nature reviews genetics

https://doi.org/10.1038/s41576-024-00720-2

Review article

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# Targeted genome-modification tools and their advanced applications in crop breeding

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## Abstract

Crop improvement by genome editing involves the targeted alteration of genes to improve plant traits, such as stress tolerance, disease resistance or nutritional content. Techniques for the targeted modification of genomes have evolved from generating random mutations to precise base substitutions, followed by insertions, substitutions and deletions of small DNA fragments, and are finally starting to achieve precision manipulation of large DNA segments. Recent developments in base editing, prime editing and other CRISPR-associated systems have laid a solid technological foundation to enable plant basic research and precise molecular breeding. In this Review, we systematically outline the technological principles underlying precise and targeted genome-modification methods. We also review methods for the delivery of genome-editing reagents in plants and outline emerging crop-breeding strategies based on targeted genome modification. Finally, we consider potential future developments in precise genome-editing technologies, delivery methods and crop-breeding approaches, as well as regulatory policies for genome-editing products.

## Sections



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## Introduction

Ensuring food security in the face of massive population growth, climate change and geopolitical conflicts is a vital challenge for agricultural scientists worldwide[1]. Germplasm improvement with the use of genetic resources is a key component of strategies aimed at responding to these challenges. To meet the changing needs of a growing global population in a sustainable manner, agricultural crop varieties should be developed and bred that are high-yielding, resilient and adaptable.

Crop breeding relies on improving desirable traits by producing and combining advantageous genetic variation, but awaiting the occurrence of spontaneous beneficial mutations is time-consuming[2]. Hence, artificial ways of rapidly generating mutations have become increasingly important. Traditional physical and chemical mutagenesis methods can generate random mutations but require large-scale screening to identify those that are beneficial[3]. The advent of targeted genome modification (TGM) technologies, which are based on the application of targeting 'modules', has largely solved this problem. The term 'targeted' describes the specificity of a genome-editing tool in recognizing and interacting with a particular DNA sequence. The three main targeting modules currently in use are zinc finger (ZF) DNA-binding domains, transcription activator-like effector (TALE) DNA-binding domains and clustered regularly interspaced short palindromic repeat (CRISPR) systems[4,5]. The targeting capabilities of ZF and TALE modules depend on the design of custom DNA-binding amino acid sequences; the construction process is relatively complicated and has been reviewed in detail elsewhere[6]. By contrast, CRISPR systems possess simplicity, powerful programmability and excellent targeting activity, making CRISPR the most frequently used targeting module[7]. The CRISPR targeting module consists of two components: a guide RNA (gRNA), designed to be complementary to a specific DNA sequence, and a CRISPR-associated protein (Cas); the resulting complex searches for a protospacer adjacent motif (PAM) and binds the target DNA sequence. By combining targeting modules with different effectors[8–10] — such as DNA endonucleases, deaminases, reverse transcriptases, transposases and recombinases, among others — TGM technologies have been developed that can introduce distinct types of modification into the genome. In general, nuclease editing can generate random small insertions and deletions (indels); base editing achieves precise base substitutions; prime editing enables precise base substitutions and small indels; and editing of large DNA (kilobase-scale) segments includes strategies that use prime editing, CRISPR-associated transposases or recombinases.

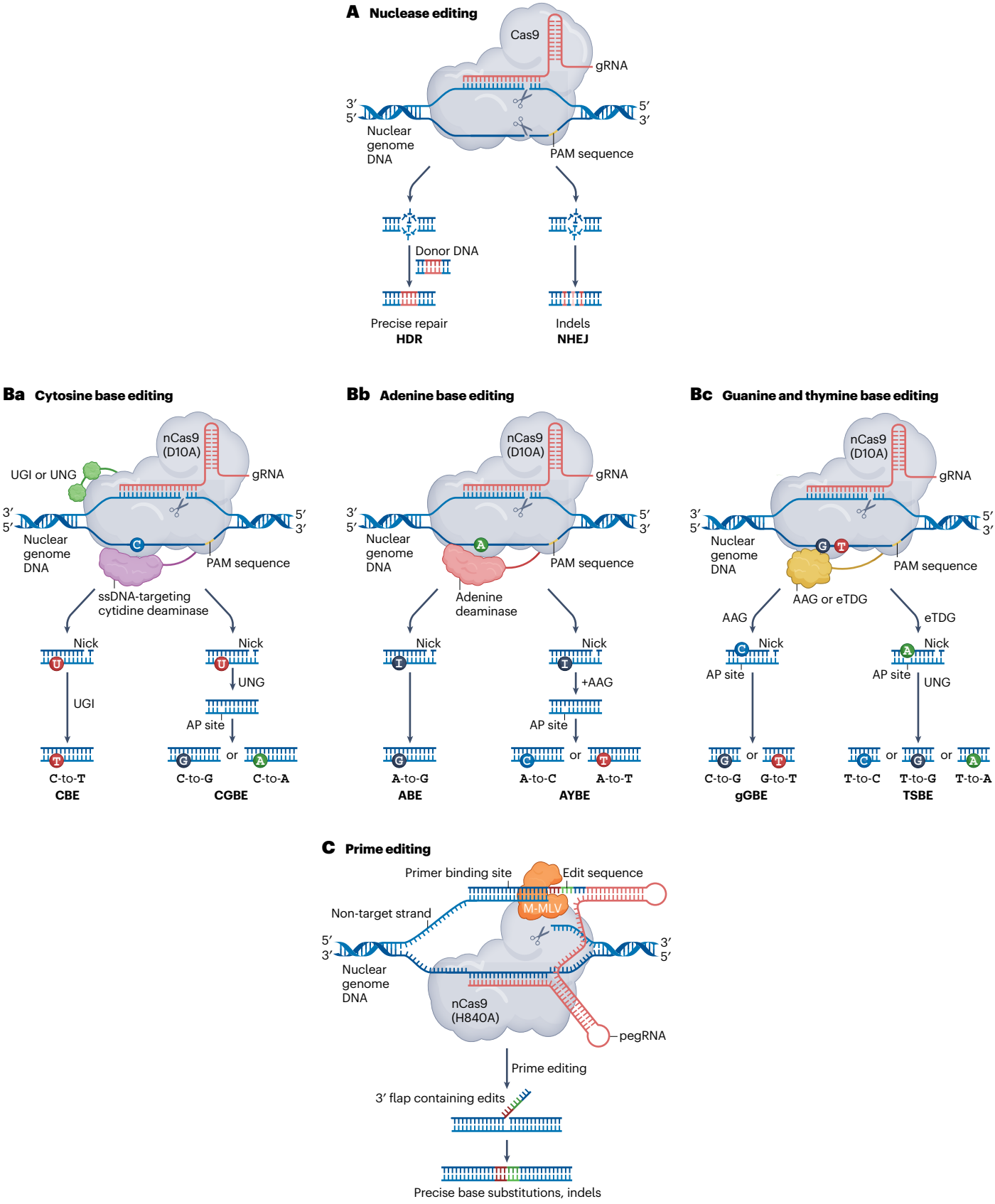
Recent advances in TGM systems have driven innovation in breeding strategies and substantially enhanced crop genetic improvement, providing considerable advantages over traditional breeding in terms of efficiency and precision[4]. In particular, TGM technologies have generated substantial mutations that are challenging to obtain without these techniques, with a broad impact on key agronomic traits such as crop yield, disease resistance, quality and others[11,12]. The delivery of TGM reagents has been a key technological bottleneck in plant research[13,14], but the recent development of novel plant delivery systems has expanded options for streamlining the use of TGM for crop design.

In this Review, we first provide a comprehensive summary of precise TGM tools used in plant research, including base editing, prime editing and technologies for editing DNA segments at the kilobase scale. We then review traditional and emerging technologies that are used for the delivery of TGM components into plants. Finally, we review advanced crop-breeding strategies based on TGM and offer a perspective on future technical innovations and applications of TGM in crop breeding.

## Targeted and precise genome modification

A highly effective genome-modification tool must be not only specific but also precise. Once it has been directed to a target gene or genomic region, it should undertake desired modifications accurately, without generating by-products at target loci or causing unintended modifications at other genomic locations (called off-target effects). Traditional CRISPR–Cas genome editing harnesses nucleases such as Cas9, which acts not only as a targeting module but also as an effector, to generate targeted DNA double-strand breaks that serve as substrates for cellular DNA-repair mechanisms, including homology-directed repair and non-homologous end joining[15,16] (Fig. 1A). Although homology-directed repair can generate precise modifications, this approach is limited by the inherent inefficiency of homology-directed repair mechanisms in higher organisms[11], and non-homologous end joining typically generates targeted mutations that are imprecise. Moreover, nuclease editing often causes some cellular damage owing to the generation of double-strand breaks[17,18]. Nonetheless, CRISPR–Cas genome editing has found widespread application in plant research and agriculture[19],

**Fig. 1 | Precise DNA editing at the base pair level. A**, Nuclease-mediated editing via DNA double-strand breaks. CRISPR–Cas genome editing harnesses nucleases, such as Cas9, to generate targeted double-strand breaks that serve as substrates for cellular DNA-repair mechanisms, including homology-directed repair (HDR) and non-homologous end joining (NHEJ). A guide RNA (gRNA) designed to be complementary to a specific DNA sequence guides the Cas9 protein, which binds the target DNA sequence and cleaves it near a protospacer adjacent motif (PAM). Homology-directed repair requires a donor DNA containing the sequence of the desired modification and flanking homologous sequences. Non-homologous end joining leads to random insertions and deletions (indels). The sequence in red represents the edited sequence. **B**, Base editing. **Ba**, Cytosine base editing. A Cas9 nickase (nCas9; D10A) creates an R-loop at the target site, thereby exposing single-stranded DNA (ssDNA) to the ssDNA-targeting cytidine deaminase, which converts cytosine (C) in this region to uracil (U). A uracil DNA glycosylase inhibitor (UGI) inhibits uracil-*N*-glycosylase (UNG)-mediated excision of U, which is subsequently recognized as thymine (T) during DNA repair, thus producing a C-to-T substitution; in the absence of UGI or with the addition of UNG, U is converted to an apurinic or apyrimidinic (AP) site, which results in C-to-guanine (G) or C-to-adenine (A) conversions. **Bb**, Adenine base editing. Adenine deaminase converts A to inosine (I), enabling A-to-G transitions; if alkyladenine DNA glycosylase (AAG) is added, I is converted to an AP site, enabling A-to-C or A-to-T conversions. **Bc**, Guanine and thymine base editing. G is converted to an AP site by AAG, achieving G-to-C or G-to-T changes; T is converted to an AP site by enhanced thymine DNA glycosylase (eTDG), achieving T-to-C, T-to-G or T-to-A changes. **C**, Prime editing. Prime editors use nCas9 H840A fused to a reverse transcriptase from the Moloney murine leukaemia virus (M-MLV). A prime editing gRNA (pegRNA) includes a spacer in its 5′ end, a reverse transcription template and an additional primer binding site in its 3′ end. nCas9 (H840A) induces a nick in the non-target strand. The primer binding site sequence then hybridizes with the 5′ end of the nick, initiating reverse transcription. Reverse transcription generates a 3′ flap containing the desired edits, which can be incorporated during DNA repair. The green and dark-red sequences represent the desired edits. ABE, adenine base editor; AYBE, adenine transversion base editor; CBE, cytosine base editor; CGBE, C-to-G base editor; gGBE, glycosylase-based guanine base editor; TSBE, T-to-S (G/C) base editor.


