficiencies of up to 100% in the progeny[110] (FIG. 3c).

Gene editing with haploid inducers. In some crops, genetic transformation is still restricted to particular genotypes, thus drastically limiting breeding procedures. To solve this problem, two delivery systems were recently developed, named 'haploid induction editing'[111] and 'haploid-inducer mediated genome editing'[112] (FIG. 3d). In both strategies, elite maize lines are pollinated with haploid inducer lines that harbour CRISPR–Cas systems. After fertilization, the paternal genome from haploid inducer lines induces mutations in the maternal genome and is subsequently eliminated

Box 1 | Genome editing in plants without the use of exogenous DNA

Genome editing in plants without the use of exogenous DNA is preferable to traditional genome editing, because it produces fewer off-target muta… statutory regulatory concerns. Although exogenous … can be eliminated by crossing, undetected sequences of the vector may … genome, and crossing is not practical for asexually propagated specie… studies using CRISPR–Cas9 ribonucleoprotein or mRNA have demonstrated … editing can be performed in a DNA-free manner in rice[161] … and wheat[162,163]. However, generalization of DNA-free genome editing is impeded by its … and incompatibility with introducing exogenous selection markers. One … these problems is to create endogenous selectable markers. As certain … substitutions in herbicide-target genes such as the acetolactate s… (*ALS*) gene can bestow tolerance to herbicides, co-targeting a gene of interest a… *ALS* with single guide RNAs and selecting for herbicide resistance can greatly enrich … desired gene-[14,64]. This selectable co-editing … facilitate DNA-free editing if every component were delivered in RNA or protein … way to facilitate DNA-free gene editing is to use morphogenetic regu… (BOX 2) to increase editing efficiency by delivering them with DNA-free CRISPR–C… morphogenetic regulators can boost the regeneration of transformants[162,164]. With the future progress, DNA-free editing may become the main method of plant genome …