A Nuclease editing
Cas9
gRNA
3′
5′
5′
3′
Nuclear genome DNA
PAM sequence
Donor DNA
Precise repair
HDR
Indels
NHEJ
Ba Cytosine base editing
nCas9 (D10A)
UGI or UNG
gRNA
Nuclear genome DNA
PAM sequence
ssDNA-targeting cytidine deaminase
Nick
UGI
UNG
AP site
or
C-to-T
CBE
C-to-G
C-to-A
CGBE
Bb Adenine base editing
nCas9 (D10A)
gRNA
Nuclear genome DNA
PAM sequence
Adenine deaminase
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AYBE
Bc Guanine and thymine base editing
nCas9 (D10A)
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PAM sequence
AAG or eTDG
AAG
eTDG
Nick
AP site
UNG
or
C-to-G
G-to-T
gGBE
T-to-C
T-to-G
T-to-A
TSBE
C Prime editing
Primer binding site
Edit sequence
M-MLV
Non-target strand
Nuclear genome DNA
nCas9 (H840A)
pegRNA
Prime editing
3′ flap containing edits
Precise base substitutions, indels

**Table 1 | Precise targeted genome-modification tools used in plants**

| Editing system | Editing type | PAM | Plant species | Ref. |
|---|---|---|---|---|
| **Cytosine base editors** | | | | |
| PBE | C-to-T | NGG | Rice, wheat, maize | 23 |
| BE3 | C-to-T | NGG | Rice, *Arabidopsis*, watermelon, cotton | 177–180 |
| pCXUN-BE3 | C-to-T | NGG | Rice | 181 |
| Target-AID | C-to-T | NGG | Tomato | 182 |
| rBE3, rBE4 | C-to-T | NGG, NAG, NGA, AGTG and AGCG | Rice | 24 |
| rBE5, rBE9 | C-to-T | NGG | Rice | 183 |
| A3A–PBE | C-to-T | NGG, NGA, NGCG and NNNRRT | Rice, wheat, potato | 25 |
| Anc689BE4max | C-to-T | NGN | Rice | 184 |
| CBE-P1/P3/P5 | C-to-T | NGG, GAG, CGA, GGA and TGCAGT | Rice | 185 |
| rBE22 | C-to-T | NGN, NAC, NTT, NTG and NCG | Rice | 186 |
| CBE | C-to-T | NGG | Potato, tomato, rape | 187–189 |
| rBE25 | C-to-T | NNG | Rice | 190 |
| xCas9n-epBE | C-to-T | GAN and NGN | Rice | 191 |
| PhieCBEs | C-to-T | NGN | Rice | 192 |
| nCas9–Sdd7 | C-to-T | NGG | Soybean | 29 |
| **Adenine base editors** | | | | |
| PABE | A-to-G | NGG | Rice, wheat | 35 |
| rBE14 | A-to-G | NGG | Rice | 193 |
| pcABE7.10 | A-to-G | NGG | Rape | 36 |
| ABE-P1/P2/P3/P4/P5 | A-to-G | NGG, GAG, CGA, GGA, GCG and TGCAGT | Rice | 185 |
| ABEmax | A-to-G | NGN | Rice, rape | 184,194 |
| rBE23 | A-to-G | NGN, NAC, NTT, NTG and NCG | Rice | 186 |
| ABE-P1S | A-to-G | NGG and NNNRRT | Rice | 195 |
| rBE26 | A-to-G | NNG | Rice | 190 |
| rABE8e | A-to-G | NGN | Rice | 38 |
| rBE49b, rBE53, rBE57, rBE65 | A-to-G | NGN, NAN, NCG | Rice | 196 |
| PhieABEs | A-to-G | NNN | Rice | 197 |
| **Dual-base editors and other types of base editor** | | | | |
| STEME | C-to-T and A-to-G | NGN | Rice | 113 |
| pDuBE1 | C-to-T and A-to-G | NGG | Rice | 198 |
| CGBE | C-to-G | NGG | Rice | 199 |
| Cas12a-BE | C-to-T or A-to-G | NTTN | Rice | 200 |
| SCGBE2.0 | C-to-G | NGG and NAG | Watermelon | 201 |
| pAKBE | A-to-T or A-to-G | NGG | Rice | 202 |
| AKBEs | A-to-T or A-to-G | NNN | Rice, potato | 203 |
| ZmAYBEv3 | A-to-T or A-to-C | NGG | Maize | 204 |
| **Tools for editing small DNA fragments** | | | | |
| PPE2, PPE3 | 2-bp subs | NGG | Rice, wheat | 205,206 |
| PE-2, PE-3 | 2–4-bp subs, 2-bp ins | NGG | Rice | 207 |
| Sp-PE2, Sp-PE3 | 1–2-bp subs | NGG | Rice | 208 |
| pPE2, pPE3 | 1–3-bp ins, 1-bp subs | NGG | Rice | 209 |
| pZ1WS | 2–3-bp subs | NGG | Maize | 210 |
| pCXPE03 | 2-bp subs, 2-bp ins | NGG | Tomato | 211 |

**Table 1 (continued) | Precise targeted genome-modification tools used in plants**

| Editing system | Editing type | PAM | Plant species | Ref. |
|---|---|---|---|---|
| **Tools for editing small DNA fragments (continued)** | | | | |
| ePPE | 1–2-bp subs, 3-bp ins, 2-bp del, 15–90-bp ins or del | NGG, NGC, NGA | Rice, wheat | 45 |
| enpPE2 | 1-bp ins, 1–2-bp subs | NGG | Rice | 212 |
| PE3-HS/AS/DS | 1–7-bp subs | NGG | Rice | 213 |
| PPE3-evopreQ/mpknot | 1–3-bp subs, 3-bp ins | NGG | Rice | 214 |
| PE-P3-RT-M/S, PE-P2-RT-M/S | 1–4-bp subs | NGG | Rice | 55 |
| ePE3max, ePE5max | 1-bp ins, 1–3-bp subs | NGG | Rice | 57 |
| PE2 (v2) | 1–2-bp subs, 4-bp ins or del | NGG | Rice | 215 |
| ePPEplus | 1–3-bp subs, 3–6-bp del, 4-bp ins | NGG | Wheat | 46 |
| DPE, TPE, QPE | 2–3-bp subs, 28-bp ins | NGG | Rice | 216 |
| PE5max | 30-bp ins | NGG | Rice | 217 |
| AFID-1/2/3 | 1–16-bp del | NGG | Rice, wheat | 60 |
| GRAND | 46-bp subs | NGG | Rice | 218 |
| **Tools for manipulating large DNA segments** | | | | |
| PrimeRoot | 720-bp, 1.4-kb, 4.9-kb, 7.7-kb, 11.1-kb ins | NGN | Rice, maize | 66 |
| Cas9–Pong | 430-bp, 444-bp, 1-kb, 1.5-kb ins | NGG | *Arabidopsis*, soybean | 71 |

ABE, adenine base editor; CBE, cytosine base editor; bp, base pair, del, deletion; ins, insertion; subs, substitution.

where it has been used to accelerate breeding or to enhance desirable traits including yield and nutritional content in crops such as wheat, rice, maize and tomato[12]. By contrast, base editing, prime editing and some strategies for editing large DNA segments avoid double-strand breaks by using modified or partially catalytically inactive Cas variants with additional functionalities to generate targeted modifications. Given their potential for achieving greater precision and larger editing scale in plant genome editing, we will focus on these technologies here (Table 1).

## Base editing

Base editors achieve precise base substitutions without the need for double-strand breaks and the use of donor DNA, thereby avoiding error-prone repair processes[20]. Base editors consist of a modified Cas9 protein to enable recognition of its target locus under direction of a gRNA, where they generate an R-loop. The modified Cas9 is fused to a deaminase that chemically modifies DNA bases within a small 'editing window' of exposed single-stranded DNA (ssDNA) on the non-targeted strand, resulting in the desired changes of the target sequence via the endogenous DNA-repair mechanisms of the cell. The mainstream base editors all harness a Cas9 nickase (nCas9) containing the point mutation Asp10Ala (D10A), which can inactivate one of the three RuvC nuclease domains of *Streptococcus pyogenes* Cas9 (SpCas9), rather than catalytically dead Cas9 (dCas9). nCas9 generates single-strand breaks ('nicks') in the targeted strand and stimulates endogenous DNA-repair mechanisms that tend to use the edited strand as a template, thereby enhancing editing efficacy. The two main types of base editor are cytosine base editors and adenine base editors, and more derivative base editors have also been developed that can target the nuclear genome as well as organelle genomes (Table 1, Box 1).

**Targeted cytosine base editing.** Cytosine base editors use a ssDNA cytosine deaminase, such as rat APOBEC1 (rAPOBEC1) or *Petromyzon marinus* cytidine deaminase 1 (PmCDA1), tethered to a uracil DNA glycosylase inhibitor (UGI), together with nCas9 (D10A), to achieve precise conversion of C•G base pairs to T•A base pairs via a uracil intermediate (U•G mismatch)[21,22] (Fig. 1Ba). Initially, the efficiency of a cytosine base editor was relatively low in some endogenous target sites in plants, such as rice and wheat, and it showed sequence preference for TC motifs, which affected the ability to target broadly across the genome[23,24]. Replacing rAPOBEC1 with human APOBEC3A increased the average efficiency of a cytosine base editor more than 10-fold, eliminated the sequence preference and enlarged the editing window from positions 3–9 to 1–17, thus greatly expanding the utility of cytosine base editors in plants[25].

A series of cytosine base editor variants has been developed by directed evolution and rational design of deaminases that exhibit different sequence preferences and editing-window sizes, greater specificity and lower off-target effects than the original cytosine base editor[26,27] (Table 1). Because deaminases are key functional elements in cytosine base editors, harnessing diverse deaminases has been critical to their optimization. The assistance of AlphaFold2 (ref. 28), a structure-based (rather than sequence-based) protein clustering method, has improved our ability to identify novel deaminases and expanded the utility of

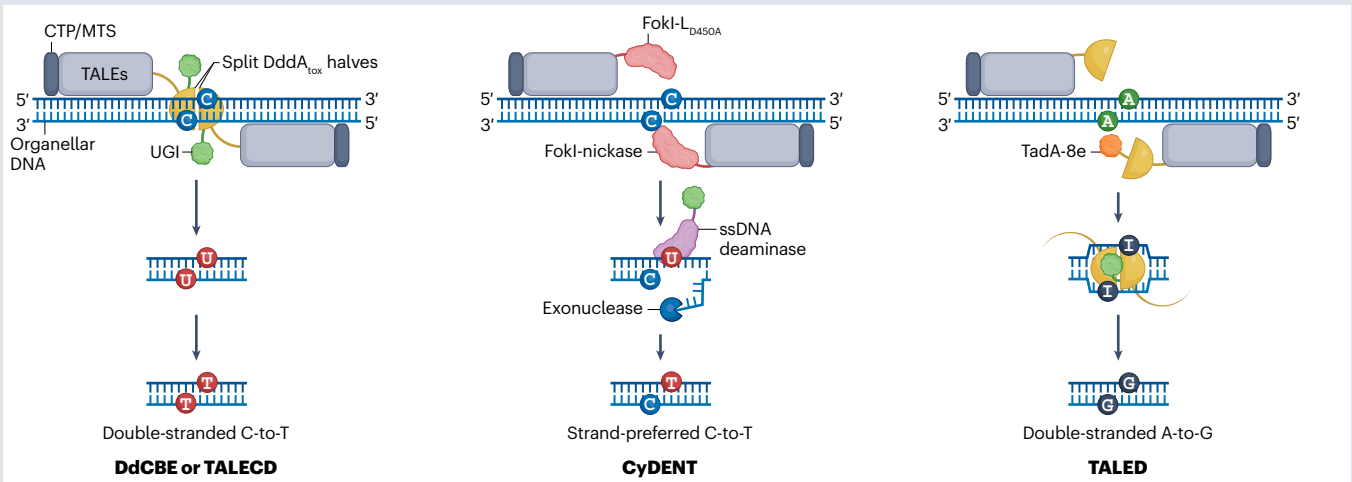
## Box 1

# Targeted base editing of organelle genomes

Apart from the nuclear genome, organelle genomes, such as from the mitochondria and chloroplasts, encode for genes that have great influences on cellular processes such as respiration and photosynthesis. There exist important target genes in which precise targeted base editing could uncover fundamental biological research processes and facilitate molecular crop breeding[219]. However, editing the DNA within these organelles is especially challenging when using CRISPR–Cas systems because the double-membrane structure of these organelles hinders single-guide RNA penetration and delivery[220]. By contrast, protein-based DNA targeting enzymes such as transcription activator-like effectors (TALEs) can readily be made to enter mitochondria or chloroplasts by the addition of their respective targeting signals. MitoTALEN, which is comprised of a pair of TALE proteins each fused with a FokI nuclease and localized by a mitochondrial targeting signal (MTS), has already been demonstrated to be capable of disrupting genes associated with cytoplasmic male sterility in the mitochondria of crops such as rice, rape[221] and tomato[222], producing male infertility, which is useful for crop breeding.

To further mitigate the impact of DNA double-strand breaks on organelle genome stability and achieve even more precise targeted modifications, base editors tailored for plant organelles have been developed (see the figure). The double-stranded DNA deaminase (DddA)-derived cytosine base editor (DdCBE)[223] combines split versions of the double-stranded DNA-targeting cytidine deaminase DddAtox (the cytosine deamination functional domain of DddA) with TALEs and a uracil DNA glycosylase inhibitor (UGI) peptide, enabling precise C-to-T changes on both strands of human mitochondrial DNA. The addition of either a MTS or chloroplast transit peptide (CTP) permits DdCBE to perform cytosine base editing in the mitochondrial or chloroplast genome of lettuce, respectively[220]. Similarly, TALECD can edit cytosines in the plastid genomes of *Arabidopsis* based on a similar working model[224]. Nevertheless, DddA is a double-stranded DNA deaminase that presents difficulty in inducing mutations on a specific DNA strand. Recently, the combination of using single-strand DNA (ssDNA) deaminases, TALE effectors, a UGI peptide, FokI nickases and an additional exonuclease resulted in the cytidine deaminase–exonuclease–nickase–TALE (CyDENT) base editing system[225]. CyDENT enables strand-preferred C-to-T conversion in rice nuclear and chloroplast genomes without the addition of DddA, thus further enhancing precision in organelle genome editing. A-to-G editing in organelles is also feasible by a similar design but with an adenosine deaminase. TALEDs, which are composed of TALEs, split DddAtox and the adenosine deaminase TadA-8e, were shown to edit adenine bases on double-stranded DNA at target sites in *Arabidopsis* and lettuce chloroplast genomes[226]. In this system, DddA was involved in the unwinding of the DNA double helix to provide transient-state single-stranded DNA substrates for TadA-8e deamination. Other organellar base editors, such as mitoBEs[227], are also promising for use in plants despite only having been tested in mammalian cells so far.

Compared to editing the nuclear genome, research on organelle editing still needs further development, which will facilitate the manipulation of important crop traits such as photosynthetic efficiency and fertility. Furthermore, precise editing of chloroplasts, given their matrilineal inheritance and multi-copy nature within cells, has the potential to positively affect the generation of plant bioreactors and synthetic biology research.




cytosine base editors to more species, including soybean[29]. Because the structure of a protein determines its function, this work provides a direct and simple approach for better differentiating and discovering deaminases with diverse functions than the original sequence-based clustering method.

In addition, researchers have found that cytosine base editors without UGI show a preference for converting cytosine to guanine via the base excision repair pathway[30]. Fusing *Escherichia coli*-derived uracil DNA *N*-glycosylase (eUNG), which can convert uracil to an apurinic/apyrimidinic (AP) site and so facilitate the base excision repair pathway,

with rAPOBEC deaminase and nCas9 (D10A) permits efficient C-to-G editing and a low frequency of C-to-A editing[31–33] (Fig. 1Ba). We note that this approach is generally accompanied by more by-products, such as small indels, than conventional C-to-T editing.

**Targeted adenine base editing.** Adenine base editors achieve A-to-G base substitutions by fusing nCas9 (D10A) to an adenine deaminase (Fig. 1Bb). Few natural ssDNA adenine deaminases exist, so multiple rounds of directed evolution of the tRNA deaminase TadA were required to obtain the first ssDNA adenosine deaminase TadA7.10, thus producing ABE7.10 (ref. 34). However, ABE7.10 is still less efficient at deaminating many endogenous target sites than cytosine deaminases, especially in plants[35,36]. Subsequently, researchers carried out phage-assisted non-continuous and continuous evolution of TadA7.10, ultimately obtaining TadA8e, which has 590-fold better deamination activity than TadA7.10 (ref. 37). Using the ABE8e system based on TadA8e, more efficient A-to-G editing has been achieved in both animals and plants, with an improvement of over 10-fold in plants compared with ABE7.10 (ref. 38). Additionally, combining ABE8e with alkyladenine DNA glycosylase (AAG), which can convert inosine to an AP site, allowed for efficient A-to-C and A-to-T base substitutions, and the purity of the A-to-C and A-to-T editing products could be improved by the rational design of TadA8e and mAAG, thus optimizing editing precision[39–41] (Fig. 1Bb).

**Targeted guanine and thymine base editing.** In addition to cytosine and adenine editing, high-efficiency G-to-T and G-to-C substitutions have been created in mammalian cells without relying on any deaminase by using CRISPR to directly reprogramme AAGs[42] (Fig. 1Bc). Recently, researchers have engineered the UNG by rational design, obtaining an enhanced uracil *N*-glycosylase variant with specificities towards thymines (enhanced thymine DNA glycosylase, eTDG). Integrating eTDG with nCas9 protein led to the development of editors for programmable T-to-G or T-to-C substitutions[43]. However, this approach requires further validation in plants, and the purity of the products generated by these editors needs to be improved.

## Editing of small DNA fragments

Although optimized base editors are highly effective in eukaryotic cells, the types of modification they can produce are limited. The development of prime editing represented a milestone in TGM: its creative design allows the production of precise DNA substitutions and small indels. Prime editors use nCas9 (H840A) protein fused with Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) and an engineered prime editing gRNA (pegRNA), which includes a spacer in its 5′ end, a reverse transcriptase template as well as an additional primer binding site in its 3′ end[44] (Fig. 1C). nCas9 (H840A) contains the point mutation His840Ala, which inactivates the HNH nuclease domain of Cas9 so that it primarily generates single-strand breaks (nicks) in the non-targeted strand. The initial prime editor 1 (PE1) system has been enhanced in several ways: first, by optimizing the protein components; second, by engineering the pegRNA; third, by manipulating DNA repair; and finally, by harnessing the DNA polymerase, as discussed below.

The M-MLV RT of PE2 carries five amino acid mutations introduced to improve its thermal stability and reverse transcription activity, which resulted in a five-fold increase in editing efficiency[44]. Furthermore, the engineered plant prime editor (ePPE) was developed by deleting the RNaseH domain of M-MLV RT as well as by incorporating the viral nucleocapsid protein[45]. ePPEplus was generated by introducing additional effective amino acid mutations on M-MLV RT and nCas9 based on ePPE[46]. These changes substantially increased the efficiency of prime editing in plants[45,46]. Prime editing efficiency has also been increased by using phage-assisted continuous evolution for directed evolution of M-MLV RT and other reverse transcriptases, such as Tf1[47]. Recently, a study used the virion-associated protein Vpx to degrade the phosphohydrolase SAMHD1, thereby increasing the cellular concentration of deoxynucleoside triphosphates (dNTPs) and promoting reverse transcription, thus improving prime editing efficiency[48]. Moreover, introducing point mutations in Cas9 to enhance its nickase activity[49], as well as using more effective nuclear localization signal sequences are also feasible approaches for optimizing prime editing efficiency[50].

Adding RNA secondary structures (such as evopreQ, G-quadruplex, Zika xrRNA) to the 3′ end of the pegRNA can enhance efficiency by improving its stability and stimulating reverse transcription[51–53]. Prime editing efficiency can also be increased by introducing synonymous mutations of the host genome sequence in the reverse transcriptase template[54,55]. Moreover, deep learning can be applied to predict the optimal lengths of the primer binding site and reverse transcriptase template[56].

The PE3 system uses the same principle as base editors, incorporating an additional gRNA to generate another nick in the target strand, which stimulates DNA repair and increases prime editing efficiency[44]. However, this strategy also leads to an increase of by-products. Through large-scale CRISPR interference (CRISPRi)-based screening, researchers have identified a mismatch-repair-related factor, MLH1, that inhibits prime editing. By fusing a dominant negative mismatch-repair protein (MLH1dn) with PE2/PE3, the PE4/PE5 system was developed, which has improved prime editing efficiency in mammalian cells and plants[49,57].

Prime editing can also be achieved by using RNA–DNA or purely DNA forms of the primer binding site–reverse transcriptase template and by replacing M-MLV with DNA polymerase[58,59], although efficiency remains relatively low. The strategy of using DNA polymerases is likely to be adapted for editing larger segments of DNA because of its high fidelity and greater capacity to synthesize DNA. A complete understanding of the dynamics of prime editor complexes and the intended repair mechanisms should lead to further optimization of prime editing systems.

Other methods of achieving precise small DNA changes have been developed. One of these, the AFID (APOBEC–Cas9 fusion-induced deletion) system creates predictable small DNA deletions in plants[60]. AFID uses the powerful APOBEC deaminases and uracil DNA glycosylase, along with AP lyase, to introduce precise deletions between deaminated cytosines and the Cas9 cleavage site. Apart from deletions, the ssDNA extensions generated by AFID editing may also be used to create precise insertions or replacements of small DNA fragments.