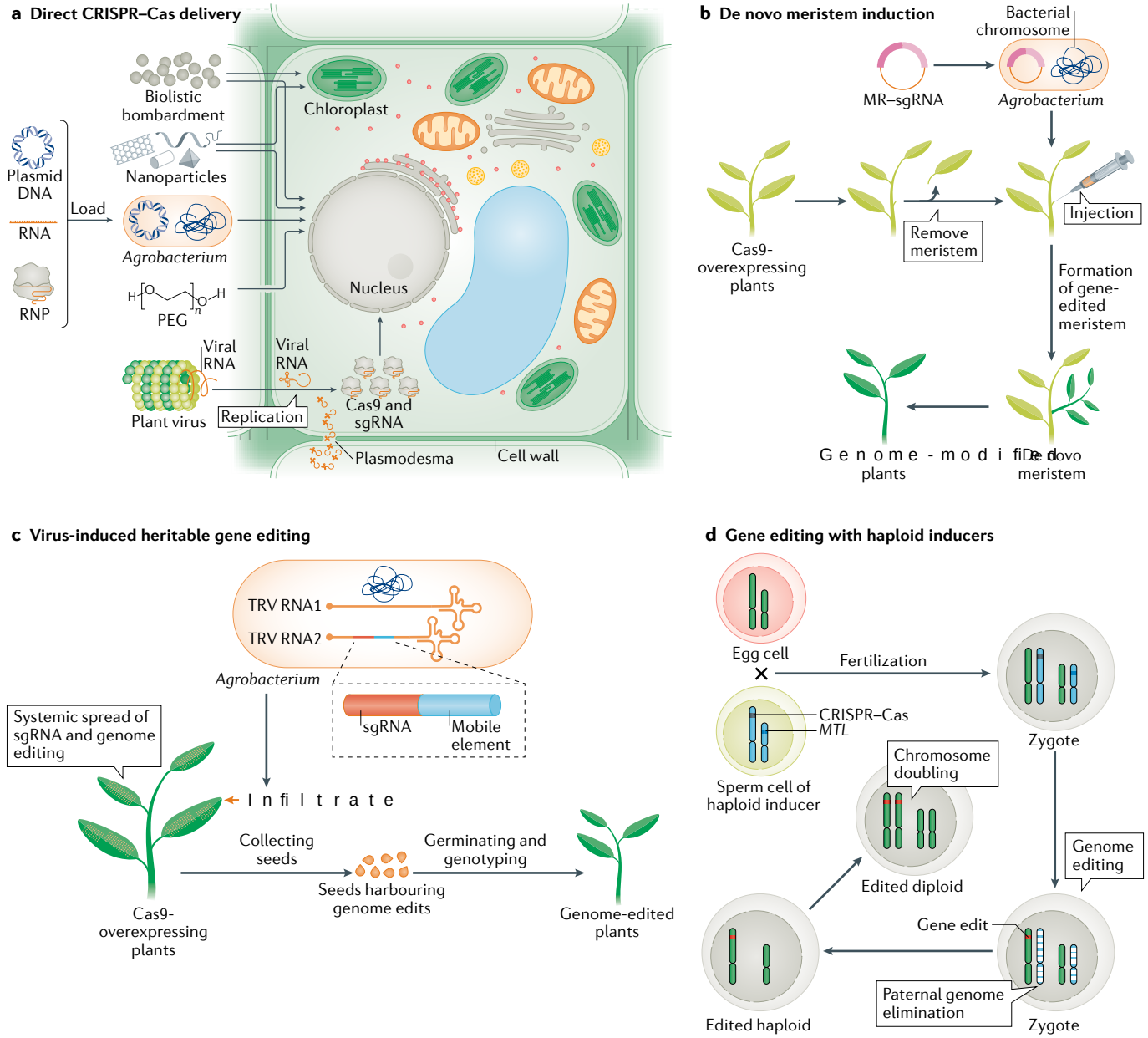

Fig. 3 | **Strategies for CRISPR–Cas delivery. a** | Delivery of CRISPR–Cas reagents into plant cells. DNA or RNA that encode CRISPR–Cas reagents (Cas and single guide RNAs (sgRNAs)) or the CRISPR–Cas–sgRNA ribonucleoprotein (RNP) can be delivered into plant cells using *Agrobacterium*, nanoparticles and biolistic bombardment, which can all pass through the rigid cell wall. Polyethylene glycol (PEG) can mediate the transformation of plant protoplasts. DNA or RNA encoding CRISPR–Cas reagents can be introduced into the genomes of plant viruses, which infect plant cells, replicate in them and move to other cells through plasmodesmata. Chloroplast-targeted delivery can be achieved using biolistic bombardment and nanoparticles. **b** | Gene editing induction of meristems. The meristems of Cas9-overexpressing plants are firstly removed, and *Agrobacterium* cultures that contain morphogenetic regulators (MRs) and sgRNAs are injected into the pruning sites. Morphogenetic regulators can induce the formation of new, gene-edited meristems, and gene-edited plants can be harvested from the newly generated shoots. **c** | Use of plant RNA viruses to induce heritable genome editing. sgRNA fused with an RNA mobile element is introduced into tobacco rattle virus (TRV) RNA2. Following *Agrobacterium*-mediated infiltration into Cas9-overexpressing plants, the sgRNA can be spread systemically in the plant by the mobile element and thus introduces inheritable mutagenesis. **d** | Delivering CRISPR–Cas by means of a haploid inducer. Transformation-r by haploid inducer lines expressing editing occurs and the paternal genome, which carries a mutation in the *MATRILINEAL* (*MTL*) gene, is eliminated, thereby creating an elite genetic background. Homozygous mutant lines could be generated by chromosome doubling.

from the zygote, generating gene-edited maize haploids with maternal backgrounds. Similarly, through pollination of wheat cultivars with maize lines stably expressing CRISPR-Cas9, two wheat genes were successfully edited[113]. The chromosomes of edited haploid lines could be doubled spontaneously in nature or artificially by treating them with mitotic inhibitors. These methods not only skilfully circumvent the insurmountable transformation problem, but also produce homozygous transgene-free gene-edited plants.

Nanoparticles
Particles with specific nanoscale structures that can load biomacromolecules and deliver them into intact plant cells.

Polyethylene glycol
A high molecular weight polymer that enables uptake by plant protoplasts of biomacromolecules, including DNA, RNA and protein.

Gene regulation using CRISPR–Cas

Whereas knockout mutagenesis in plants using CRISPR–Cas often has pleiotropic effects or is even lethal, programmable and heritable modulation of gene expression provides a tunable and flexible way to alter phenotypes and create elite traits without changing protein coding sequences.

Transcription modulation. Although catalytically inactive Cas9 variants (known as dCas9) lack DNA-cleaving activity, they still retain sgRNA-mediated sequence-specific DNA-binding activity. By simply docking at targeted points in the genome, dCas9 can prevent the binding of transcriptional machineries or block the passage of RNA polymerases, thereby repressing transcription[114,115]. Furthermore, through fusion of dCas9 or its orthologues with transcription regulators[116–118] or epigenetic modulators[119,120], gene expression can be precisely regulated. Such gene control can be enhanced by tethering multiple effectors to the targeted loci[116,119,120]. Use of dCas9 can also alter chromatin structure to modulate gene expression by promoting or inhibiting enhancer–promoter interactions[121].