## Precise manipulation of large DNA segments

Although base editing and prime editing are currently more suitable for base-pair level changes, advances in CRISPR technology are continually expanding the capabilities for manipulating the genome, including larger-scale modifications at the kilobase level and above. We note that precise editing of large DNA segments poses greater challenges than editing single nucleotides or small DNA fragments owing to the inherent confined cell DNA-repair mechanisms and the limited manipulation ability of existing editors. Traditional strategies for manipulation of large DNA segments – such as insertion[61], deletions[62], inversions[63] and translocations[64] – rely on the generation of multiple double-strand breaks, which is imprecise and relatively inefficient. Some emerging

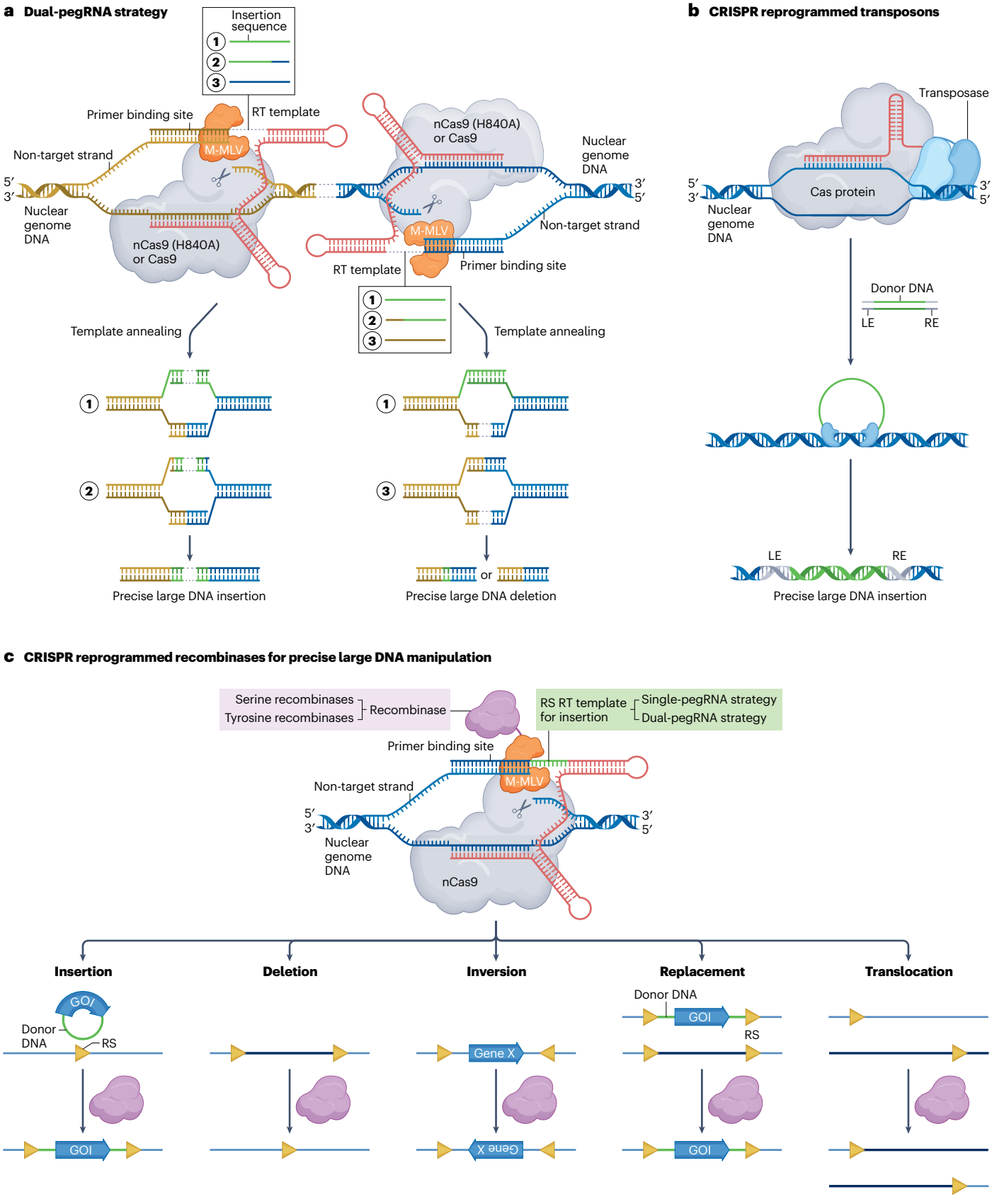

a Dual-pegRNA strategy
Insertion sequence
1
2
3
Primer binding site
RT template
M-MLV
Non-target strand
5′
3′
Nuclear genome DNA
nCas9 (H840A) or Cas9
nCas9 (H840A) or Cas9
Nuclear genome DNA
3′
5′
Non-target strand
M-MLV
RT template
Primer binding site
Template annealing
Template annealing
Precise large DNA insertion
or
Precise large DNA deletion
b CRISPR reprogrammed transposons
Transposase
Cas protein
Nuclear genome DNA
Donor DNA
LE
RE
LE
RE
Precise large DNA insertion
c CRISPR reprogrammed recombinases for precise large DNA manipulation
Serine recombinases
Tyrosine recombinases
Recombinase
RS RT template for insertion
Single-pegRNA strategy
Dual-pegRNA strategy
Primer binding site
M-MLV
Non-target strand
5′
3′
Nuclear genome DNA
nCas9
3′
5′
Insertion
Deletion
Inversion
Replacement
Translocation
GOI
Donor DNA
RS
GOI
Gene X
Gene X
Donor DNA
GOI
RS
GOI

**Fig. 2 | Precise editing of large DNA segments. a**, Dual-prime editing guide RNA (pegRNA) strategy for inserting and deleting large DNA segments (kilobase-level and above). Reverse transcription (RT) sequences of types 1 and 2 enable precise replacement of the sequence between the two target cleavage sites with desired edits in RT; the type 3 RT sequence enables precise deletion of the sequence between the two target cleavage sites. The sequence of one target site is in blue, that of the other in brown; the green sequence represents the sequence to be inserted. **b**, Insertion of large DNA segments by reprogrammed transposases. LE (left end) and RE (right end) represent the transposase recognition sites; the green sequence represents the desired donor segment. **c**, Manipulation of large DNA segments by prime-editing-reprogrammed site-specific recombinases. Inserting a recombination site (RS) into a DNA allele along with a circular donor DNA containing another RS enables precise insertion of a gene of interest (GOI); inserting two RSs head-to-tail into a DNA allele enables precise deletion between the two RSs; inserting two RSs head-to-head enables precise inversion of the intervening segment containing endogenous genes (gene X); inserting two RSs and providing a donor DNA with homologous RSs enables precise replacement of the sequence between the RSs with the GOI; and inserting RSs into two DNA alleles facilitates translocation between them. nCas9, Cas9 nickase; M-MLV, Moloney murine leukaemia virus.

methods are independent of double-strand breaks and endogenous DNA-repair mechanisms; although they remain at an early stage, their efficiency and precision are being improved.

**Prime-editing-based modification of large DNA segments.** Usage of prime-editing-based dual pegRNA systems has allowed the insertion and deletion of large DNA segments. These systems make use of two pegRNAs within a certain distance, by utilizing two reverse transcriptase templates that contain only complementary insertion sequences or another pegRNA target site sequence; these two reverse transcription products serve as substrates for an efficient and large-scale DNA-repair pathway that may involve single-strand annealing (Fig. 2a). The systems have been shown to work in animals and plants. For example, GRAND (genome editing by reverse transcriptase templates partially aligned to each other but nonhomologous to target sequences within duo pegRNA) editing performs efficient and precise 250-base-pair DNA insertions in mammalian cells[65], whereas the dual-ePPE system has permitted precise insertion of the green fluorescent protein gene (*GFP*) in plant cells[66], albeit at low efficiencies. The Prime-DEL and PEDAR (PE-Cas9-based deletion and repair) systems have achieved precise deletions of up to 10 kb in mammalian cells[67,68], but their effectiveness in plants has yet to be confirmed.

These examples demonstrate the potential of this strategy for achieving precise editing of large DNA segments. Further improvements may be expected from the discovery and rational design of the reverse transcriptases and by upgrading pegRNA structures to edit larger DNA segments.

**Transposase-based insertion of large DNA segments.** Transposons are naturally ubiquitous mobile DNA elements, and active transposons can precisely transfer large DNA cargoes from one location to another target site in the genome[69]. Diverse transposon systems can be used for precisely targeted insertion of large DNA segments in eukaryotes (Fig. 2b). Currently, most systems can be grouped into two types. The first type comprises transposons that are active, or have been remodelled to be active, in eukaryotic cells[70], which typically target simple AT motifs and produce significant off-target changes even after being reprogrammed by specific targeting modules. For example, as reported in a recent preprint[71], the rice *Pong* transposase in a synthetic CRISPR-associated transposase (CAST) system achieved efficient site-specific insertion of large DNA segments after CRISPR–Cas9 reprogramming in both a model plant (*Arabidopsis*) and a crop plant (soybean); however, this approach resulted in a large number of random insertion events. To address this issue, transposases can be modified to weaken their DNA binding ability while retaining transposition activity. For instance, the FiCAT (find and cut-and-transfer) system uses an inactivated *PiggyBac* coupled with Cas9 to increase targeting specificity[72].

The second type consists of CASTs in which the transposon system is naturally coupled with a CRISPR system[73]. In these systems, the original targeting module of transposases has been lost during evolution and replaced by a CRISPR system; as a result, they are highly specific and efficient in prokaryotic cells[73,74]. Nevertheless, despite remodelling and optimization, the transposition efficiency of CAST systems in mammalian cells is still below 1%[75,76], and further validation is required in plants. Therefore, it would be useful to expand and retrofit diverse transposon systems, thereby making eukaryotic transposases highly specific and improving the efficiency of prokaryotic CAST systems in eukaryotic cells. In addition, the development and application of retrotransposons may also provide an important route for achieving precise site-specific insertion of large DNA segments[77].

**Recombinase-based manipulation of large DNA segments.** An advantage of site-specific recombinase systems over other genome editing systems is that most of them do not rely on endogenous DNA-repair mechanisms and energy-consuming cofactors, but require only a single component to complete DNA recombination; they are thus considered to have huge potential for precisely manipulating large DNA segments[78] (Fig. 2c). However, these systems are not easily programmable, because they recognize only specific sequences called recombination sites. One solution is to insert a recombination site at the desired target site using CRISPR. For example, the prime editing system can be coupled with the serine recombinase Bxb1, such as in PASTE (programmable addition via site-specific targeting elements) and twinPE + Bxb1, to achieve precise insertions of up to 36 kb and precise inversions of 40 kb in mammalian cells[79,80]. Similarly, the PrimeRoot (prime editing-mediated recombination of opportune targets) system couples dual-ePPE with the tyrosine recombinase Cre; by pre-processing donor DNA with Cre, DNA insertions of up to 11.1 kb without the vector backbone have been achieved in plants such as rice and maize[66]. In addition, microbial recombination enzymes referred to as SSAPs (single-stranded annealing proteins) can be guided by dCas9 to achieve precise targeted insertion of large DNA segments in mammalian cells[81], but further validation is needed in plants. These studies reveal the tremendous potential of site-specific recombinases for editing large DNA segments, which indicate that in-depth characterization and optimization of recombinases are important avenues for investigation.

## Delivery technologies in plant TGM

When applying TGM in plants, it is typically necessary to deliver TGM tools in the form of DNA, RNA or ribonucleoproteins into plant cells and regenerate the cells with intended genetic modifications into complete individuals. Therefore, highly efficient delivery systems are a prerequisite within the technical framework that connects advanced

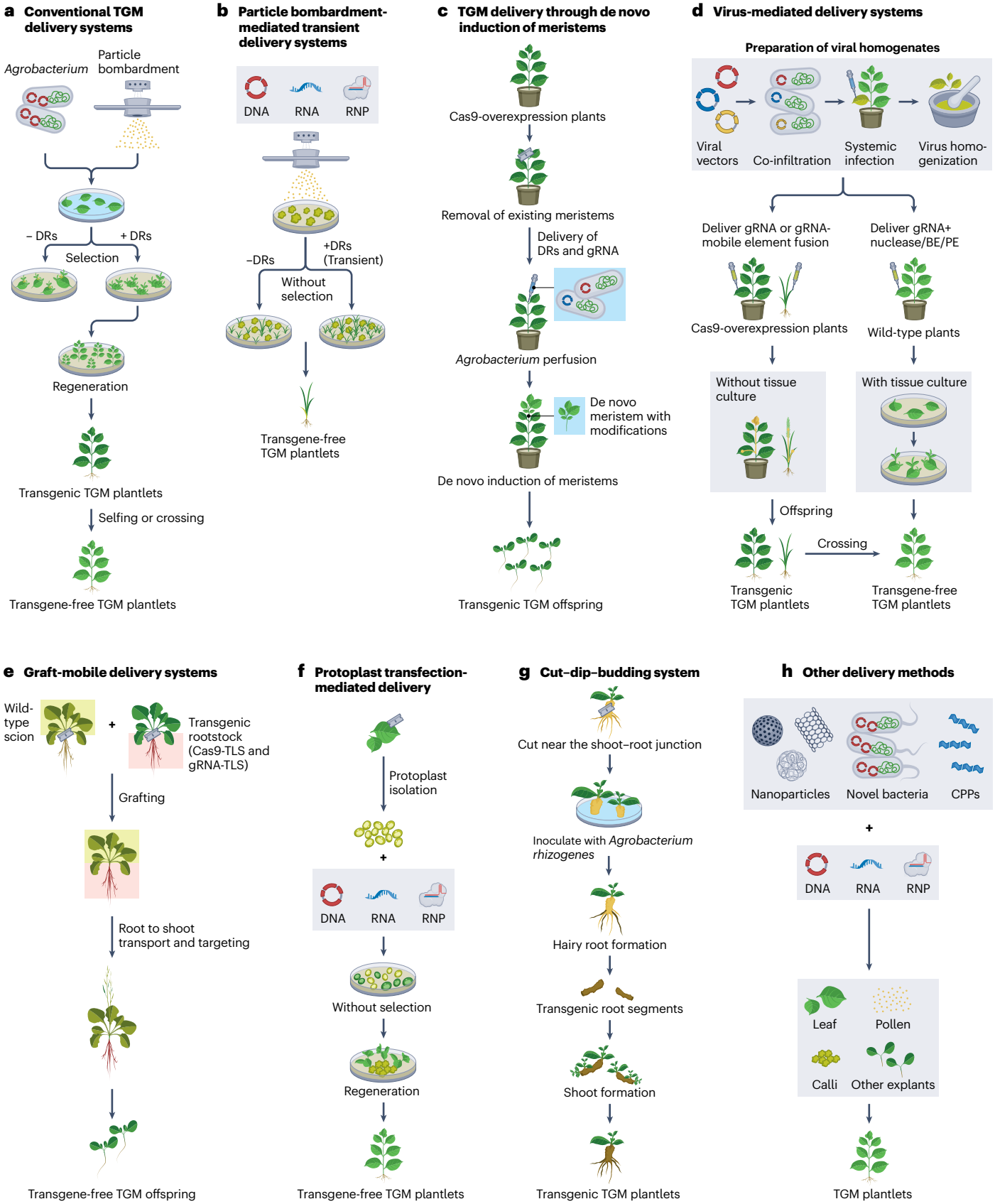

a Conventional TGM delivery systems
Agrobacterium
Particle bombardment
– DRs
+ DRs
Selection
Regeneration
Transgenic TGM plantlets
Selfing or crossing
Transgene-free TGM plantlets
b Particle bombardment-mediated transient delivery systems
DNA
RNA
RNP
–DRs
+DRs (Transient)
Without selection
Transgene-free TGM plantlets
c TGM delivery through de novo induction of meristems
Cas9-overexpression plants
Removal of existing meristems
Delivery of DRs and gRNA
Agrobacterium perfusion
De novo meristem with modifications
De novo induction of meristems
Transgenic TGM offspring
d Virus-mediated delivery systems
Preparation of viral homogenates
Viral vectors
Co-infiltration
Systemic infection
Virus homo-genization
Deliver gRNA or gRNA-mobile element fusion
Deliver gRNA+ nuclease/BE/PE
Cas9-overexpression plants
Wild-type plants
Without tissue culture
With tissue culture
Offspring
Crossing
Transgenic TGM plantlets
Transgene-free TGM plantlets
e Graft-mobile delivery systems
Wild-type scion
Transgenic rootstock (Cas9-TLS and gRNA-TLS)
Grafting
Root to shoot transport and targeting
Transgene-free TGM offspring
f Protoplast transfection-mediated delivery
Protoplast isolation
DNA
RNA
RNP
Without selection
Regeneration
Transgene-free TGM plantlets
g Cut–dip–budding system
Cut near the shoot–root junction
Inoculate with Agrobacterium rhizogenes
Hairy root formation
Transgenic root segments
Shoot formation
Transgenic TGM plantlets
h Other delivery methods
Nanoparticles
Novel bacteria
CPPs
DNA
RNA
RNP
Leaf
Pollen
Calli
Other explants
TGM plantlets

**Fig. 3 | Delivery technologies used in plant targeted genome modification.** **a**, *Agrobacterium*- or particle-bombardment-mediated conventional targeted genome-modification (TGM) delivery systems. Conventional systems rely on selection pressure to obtain transgenic TGM plants, and the foreign DNA is then segregated out by selfing or crossing. Developmental regulators (DRs) can be used to boost regeneration during tissue culture. **b**, Particle-bombardment-mediated transient DNA, RNA and ribonucleoprotein (RNP) delivery systems. Transient expression of DRs can also promote regeneration. **c**, TGM delivery via de novo induction of meristems involves the removal of existing meristems from Cas9-overexpressing plants, with DRs and guide RNA (gRNA) subsequently delivered through *Agrobacterium*-mediated transformation at the cut site. The newly induced transgenic meristem tissue with desired modifications can be obtained and the mutations are heritable. **d**, Virus-mediated delivery. *Agrobacteri*a that harbour viral vectors are co-infiltrated into *Nicotiana benthamiana* leaves. After systemic infection, the infected leaves are ground up to produce a virus homogenate for inoculating recipients. Cas9-overexpressing and wild-type plants are used as recipients for virus with gRNA or gRNA plus Cas protein, base editor (BE) or prime editor (PE), respectively. **e**, Graft-mobile delivery systems involve joining tissues of a wild-type scion and a transgenic rootstock; this enables the transportation of Cas9–tRNA-like sequences (TLS) and gRNA–TLS from root to shoot, leading to targeted modifications in offspring. **f**, Protoplast delivery and regeneration. Protoplast transfection involves introducing TGM tools into isolated plant protoplasts, followed by their regeneration into whole plantlets. DNA and RNPs are widely used forms of reagents in protoplast delivery, and RNA may also be used in future. The darker green protoplasts have undergone an editing event. **g**, Cut–dip–budding is a tissue-culture-free method that uses *Agrobacterium rhizogenes* to induce transgenic roots from the cut sites of explants. The transformed roots possess shoot-forming ability, enabling the production of TGM buds via root suckering. **h**, Other delivery methods in plants include nanoparticles, novel bacteria and cell-penetrating peptides (CPPs).

TGM tools to their applications in crop breeding[82]. However, the impediment of the cell wall and the inefficient regeneration capacity of plant cells have become key limiting factors. Additionally, how to prevent the integration of exogenous DNA into the genome during the delivery process to mitigate potential unexpected genome perturbations and avoid transgene regulation issues is a matter of concern. Recent advances have weakened the impact of the cell wall and the cell regeneration capacity on delivery efficiency through plant viruses, de novo induction of meristems and grafting. Furthermore, delivering RNA or ribonucleoproteins, rather than DNA, has circumvented limitations associated with transgenesis, leading to the improvement of plant TGM technologies. This section will provide an overview of the current plant TGM delivery technologies, while also highlighting their characteristics and potential applications.

## Conventional TGM delivery systems

Conventional TGM delivery in plants mainly relies on use of the soil bacterium *Agrobacterium tumefaciens* as a vector to transfer foreign genes into plant cells or particle-bombardment-mediated genetic transformation. Transgenic plants that have regenerated under selection pressure are submitted to genotypic and/or phenotypic analyses to identify the mutants that harbour a desired mutation. Once such a mutant has been obtained, the integrated TGM components in plant genomes become unnecessary and can be eliminated through selfing or crossing (Fig. 3a). However, these conventional methods have two drawbacks: one is the lengthy regeneration process, which is especially challenging in recalcitrant crop species such as cotton and wheat[13]; the other challenge is that eliminating stable transgenes may prolong the time needed for generating new germplasm[83]. Moreover, transgene elimination through selfing or crossing is impractical for vegetatively propagated crops such as potato, sweet potato and strawberry. Therefore, novel delivery strategies that bypass tissue culture and avoid the incorporation of transgenes are much needed to apply TGM in crops.

## Particle-bombardment-mediated transient delivery systems

Particle bombardment enables transient delivery of TGM elements in the form of DNA, RNA and ribonucleoproteins without the need to apply selection pressure[84–87] (Fig. 3b). Consequently, it can yield transgene-free mutants in the $T_0$ generation. Given that regeneration remains a potential bottleneck owing to tissue-culture requirements, transient expression of certain developmental regulators via particle bombardment should be sufficient to promote regeneration, which has been demonstrated in both wheat and maize[88,89]. Although particle bombardment can cause damage to reagents and the genome[90], it remains one of the simplest and most universally applicable methods for delivering TGM reagents in the form of RNA or ribonucleoproteins in plants.