Although these approaches are robust, both the dCas9 fusion proteins and sgRNAs have to be inserted into the genome for continuous expression to achieve stable gene regulation. Editing *cis*-regulatory elements (CREs) provides an alternative approach to expressing dCas9 fusion proteins. For example, by targeting of CREs in the promoter region of *S. lycopersicum CLV3* using eight gRNAs, a spectrum of alleles showing various transcriptional and phenotypic features were created in tomato[40]. The expression level of *O. sativa TB1*, which is a yield-related gene, was also altered by editing of its regulatory region with six gRNAs[122]. Furthermore, as the expression of some genes is controlled by multiple mechanisms, simply regulating transcription may not achieve the desired phenotype, whereas editing CREs can modify gene expression in a tissue-specific or stage-specific manner, which might also be responsive to extracellular stimuli[40,51,52,123,124]. Thus, this approach would enable the creation of multifaceted traits and identification of DNA motifs responsive to given signals.

Targeting RNA. A number of RNA-targeting CRISPR–Cas systems, such as Cas13a and Cas13b, have been established recently in plants. As the targeted RNA is cleaved and degraded, these CRISPR–Cas systems can downregulate individual transcripts with greater specificity than the widely used RNAi[125]. In addition to targeting RNA directly, CRISPR–Cas can be used to modulate pre-mRNA splicing. As most pre-mRNA splicing strictly follows the canonical GU–AG rule, editing key splicing motifs can disrupt splicing and alter gene function[23,126]. Moreover, as constitutive introns can promote gene expression through a poorly defined mechanism known as intron-mediated enhancement, editing the intronic splicing site in the 5′ untranslated region of *O. sativa GBSS1* reduced the expression of the gene and created waxy rice lines[127]. In addition to constitutive splicing, many genes can frequently give rise to distinct mRNA isoforms through alternative splicing. By mutating alternative splicing sites with a CBE, researchers managed to perturb the alternative splicing of *A. thaliana HAB1.1* and *A. thaliana RS31A* and produced plants with abscisic acid hypersensitivity and mitomycin C insensitivity, respectively[128].

Translation modulation. Upstream open reading frames (uORFs) are well-studied regulatory elements located in the 5′ untranslated regions of many eukaryotic mRNAs that generally reduce the translation of the downstream, primary ORF and can promote mRNA decay. Hence, editing of uORFs is a promising method for upregulating gene expression. By the knocking out of the initiation codons of uORFs, the translation of four genes in *A. thaliana* and lettuce was increased, and a lettuce germplasm with high ascorbate content was created[129]. Moreover, by modulating the uORFs of *Fragaria vesca* b*ZIP1.1* using a CBE in diploid strawberry, translation of the primary ORF was enhanced, and sweetness in strawberry was increased[130]. The new genotypes could be fixed in subsequent generations by asexual reproduction. Because ~40% of plant genes contain uORFs, which could be altered by CRISPR–Cas, this approach is a generally applicable and tunable way to translationally regulate gene expression. Other widespread genetic elements, such as polyadenylation signals, alternative transcription initiation sites and enhancers, also have important roles in regulating gene expression and are candidates for genome editing. As the expression of several crucial genes in plants is under tight control and knocking out or ectopically overexpressing them may undermine fitness, more nuanced regulation by disrupting or artificially creating regulatory elements through genome editing holds great promise for fine-tuning gene expression and developing highly tunable crops with minimal fitness penalties.

Conditional CRISPR–Cas systems

About 10% of the protein-coding genes in plants are indispensable[131], and their loss of function has pleiotropic effects or causes lethality. Alternatives such as gene knockdown through RNAi or CRISPR–Cas-based regulation is commonly inefficient. To overcome this

Box 2 | Use of morphogenetic regulators to boost regeneration

Regeneration is a central and unavoidable step in obtaining CRISPR–C plants. However, current regeneration systems, which rely heavily o are time-consuming, laborious and species or genotype dep Since regeneration has become a barrier to the application of CRISPR morphogenetic regulators have emerged as a promising tool to meet th Morphogenetic regulators are a group of transcription factors that, with phytohormones, have roles in meristem determination and mainte and can induce meristem morphogenesis and produce embryo-like struc Simultaneously overexpressing the genes encoding two morphogenetic *BABY BOOM 1* (*BBM1*) and *WUS2*, in maize explants induced the regeneration of transgenic plants from inbred lines and tissues that had been trans recalcitrant[99]. As morphogenetic regulators are highly conserved, this metho be easily generalized to other non-regenerable genotypes or crops su sugar cane[99,164,165]. With the use of morphogenetic regulators, gene-edited plants of recalcitrant maize inbred lines were successfully regenera CRISPR–Cas9 and *BBM1* and *WUS2* were delivered together into plant cells[166]. However, as constitutive overexpression of *BBM1* and *WUS2* results in developmental abnormalities and sterility, new tools using other morphogenetic reg greatly needed.