## TGM delivery through de novo induction of meristems

Sidestepping tissue culture greatly enhances the efficiency and speed of TGM. Previous studies have demonstrated the potential of ectopic expression of specific developmental regulators to boost regeneration[13,89,91,92]. For this reason, a technique called Fast-TrACC (fast-treated agrobacterium coculture) has been developed that utilizes *Agrobacterium* for delivering combinations of specific developmental regulators, such as *Wuschel 2* (*Wus2*) and *SHOOT MERISTEMLESS* (*STM*), as well as gRNAs to plants overexpressing Cas9[93] (Fig. 3c). Fast-TrACC achieves heritable genome editing through de novo induction of meristems, rather than via tissue culture, and this approach promises to be more widely applicable in dicotyledonous species. However, it can only be used in crops susceptible to *Agrobacterium* infection, and the resulting mutant plants are transgenic[93].

## Virus-mediated delivery systems

As plant viruses possess the ability to replicate, self-assemble and move between plant cells, they can be exploited to deliver TGM reagents[94] (Fig. 3d). Some positive-strand RNA viruses such as tobacco rattle virus (TRV)[95,96] and barley stripe mosaic virus (BSMV)[97] have been used to directly deliver gRNA to Cas9-overexpressing plant cells, thereby avoiding tissue culture. Fusing gRNA with mobile RNA elements, such as *Flowering Locus T* (*FT*) RNA or tRNA, has been shown to further facilitate the entry of gRNA into meristematic tissue and to achieve efficient editing without tissue culture, and this strategy is currently more effective in dicotyledonous than monocotyledonous plants[95,97]. Nonetheless, these methods are all limited by the cargo capacity of virus particles and fail to deliver effectors over 1,000 amino acids in size, such as Cas9. However, genetically engineered viruses, such as sonchus yellow net rhabdovirus (SYNV)[98] and tomato spotted wilt virus (TSWV)[99], are able to deliver nucleases and even larger base editors together with gRNAs into plant cells, but tissue culture is still needed to obtain desired mutant progenies (Fig. 3d). Although virus-mediated delivery provides more options for transgene-free and tissue-culture-free TGM, viruses that

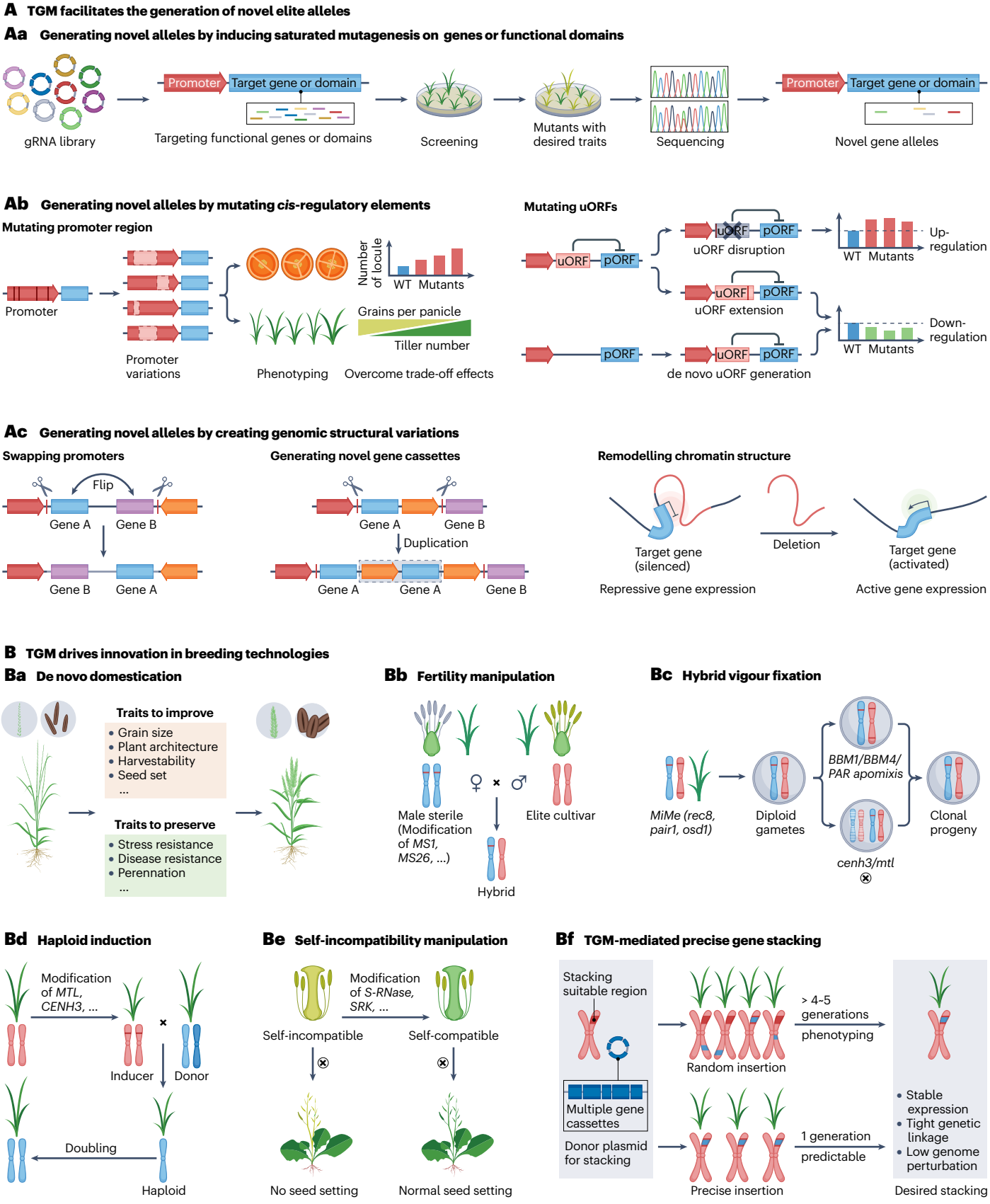

A TGM facilitates the generation of novel elite alleles
Aa Generating novel alleles by inducing saturated mutagenesis on genes or functional domains
gRNA library
Promoter
Target gene or domain
Targeting functional genes or domains
Screening
Mutants with desired traits
Sequencing
Promoter
Target gene or domain
Novel gene alleles
Ab Generating novel alleles by mutating cis-regulatory elements
Mutating promoter region
Promoter
Promoter variations
Number of locule
WT Mutants
Grains per panicle
Tiller number
Phenotyping
Overcome trade-off effects
Mutating uORFs
uORF
pORF
uORF disruption
WT Mutants
Up-regulation
uORF extension
Down-regulation
de novo uORF generation
Ac Generating novel alleles by creating genomic structural variations
Swapping promoters
Flip
Gene A
Gene B
Generating novel gene cassettes
Duplication
Remodelling chromatin structure
Target gene (silenced)
Repressive gene expression
Deletion
Target gene (activated)
Active gene expression
B TGM drives innovation in breeding technologies
Ba De novo domestication
Traits to improve
Grain size
Plant architecture
Harvestability
Seed set
...
Traits to preserve
Stress resistance
Disease resistance
Perennation
...
Bb Fertility manipulation
Male sterile (Modification of MS1, MS26, ...)
Elite cultivar
Hybrid
Bc Hybrid vigour fixation
MiMe (rec8, pair1, osd1)
Diploid gametes
BBM1/BBM4/ PAR apomixis
cenh3/mtl
Clonal progeny
Bd Haploid induction
Modification of MTL, CENH3, ...
Inducer
Donor
Doubling
Haploid
Be Self-incompatibility manipulation
Modification of S-RNase, SRK, ...
Self-incompatible
Self-compatible
No seed setting
Normal seed setting
Bf TGM-mediated precise gene stacking
Stacking suitable region
Multiple gene cassettes
Donor plasmid for stacking
Random insertion
> 4-5 generations
phenotyping
Precise insertion
1 generation
predictable
Stable expression
Tight genetic linkage
Low genome perturbation
Desired stacking

**Fig. 4 | Advanced applications of TGM in crop breeding. A**, Targeted genome-modification (TGM) facilitates the generation of novel elite alleles. **Aa**, Strategies for generating novel alleles using saturated mutagenesis. By designing and transforming a guide RNA (gRNA) library to target functional genes or domains, saturated or near-saturated mutagenesis can be achieved, resulting in mutants with desired traits. **Ab**, Strategies for generating novel alleles by mutating *cis*-regulatory elements. These strategies can be used to manipulate gene expression and create valuable phenotypes. The panel shows TGM-mediated mutations on promoters or upstream open reading frames (uORFs) that can tune gene expression and affect yield-related traits. pORF, primary open reading frame. **Ac**, Strategy for generating novel alleles by creating genomic structural variation, such as inversion, duplication, deletion and others. **B**, TGM drives innovation in breeding technologies. **Ba**, De novo domestication of wild species (*Thinopyrum intermedium* as an example) by targeting domestication genes to improve their agronomic traits while preserving the original advantageous characteristics. **Bb**, TGM-mediated manipulation of fertility by targeting male-sterility-related genes. **Bc**, TGM generates mitosis instead of meiosis (MiMe) genotypes. Incorporating *CENH3* or *MTL* mutations, or ectopically expressing apomixis-inducing genes, can fix hybrid vigour to produce clonal seeds. **Bd**, Mutating haploid-induction-related genes generates haploid inducer lines via TGM. **Be**, TGM-mediated manipulation of self-incompatibility by knocking out endogenous genes, such as *S-RNase*, *SRK* and others. **Bf**, TGM-mediated precise gene-stacking events are stable, with tight genetic linkage and low perturbation to the genome. WT, wild type.

can deliver gRNA and Cas9 together while enabling heritable editing are still lacking.

### Graft-mobile delivery systems

Graft-mobile editing systems are essentially RNA-transfer systems and provide a novel strategy for transgene-free and tissue-culture-free TGM among species and varieties. RNA molecules such as tRNA and FT have been shown to possess the ability to move long distances within plants, while grafting serves to establish extensive substance and information exchange between different varieties or species of rootstock and scion[100–102]. Combining these two processes has opened up a novel route for TGM delivery. In a recent study, researchers generated a transgenic *Arabidopsis* rootstock producing Cas9–tRNA-like sequences (TLS) and gRNA–TLS fusions to promote RNA movement from root to shoot, creating targeted deletions in offspring when wild-type *Arabidopsis* scions were grafted to the rootstock[103] (Fig. 3e). Cas9–TLS and gRNA–TLS constructs can also be transported from transgenic *Arabidopsis* roots to wild-type *Brassica rapa* shoots and induce targeted mutations[103], thus modifying the genome of a grafted plant. Nevertheless, the manufacture of rootstocks remains a rate-limiting step and needs further optimization to enhance the throughput of graft-mobile gene-editing systems. Grafting techniques are also still limited, making it challenging to implement this method in monocot plants.