RNA aptamers
RNA oligonucleotides that form a secondary structure to bind a specific protein with high specificity and affinity.

CRISPR library
A high-throughput tool for functional genomics studies, comprising a collection of single guide RNAs or CRISPR RNAs, which target a set of predefined loci.

considerable problem, conditional CRISPR–Cas systems have been developed. With use of various tissue-specific promoters, the expression of Cas9 can be restricted to specific cell types and thus gene editing can be limited to particular tissues or organs. This approach has been applied to elucidating gene function in the root cap, stomatal lineage and lateral roots[132]. Furthermore, this strategy can be combined with inducible expression systems. Using inducible tissue-specific promoters, gene editing can be both restricted to specific cell types and controlled by exogenous inducers[133]. Similarly, blue light-repressed and red light-induced CRISPR–Cas systems have also been developed[134]. Conditional systems can also synchronize expression of Cas9 with mobilization of the donor template and consequently increase the efficiency of gene targeting[135]. Conditional CRISPR–Cas systems provide great flexibility and compatibility with plant gene editing, and thus may become popular in plant genetics research.

Multiplexed genome editing in plants

Multiplexed genome editing is used for regulating gene expression, stacking traits and controlling regulatory pathways, and thus has facilitated the previously described crop improvement, breeding and domestication.

Multiplexed sgRNA expression systems. Many convenient and efficient multiplexed sgRNA systems for CRISPR–Cas9 have also been developed in plants, including RNA polymerase III (Pol III)-driven and Pol II-driven systems. The canonical method for Pol III promoter-driven systems in plants uses multiple Pol III promoters (U3 and U6) to express multiple sgRNAs in one construct[136,137]. Moreover, by use of cellular RNase P and RNase Z to process out pre-tRNAs, which serve as spacers interspersed in-between the multiple sgRNAs of a polycistronic tRNA–sgRNA transcript, multiple sgRNAs can be expressed with flanking tRNA sequences under the control of a single Pol III promoter[138]. For Pol II promoter-driven systems, strategies have been adopted for simultaneously expressing multiple sgRNAs on the basis of the expression and processing of poly-sgRNA-containing transcripts, including using ribozyme sequences flanking the sgRNAs[139], using polycistronic tRNA–sgRNA transcripts inserted into introns[140] and the addition of 6-bp or 12-bp linkers to flank the sgRNAs[141]. A more efficient Csy-type ribonuclease 4 (Csy4)-processing system, which can cleave specific 20-nucleotide sequences flanking the sgRNAs, was also developed in plants, driven by a Pol II promoter[142]. Finally, the use of CRISPR–Cas12a, which can mature its own CRISPR RNA (crRNA) by processing pre-crRNAs that are separated by direct repeats in crRNA arrays, provides more flexible multiplexed editing using crRNA arrays[143]. However, strategies for expressing multiplexed random sgRNAs, which would facilitate high-throughput sequencing, are still to be developed.

Multiplexed orthogonal editing. Most examples of multiplexed editing in plants have used a single type of editor combining one CRISPR–Cas system and multiple sgRNAs (FIG. 4a). However, a single type of Cas protein or scRNA is not suitable for performing multiplexed orthogonal editing for synthetic genome manipulation. Several strategies have been developed for performing multiplexed orthogonal editing in mammalian cells. One strategy involves using a dCas9 with various scRNAs that harbour different RNA aptamers that recruit different transcription activators (such as VP64) and repressors (such as KRAB)[144] (FIG. 4b). Others strategies involve simultaneously using an sgRNA with a full-length protospacer to direct the formation of DSBs and thus a gene knockout, with a second sgRNA that has a truncated protospacer for targeting the regulation of another gene by Cas9 (REF.[145]), Cas12a-[repressor] or Cas12a-[activator] ([repressor] and [activator] represent the effectors for gene repression and activation, respectively)[146] (FIG. 4c). The combination of Cas orthologues allows multiplexed gene knockout and transcription regulation and facilitates the analysis of complex gene networks[147] (FIG. 4d). All three multiplexed orthogonal editing strategies have been implemented in plants (FIG. 4b–d).

The recent development of CRISPR-based simultaneous and wide editing induced by a single system (SWISS) enables the multiplexed and orthogonal production of simultaneous base edits and gene knockouts in rice[148] (FIG. 4e). In SWISS, RNA aptamers in the engineered scRNAs recruit their cognate binding proteins, which are fused with a cytidine deaminase and an adenosine deaminase to create simultaneous CBE and ABE edits on nCas9-targeted sites. The use of a pair of sgRNAs also allows nCas9 to introduce a third type of edit, namely indels (small DNA insertions or deletions). In another study, a dual-function system combining a full-length protospacer and a truncated protospacer to control the activity of enhanced specificity SpCas9 variant 1.1 (referred to as eSpCas9(1.1))-based CBE to create an indel and C:G>T:A base transitions has also been adopted in plants[149]. In a Cas orthologue strategy, the SaCas9-based ABE and SpCas9-based CBE were combined to perform multiplexed orthogonal base editing in rice[20]. These multiplexed orthogonal editing systems pave the way for manipulating the genome in a synthetic manner.

Mutagenesis and directed evolution

In addition to multiplexed gene editing, CRISPR–Cas can also be used for high-throughput genetic studies. Because the spacer sequence is the only determinant of programmable gene editing, CRISPR–Cas-based platforms can be easily scaled up to use sgRNA pools, and are promising tools for high-throughput functional genomics screening and directed evolution in plants.

CRISPR–Cas-based functional genomics screening. Functional genomics screening is a powerful approach for identifying genes responsible for particular phenotypes, and CRISPR–Cas, owing to its programmable and robust properties, allows high-throughput genome-scale screening within a single generation in plants. In rice, a CRISPR library containing 25,604 pooled sgRNAs targeting 12,802 genes was designed and constructed, and more than 14,000 independent T0 lines displaying a high frequency of edits were regenerated[150]. Among the 200 lines tested, 54 had altered morphological phenotypes, and their genotypes could be easily identified by sequencing