Innovative work is attempting to expand plant-delivery systems. Cell-wall-free protoplasts exhibit improved transformation characteristics that resemble those of cultured cell lines (Fig. 3f). However, regeneration is challenging and is currently only feasible in a limited number of species[104–106]. The cut–dip–budding delivery method seems promising for transforming recalcitrant species such as *Taraxacum kok-saghyz*, *Coronilla varia* and *Aralia elata*, using *Agrobacterium rhizogenes* (now *Rhizobium rhizogenes*) without the need for sterile conditions and tissue culture[107] (Fig. 3g). Additionally, genetically modified *Agrobacterium*[108], cell-penetrating peptides[109], magnetic nanoparticles[110,111] and other methods[112] are potential approaches for expanding TGM delivery methods (Fig. 3h).

## Advanced applications of TGM in crop breeding

Advances in TGM technologies and delivery systems have consistently helped to make plant genome manipulation more precise and flexible. This has made it possible to create mutations that are difficult to achieve using other techniques, and to reproduce known beneficial variations swiftly and precisely through targeted knockouts, base substitutions and precise manipulation of genomic segments at different scales, thereby optimizing the traits of crops. The integration of TGM with other breeding methods has driven the iterative progress of breeding technologies, with the complementary advantages of such techniques greatly boosting breeding efficiency. In this section, we showcase the innovative applications of TGM in crop breeding in two areas: generating novel mutations and fostering innovation in breeding technologies (Fig. 4).

### Generation of novel elite alleles

Heredity and variation form the cornerstones of crop genetic improvement. The programmable, precise and highly efficient targeting capacity of TGM has substantially enhanced the speed and efficiency of genome mutagenesis, leading to the creation of a substantial number of novel approaches for driving germplasm enhancement. This section will introduce methods for generating elite alleles through TGM and describe their successful applications in breeding.

**Generating novel alleles by inducing saturated mutagenesis on genes or functional domains.** Research in crop genomics has provided vast genetic resources for breeding work. However, breeders face challenges in quickly obtaining the most suitable allele of a target gene for breeding applications. TGM technologies possess great advantages over conventional mutagenesis methods in terms of targeting, enabling rapid saturated mutagenesis of a specific gene or functional domain[113,114] (Fig. 4Aa), thereby overcoming the limited genetic diversity of the natural gene pool. In rice, by using dual-base editors, or adenine base editors and cytosine base editors separately, near-saturated mutagenesis has been achieved in the target domains of rice *acetyl-coenzyme A carboxylase* (*OsACC*)[113,115], rice *acetolactate synthase 1* (*OsALS1*)[114] and *enzyme 5-enolpyruvylshikimate-3-phosphate synthase* (*OsEPSPS*)[116], thereby identifying novel herbicide resistance mutations[113–116]. Moreover, a recent study further enriched this strategy by harnessing prime editing to achieve saturated mutagenesis of six important herbicide resistance-related residues in rice ACCase[117]. These achievements underline the remarkable abilities of TGM to generate novel beneficial alleles.

**Generating novel alleles by mutating *cis*-regulatory elements.** Gene expression patterns have been shown to play a vital part in shaping traits[118], and variations within *cis*-regulatory elements can affect the fine regulation of gene expression. Therefore, generating novel *cis*-regulatory element variants could serve as an effective strategy for precise shaping of crop traits[119–121] (Fig. 4Ab). In tomato, targeted mutagenesis of the regulatory elements of stem-cell-proliferation-related genes, such as *CLAVATA3* (*SlCLV3*) and *WUSCHEL* (*SlWUS*), has been shown to enlarge the fruit size[122,123]. Using CRISPR–Cas9 for

## Glossary

*Agrobacterium rhizogenes*
A bacterium used for plant delivery. It can induce the formation of hairy roots in the infection site. It contains a root-inducing plasmid that carries a T-DNA segment capable of integrating into the plant genome. Typically, this T-DNA harbours the desired sequences intended for transfer into the plant genome.

*Agrobacterium tumefaciens*
A bacterium used for plant delivery. It contains a modified tumour-inducing plasmid that carries a T-DNA segment capable of integrating into the plant genome. Typically, this T-DNA harbours the desired sequences intended for transfer into the plant genome, as well as marker genes for selecting positive events.

CRISPR interference
(CRISPRi). CRISPR interference utilizes dCas9 either alone or with a transcription repressor to inhibit gene expression by targeting specific DNA sequences without altering the genetic code, offering precise control for studying gene functions and regulatory processes within cells.

Guide RNA
(gRNA). An RNA molecule used to direct Cas9 or similar enzymes to a specific DNA or RNA sequence for precise modification.

Hybrid vigour
A phenomenon in which the offspring of two different inbred lines or varieties exhibit improved traits compared to their parents, such as increased yield, growth or biotic or abiotic resistance. Also known as heterosis.

Non-targeted strand
The DNA strand that is not complementary to the guide RNA sequence. DNA nicking by PE2 and base deamination by base editors occur on the non-targeted DNA strand.

Particle bombardment
A genetic transformation technique, also known as gene gun or biolistic delivery, that involves loading exogenous DNA onto microscopic metal particles that are accelerated and propelled into plant cells or other target cells by compressed gas or physical force.

Protospacer adjacent motif
(PAM). A short DNA sequence immediately adjacent to the target site that is essential for the recognition and binding of Cas protein to the target DNA.

R-loop
A specific structure consisting of one DNA strand, its complementary DNA strand and an RNA strand located between them.

Targeted strand
The DNA strand that is complementary to the guide RNA sequence. DNA nicking by base editors occurs on the targeted DNA strand.

generating high-coverage fragment deletion on the *cis*-regulatory region of *Ideal Plant Architecture 1* (*IPA1*) solved the trade-off between grains per panicle and tiller number, leading to substantially increased yield[124] (Fig. 4Ab). Another strategy involves editing the upstream open reading frames (uORFs) in the 5′ untranslated region (UTR). uORFs have been shown to bind competitively to ribosomal complexes and to reduce the translation of the corresponding primary open reading frames (pORFs)[125]. By deleting, extending or inserting uORFs, the translational level of gene transcripts has been fine-tuned (Fig. 4Ab), generating graded levels of commercially important properties such as ascorbic acid content in lettuce, sugar content in strawberry and the architecture of rice plants[126–128]. Mutating inhibitory regions in 3′ UTRs also provides a feasible approach for enhancing protein levels[129]. Additionally, TGM has the potential to modify other types of *cis*-regulatory elements, such as enhancers or silencers[130], thereby creating novel alleles with desired spatiotemporal expression patterns.

**Generating novel alleles by creating genomic structural variation.** A series of genomic studies have revealed that large-scale genomic structural variation, including duplications, deletions, inversions and inter-chromosomal translocations, can play an important part in modifying crop traits[131,132] (Fig. 4Ac); hence, designing genomic structural variation to generate novel beneficial changes is becoming a feasible method of crop improvement. TGM can assist this goal by generating structural variation that can hardly be produced by traditional approaches. For instance, in rice, creating a 911-kb inversion and a 338-kb duplication enhanced the expression of *protoporphyrinogen IX oxidase* 1 (*OsPPO1*) and *4-hydroxyphenylpyruvate dioxygenase* (*OsHPPD*) respectively, resulting in herbicide resistance[133] (Fig. 4Ac). In another example, CRISPR–Cas9-mediated knockout of the *Mildew resistance locus O* (*MLO*) gene in the A and D genomes of allohexaploid wheat, along with a 304-kb large deletion at the *MLO* locus in the B genome, altered the structure of chromatin and activated the expression of *Tonoplast monosaccharide transporter 3* (*TaTMT3B*), conferring both resistance to powdery mildew and high-yield traits to new germplasm (Fig. 4Ac). Moreover, such genomic modifications can be rapidly transferred into other elite varieties via TGM[134,135]. The role of structural variation in shaping crop traits is being uncovered[131,136], and utilizing precise large-scale DNA manipulation tools to generate beneficial-trait-associated structural variation will be one of the key strategies in generating novel alleles for future crop design.

### Innovation in breeding technologies

The application of TGM has improved existing crop-breeding methods and led to new breeding strategies, thus greatly benefiting agricultural production. This section will summarize the applications of TGM in upgrading breeding technologies.

**De novo domestication.** Highly uniform domesticated crops are under severe threat from environmental changes and biotic stresses, creating an urgent need for novel crops that are more environmentally adaptable. The concept of de novo domestication offers an excellent solution to this problem[137] (Fig. 4Ba). Wild species retain a diverse gene pool owing to the absence of artificial selection and may be inherently resistant to unfavourable conditions. By specifically targeting domestication genes, these species can be swiftly domesticated while preserving their original advantages. This strategy has been used in wild tomato, groundcherry and allopolyploid wild rice[138–141]. Of around 2,500 crop species that are currently exploited by humans, only about 10% have been fully domesticated[142], underlining the enormous potential of this strategy to expand the crop list. Furthermore, some perennial wild species and orphan crops are also promising candidates

for conversion into novel crops[143,144] (Fig. 4Ba). It seems likely that de novo domestication will stimulate the emergence of new crops. However, genome analysis as well as the establishment of efficient TGM and delivery systems are crucial issues that need to be tackled in advance.

**Fertility manipulation.** Leveraging hybrid vigour is currently a vital approach to optimizing crop traits, and the use of male sterile lines helps to avoid self-pollination, thus greatly simplifying the process of hybrid seed production (Fig. 4Bb). However, transferring male sterility traits among elite varieties by traditional crossing is time-consuming and laborious. TGM technology can rapidly generate male sterile germplasms by knocking out male-fertility-related genes such as *male sterile 1* (*MS1*) from wheat, *MS26* and *MS45* from maize, *thermo-sensitive genic male sterility 5* (*TMS5*) from rice, *APETALA3* (*SlAP3*) from tomato, and their homologous male-sterile lines have been generated in rice, wheat, maize and tomato, among others, thereby facilitating hybrid seed production[145–147].

**Hybrid vigour fixation.** The prospect of employing TGM to fix hybrid vigour is equally attractive. Because hybrid vigour is easily lost in subsequent generations, many researchers are attempting to fix it by producing clonal seeds. Based on mitosis instead of meiosis (MiMe) genotypes generated by CRISPR–Cas9, incorporating *Centromeric histone H3* (*CENH3*) or *MATRILINEAL* (*MTL*) mutations, or ectopically expressing *Baby boom 1* (*OsBBM1*), *OsBBM4* or dandelion's *PARTHENOGENESIS* (*PAR*) gene, can fix hybrid vigour by chromosome elimination or parthenogenesis[148–153] (Fig. 4Bc). TGM has paved the way towards rapid fixation of desirable agronomic traits in hybrid seeds. However, further optimization of this strategy is required to increase the yield of cloned seeds while ensuring a high fertility rate, which will rely on more advances in fundamental research to provide new targets.

**Haploid induction.** Haploid breeding can reduce the time required to obtain pure germplasm from about seven to two generations. One of the core issues it raises is how to produce efficient haploid-inducer lines. Utilizing TGM for targeted modification of haploid-induction-associated genes, such as *PLA1/MTL/NLD*, *DUF679 domain membrane protein* (*DMP*) and *CENH3*, enables the rapid transformation of specific germplasm into haploid-induction lines. This has been demonstrated in multiple species, including maize, rice and wheat[154–159] (Fig. 4Bd). Furthermore, by combining haploid-induction technology with TGM, strategies such as HI-Edit (haploid induction editing technology) and IMGE (haploid-inducer-mediated genome editing) have overcome the limitations of crop genotypes in terms of delivery[160,161], enabling trait improvement in recalcitrant cultivars.