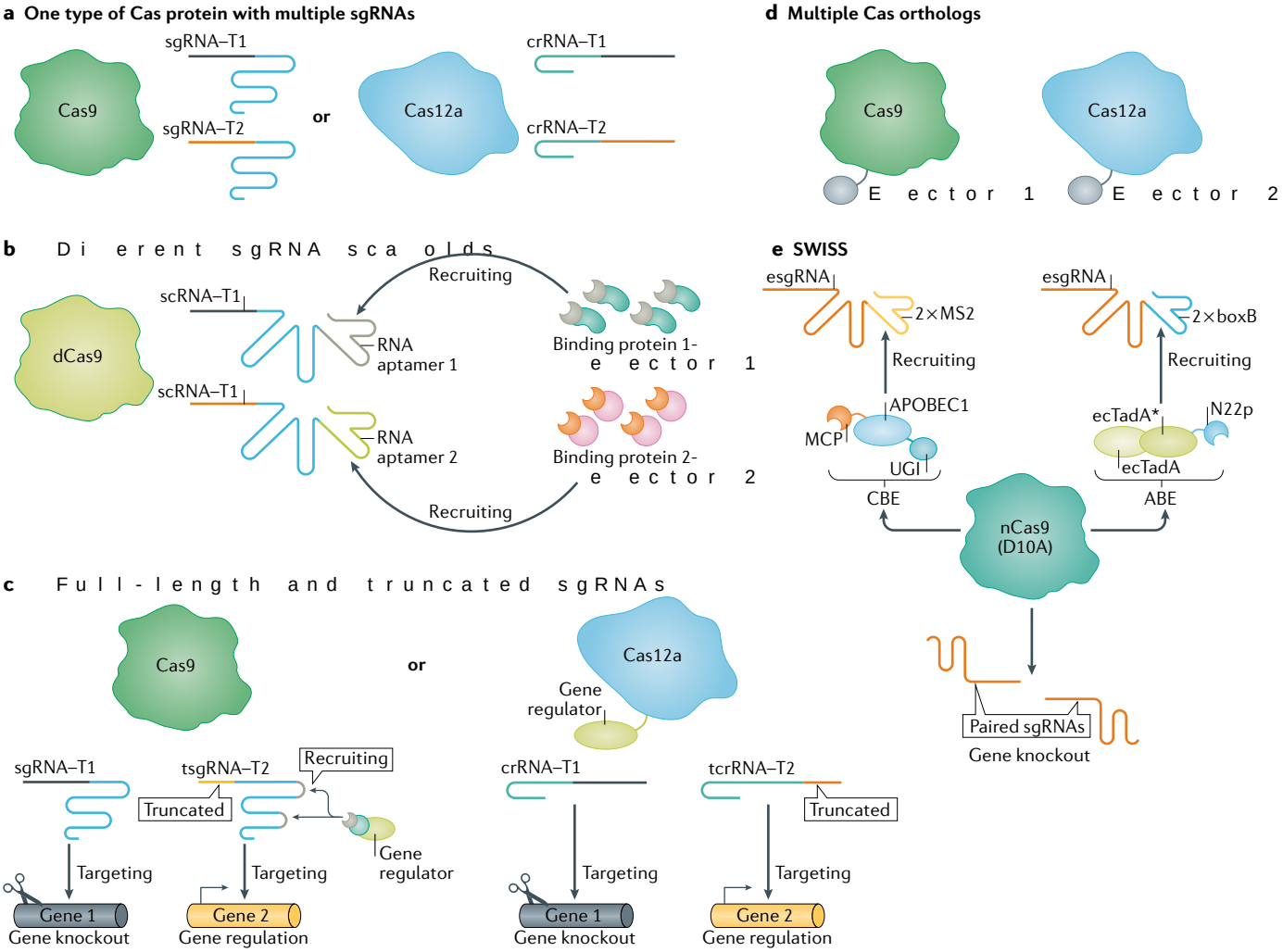

Fig. 4 | **Multiplexed genome editing strategies. a** | Multiplexed genome editing using one type of Cas protein with multiple single guide RNAs (sgRNAs). Two sgRNAs (targeting Cas9) or CRISPR RNAs (crRNAs; targeting Cas12a) can be used simultaneously to target two (or more) different sites (T1 and T2). **b** | The multiplexed orthogonal genome editing strategy uses catalytically inactive Cas9 (dCas9) with different sgRNA scaffolds (scRNAs). For example, scRNA 1 and scRNA 2 harbour different RNA aptamers, which can recruit different effector proteins such as those regulating transcription, or fluorescent proteins. **c** | A multiplexed orthogonal genome editing strategy of using one Cas protein with two sgRNAs or crRNAs, one with a full-length protospacer and one with a truncated protospacer. The full-length sgRNA–T1 or crRNA–T1 targets gene 1 for deletion by the respective Cas protein. The truncated protospacer version — tsgRNA–T2 or tcrRNA–T2 — does not support full catalytic activity of Cas and enables Cas to regulate the expression of gene 2. **d** | Multiplexed orthogonal genome editing by Cas orthologues. Orthologues of Cas9 and/or Cas12a using orthogonal sgRNA and/or crRNA scaffolds (not shown) can be applied for multiplexed orthogonal editing. The effectors can be deaminases or regulators of gene expression. However, in some cases effectors may not be needed, for example to create gene knockouts. **e** | Simultaneous and wide editing induced by a single system (SWISS). The RNA aptamer of MS2 is used to recruit the cytidine deaminase module, boxB is used to recruit the adenosine deaminase module and paired sgRNAs are used to create insertions and deletions. boxB, the RNA aptamer hairpin recruiting N22p; CBE, cytosine base editor; ecTadA, *Escherichi coli* tRNA-specific adenosine deaminase (wild-type); ecTadA*, ecTadA variant; esgRNA, enhanced sgRNA; UGI, uracil DNA glycosylase inhibitor; MCP, MS2 coat protein; MS2, the RNA aptamer from bacteriophage MS2; N22p, bacteriophage-λ N peptide with affinity enhancement.

the sgRNA spacers. Similarly, 91,004 rice mutants were created with a library of 88,541 sgRNAs[151]. Genomic screening using sgRNA libraries can also assist functional gene validation. Through screening 1,244 candidate loci using high-throughput CRISPR–Cas editing in maize, genes related to agronomic performance were accurately mapped[152]. Similar work has been done in tomato[153] and soybean[154], and these techniques will no doubt be further refined. Di-sgRNA or tri-sgRNA libraries (targeting each gene with more than one sgRNA) would be more practicable than mono-sgRNA libraries for studying phenotypic changes resulting from multiple mutations of non-coding regions (such as non-coding RNA). Moreover, instead of high-throughput induction of loss-of-function mutations, CRISPR–Cas-derived transcription modulation platforms could be used to screen for phenotypes associated with more nuanced differences in gene expression.

CRISPR–Cas-directed evolution. Directed evolution has emerged as a powerful approach for modifying genes of interest (GOIs) to acquire enhanced or novel properties. Although a number of directed evolution systems have been devised in microorganisms, the GOIs evolved using those systems may not behave the same way in

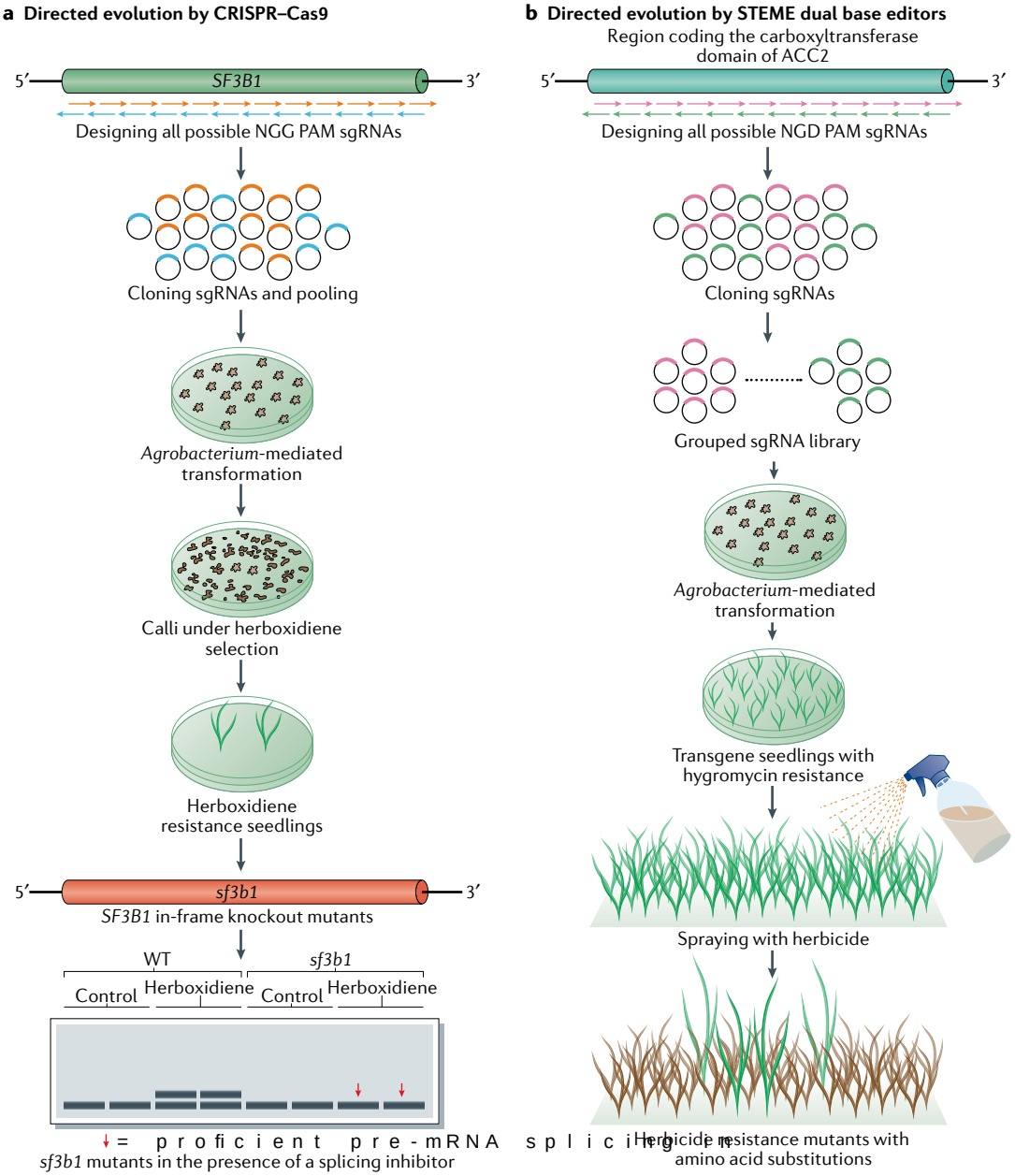

Fig. 5 | **Directed evolution of herbicide resistance genes using CRISPR–Cas based technologies. a** | CRISPR–Cas9-based directed evolution in *Oryza sativa* of the splicing factor 3B subunit 1 (*SF3B1*) gene for resistance to the splicing inhibitor herboxidiene. **b** | Directed evolution of the *O. sativa* acetyl coenzyme A carboxylase 2 (*ACC2*) gene using saturated targeted endogenous mutagenesis editor (STEME) dual base editors increases the herbicide resistance of rice. PAM, protospacer adjacent motif; sgRNA, single guide RNA; WT, wild type.

plants because of differences in cellular environment and structure. Therefore, directed evolution methods actually established in plant systems will be of great value. A basal directed evolution system includes two processes: mutagenesis, to generate various genotypes, and selection, to enrich for desired genotypes under particular selective pressures. Whereas the commonly used directed evolution methods are based on error-prone PCR and DNA shuffling methods that introduce random mutations, use of a CRISPR–Cas sgRNA library in combination with selection for the desired trait (for example, herbicide resistance) enables high-throughput saturated mutagenesis within a GOI in vivo.