**Self-incompatibility manipulation.** Self-incompatibility, which is prominent among flowering plants, maintains a high level of heterozygosity within populations by promoting cross-pollination, thus reducing the presence of deleterious homozygous recessive genes. However, this trait can sometimes be disadvantageous for crop breeding. For example, some varieties of crop species, such as diploid potato, rape

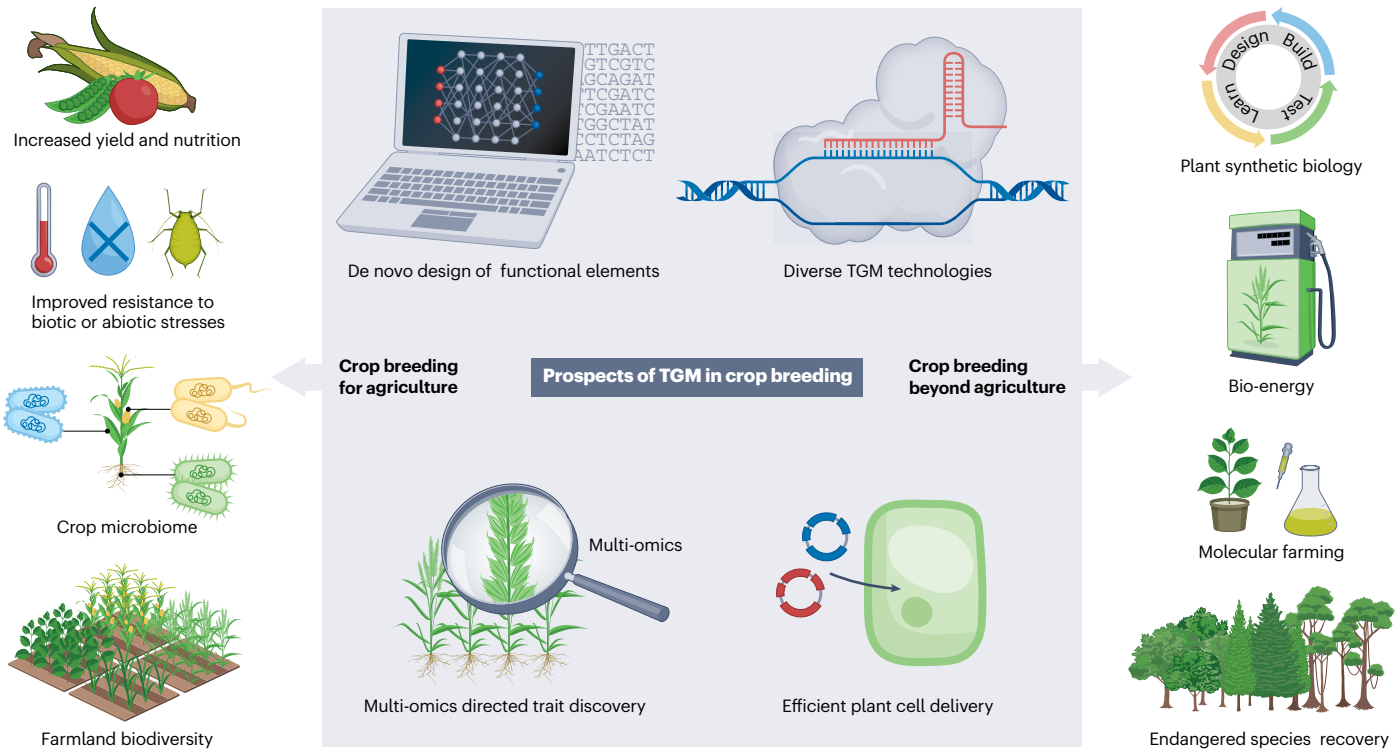



**Fig. 5 | Prospects for TGM technologies in crop breeding.** Cutting-edge technologies, such as artificial intelligence, are making it possible to design functional elements de novo, which will greatly improve the development of the underlying components of targeted genome-modification (TGM) and enable continuous iteration of TGM tools. Multi-omics-directed trait discovery is expected to identify more potential targets for trait improvement. Moreover, additional developments in delivery systems will probably facilitate the widespread adoption of TGM technologies across diverse plant species. This progress will fast-track crop breeding into a wider range of applications.

and cabbage, exhibit a self-incompatibility that makes it difficult for breeders to obtain stable homozygous inbred lines, creating obstacles to basic research and crop breeding. The mechanism of plant self-incompatibility is gradually being elucidated, especially in some *Solanaceae* and *Brassicaceae* species, and studies have shown that TGM-mediated knockout of *S-RNase* in diploid potatoes or *S-receptor kinase* in cabbage can transform them from self-incompatible to self-compatible[162,163], thus overcoming the technical barriers to obtaining pure lines through self-pollination (Fig. 4Be). Further in-depth research into the mechanism should allow self-incompatibility to be manipulated in more species using TGM.

**TGM-mediated precise gene stacking.** Gene stacking is an effective approach with which to endow crops with multiple desirable traits simultaneously or to carry out plant metabolic engineering[164,165]. However, it relies on single or multiple events of random T-DNA integration, and it can be difficult to co-segregate the target genes and to address problems arising from gene silencing owing to position effects at insertion sites. Therefore, obtaining ideal and stable gene-stacking events typically requires screening in large transgenic populations over several generations[164,166] (Fig. 4Bf). Precise insertion of large DNA segments should be able to address these issues. More recently, the emergence of PrimeRoot has enriched the toolbox for precise manipulation of large DNA segments and achieves targeted insertions of up to 11 kb (ref. 66), demonstrating its potential for applications in gene stacking (Fig. 4Bf). TGM-based gene stacking promises to speed up the development of plant synthetic biology and facilitate its applications in agriculture.

## Conclusions and prospects

Modern crop breeding relies on the rapid emergence of genetic variants, and the transition from traditional random mutagenesis to efficient targeted mutagenesis represents a milestone in this evolution. Cutting-edge TGM, along with plant delivery technologies, has a dominant role in this progress by giving rise to a series of advanced and powerful breeding techniques that will be indispensable to meet the future demands of crop breeding.

Despite the promising outlook, there are still limits to the development of TGM technologies. The modular components of existing tools are derived or modified from natural proteins, which are restricted by their intrinsic properties. Moreover, most protein elements originate from prokaryotic environments, which renders them difficult to align seamlessly with eukaryotic cell applications. Additionally, current enzyme-discovery and protein-engineering methods are time-consuming and inefficient, which makes it difficult to design protein elements with the desired editing function. The recent addition of artificial intelligence has greatly facilitated the development of protein de novo design, and algorithms based on generative modelling have successfully designed a variety of functional proteins, such as transmembrane pores or sequence-specific recognition proteins[167–169]. The development of novel TGM tools through de novo design is a potentially viable pathway. By leveraging artificial intelligence, we can perform deep mining or de novo design, retrofitting and modularizing of powerful and new targeted modules and potential enzymes such as nucleases, deaminases, site-specific recombinases and transposases, which promise to yield a series of novel TGM technologies. Given that designing and engineering complex genes and regulatory pathways are pivotal steps in molecular breeding, large-scale genomic manipulation tools are urgently needed to facilitate precise gene stacking, genomic rearrangements, chromosome remodelling and more. The use of artificial intelligence will promote the development of new tools for editing kilobase-scale and even megabase-scale DNA segments, overcoming the problem of inefficiency and the limited natural sources of components, thereby making it possible to achieve genome manipulation at larger scales.

Progress in various TGM technologies will strengthen our ability to shape crop traits, expanding the scale of manipulation from gene-level to genetic pathways and even more complex regulatory networks, thus achieving more efficient and comprehensive crop improvement. Moreover, the positive impact that TGM technologies will probably have on the entire agricultural system needs attention. TGM technologies have the capability to achieve rapid improvement of farmland biodiversity through de novo domestication and re-domestication, aiming to enhance crop diversity in different regions. This will enhance the stability of agricultural ecosystems, increase crop yield and quality, and potentially mitigate the negative effects of climate change[170]. Advanced TGM technologies could also be used to modify microbial communities[171–173], retrofitting the interactions between plants and associated microbes; this should not only enhance plant productivity, disease resistance and stress tolerance but might also contribute to soil remediation, providing substantial benefits for sustainable agriculture. Improved TGM technologies provide possibilities also for the development of plant synthetic biology[174], including for the development of drug bioreactors, new forms of bioenergy and the application of plant bio-factories, and even for assembling and remodelling artificial chromosomes of higher organisms (Fig. 5).

TGM techniques are the most precise, efficient and rapid means of generating mutations. Although the mutations are essentially no different from those that exist as natural variation or those produced by physical and chemical mutagenesis, the speed of utilization of TGM crops does not match the pace of their development. In recent years, attitudes towards the regulation of TGM crops have become more accepting, with many countries and regions consciously differentiating the regulation of TGM crops from those of genetically modified organisms, gradually easing regulatory barriers for TGM while ensuring biosafety[175,176]. For example, Japan and the USA have recently permitted the commercialization of tomatoes containing high levels of gamma-aminobutyric acid (GABA) and non-bitter mustard, respectively. Such policy changes greatly benefit the use of TGM crops. However, public acceptance of such crops is another factor determining their adoption. Insights gained from previous endeavours to promote the use of genetically modified organisms emphasize the importance of public scientific popularization of novel technologies prior to their implementation. It is important that scientists and professionals are actively engaged with the public through science outreach and educational initiatives to build a conducive and tolerant social milieu that facilitates the application of TGM crops. Against the backdrop of a world where population and environmental issues are increasingly prominent, the application of TGMs in agriculture will undoubtedly provide a more reliable guarantee of food security and help to establish a more sustainable agricultural production system.

Published online: 24 April 2024

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## Acknowledgements
This work was supported by the National Natural Science Foundation of China (32388201), the National Key Research and Development Program (2022YFF1002802), the Ministry of Agriculture and Rural Affairs of China, the Strategic Priority Research Program of the Chinese Academy of Sciences (Precision Seed Design and Breeding, XDA24020102), and the New Cornerstone Science Foundation. The authors thank K. T. Zhao, C. Xue, R. Liang, G. Liu, J. Hu, H. Li, Y. Li, F. Qiu, S. Li, Y. Lei and X. Jiang for their insightful comments on the manuscript.


## Author contributions
B.L., C.S. and C.G. researched the literature. All authors substantially contributed to discussions of the content and wrote the article. J.L. and C.G. reviewed and/or edited the manuscript before submission.

## Competing interests
The authors declare no competing interests.

## Additional information
**Peer review information** *Nature Reviews Genetics* thanks José R. Botella, who co-reviewed with Zheng (Tommy) Gong; Yuriko Osakabe; and Yiping Qi for their contribution to the peer review of this work.

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