Several excellent studies have demonstrated the feasibility of CRISPR–Cas-directed evolution in plants. The gene encoding rice splicing factor 3B subunit 1 (*O. sativa SF3B1*) has been evolved to confer resistance to the splicing inhibitor GEX1A[68]. A CRISPR–Cas library containing all possible 119 sgRNAs targeting *O. sativa SF3B1* was used to identify 6 of 15,000 transformants carrying in-frame knockout mutations conferring GEX1A resistance, with no observed fitness cost (FIG. 5a). In another study, using C>T and A>G dual base editors (STEME-1 and STEME-NG), saturated mutagenesis of the coding region of the carboxyltransferase domain of *O. sativa* acetyl-CoA carboxylase 2 (*ACC2*) was performed using

a library of 200 sgRNAs, and new *ACC2* gene variants conferring herbicide resistance were evolved[19] (FIG. 5b). Directed evolution of *O. sativa ALS1* (REF.[155]) and the *O. sativa ACC2* gene[156] has also been performed using CBE and ABE. These evolved herbicide resistance mutant plants have great potential to be directly used in weed control to increase food production.

CRISPR–Cas-directed evolution methods are still in their infancy. Because of a lack of versatile selection methods, evolvable GOIs are currently limited to herbicide resistance genes. Therefore, elaborate genetic circuits that couple genotypes with easily detectable phenotypes need to be designed to artificially evolve other GOIs. In addition, iterative mutagenesis and selection platforms are required to generate genotypes harbouring multiple mutations and to reduce labour. With further progress, we envision that these approaches will not only assist in identifying gene functions but will also enlarge the toolbox of plant synthetic biology and create valuable alleles for agriculture.

Conclusions and future prospects

CRISPR–Cas has emerged as a game-changing tool for basic and applied plant research. In addition to the indel mutations induced by the CRISPR–Cas nuclease, a series of CRISPR–Cas-derived editors have been designed that can perform precise genome manipulations. With their incomparable capability to edit genes, these tools have helped create hundreds of crop varieties with improved agronomic performance, and revolutionized breeding technologies.

CRISPR–Cas raises the possibility of domesticating orphan crops or wild species in a short time to promote global food security and poverty eradication. Numerous plant biotechnologies related to CRISPR–Cas have also been developed or updated, including delivery methods to facilitate plant gene editing; methods for precise gene regulation at different expression stages; and multiplexed and high-throughput gene editing approaches that have enabled genome editing in multiple sites, functional genomics screening and plant directed evolution.

However, these versatile tools have not yet met all the needs for plant genome manipulation, and further developments will be vital for the application of CRISPR–Cas in plants. As some agricultural traits are the products of a number of quantitative trait loci, and editing individual genes may not produce sufficient phenotypic change, it would be advantageous to develop efficient CRISPR–Cas-mediated targeted insertion and chromosome rearrangement technologies to combine or 'stack' mutated alleles. As disrupting specific genes may bring about fitness penalties, further progress in regulating gene expression and precision genome editing will be needed to fine-tune gene function efficiently and specifically. Furthermore, as directly transforming some exogenous proteins into plants might be problematic, it would be helpful to refine CRISPR–Cas-derived directed evolution platforms to make them better suited to plant systems. As the delivery of CRISPR–Cas reagents is still a major obstacle to plant genome editing, developing additional novel delivery methods would be desirable; nanomaterials (such as carbon nanotubes[157,158], DNA nanosturctures[159] and cell-penetrating peptides[160]) are promising vehicles for delivery of CRISPR–Cas reagents in various forms, because they can diffuse into walled plant cells without mechanical aid and without causing tissue damage. Advances in basic genetic research are also much needed for identifying genes related to particular desirable agronomic traits.

In addition to the aforementioned applications, CRISPR–Cas may be repurposed for new applications, such as editing the genomes of mitochondria and chloroplasts, tracing cell lineages to elucidate the patterns underlying plant development, building genetic circuits to integrate and transduce signals, developing plant biosensors to detect internal and external signals, and other applications of plant synthetic biology. All in all, CRISPR–Cas technology has undoubtedly revolutionized, and will continue to revolutionize, both agriculture and plant biotechnology.

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