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Precise plant genome editing using base editors and prime editors

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The development of CRISPR–Cas systems has sparked a genome editing revolution in plant genetics and breeding. These sequence-specific RNA-guided nucleases can induce DNA double-stranded breaks, resulting in mutations by imprecise non-homologous end joining (NHEJ) repair or precise DNA sequence replacement by homology-directed repair (HDR). However, HDR is highly inefficient in many plant species, which has greatly limited precise genome editing in plants. To fill the vital gap in precision editing, base editing and prime editing technologies have recently been developed and demonstrated in numerous plant species. These technologies, which are mainly based on Cas9 nickases, can introduce precise changes into the target genome at a single-base resolution. This Review provides a timely overview of the current status of base editors and prime editors in plants, covering both technological developments and biological applications.

A major challenge of our times is learning how to feed a global population that is estimated to reach >9.5 billion people over the next 30 years. To overcome this challenge, the power of inducing precise genetic variation must be harnessed for crop improvement. As RNA-guided sequence-specific nucleases, CRISPR–Cas systems can install genetic variation in target loci of the genomes[1–3]. We have witnessed an explosion of interest in applying this new technology for basic biology, human therapeutics and agriculture. In the CRISPR–Cas system, Cas nuclease induces a double-stranded break (DSB) at a target site specified by a short single-guide RNA (sgRNA). The target DNA locus (usually 20 nucleotides long) is commonly known as the protospacer sequence. The frequent outcome of a DSB in the genome is the generation of random insertions or deletions (indels) by NHEJ, the predominant DSB repair pathway in plants. Although NHEJ-mediated mutagenesis is highly efficient in plants, it is typically used to generate gene knockouts and alter promoter or enhancer strength. For developing novel agronomic traits, the precise modification of genomic information is necessary. DSBs in the plant genome can be precisely repaired through the homology-directed repair (HDR) pathway when DNA donor templates are supplied[4]; however, HDR is rarely used in basic research and crop improvement[5] due to its extremely low efficiency in higher plants. Nevertheless, HDR is a valuable and flexible tool for plant breeding applications that require precise knock-in and complex DNA modifications. Recently, substantial advancements have been made in increasing the efficiency of HDR-mediated editing by different approaches[6], such as tandem repeat-HDR (TR-HDR)[7] and transcript-templated HDR (TT-HDR)[8].

The recent invention of two powerful technologies, base editing and prime editing, has partly overcome such critical barriers of precise genome editing and greatly enhanced crop breeding opportunities. Both base editors (BEs) and prime editors (PEs) install desired changes without the donor DNA and a DSB introduction in the genome. BEs can exhibit about 10-to-100-fold higher efficiency than HDR in obtaining desired mutations[9,10]. As DSB occurrence is generally low during the editing process, BEs and PEs greatly curb the formation of undesired indels, generating edited plants with nucleobase precision. However, BEs and PEs, which are dependent on DNA nick formation, may be locally mutagenic in some cases due to the existence of nick repair pathways.

This Review focuses on base editing and prime editing platforms that enable single-nucleotide conversions, small insertions, deletions, sequence replacements and a combination thereof in plant genomes. We first present a comprehensive overview of the available and forthcoming base editing platforms in both nuclear and organelle genomes. We then navigate to recently developed prime editing tools, review their performance in plants and discuss the crucial parameters of enhancing editing efficiency. We also summarize the available web tools for designing, predicting the outcomes and analysing data of base editing and prime editing experiments. Applications of BEs and PEs in basic plant biology and crop improvement are highlighted.

Base editing

Base editing is a new technology for the precise modification of genomes (DNA) or transcriptomes (RNA) of living cells at the single-base resolution. BEs are composed of a catalytically impaired Cas nuclease that is fused to a nucleotide deaminase and, sometimes, to DNA repair proteins. BEs can introduce single-nucleotide variants at desired loci in DNA (nuclear or organellar) or RNA of both dividing and non-dividing cells. Broadly, there are two types of BEs—DNA BEs that directly induce targeted point mutations in DNA, and RNA BEs that convert one ribonucleotide to another in RNA. Currently available DNA BEs can be further categorized into cytosine BEs (CBEs), adenine BEs (ABEs), C-to-G BEs (CGBEs), dual-base editors and organellar BEs. Each of these categories is discussed below.

CBEs. CBEs were the first DNA BEs developed to enable C•G to T•A transitions[11,12]. The two seminal studies have introduced CBEs with different architectures containing a Cas9 nickase (nCas9, for example, with a D10A mutation) fused to cytidine deaminase and uracil glycosylase inhibitor (UGI)[11,12] (Fig. 1a,b). Similar to the canonical CRISPR–Cas systems, CBEs are guided to the target

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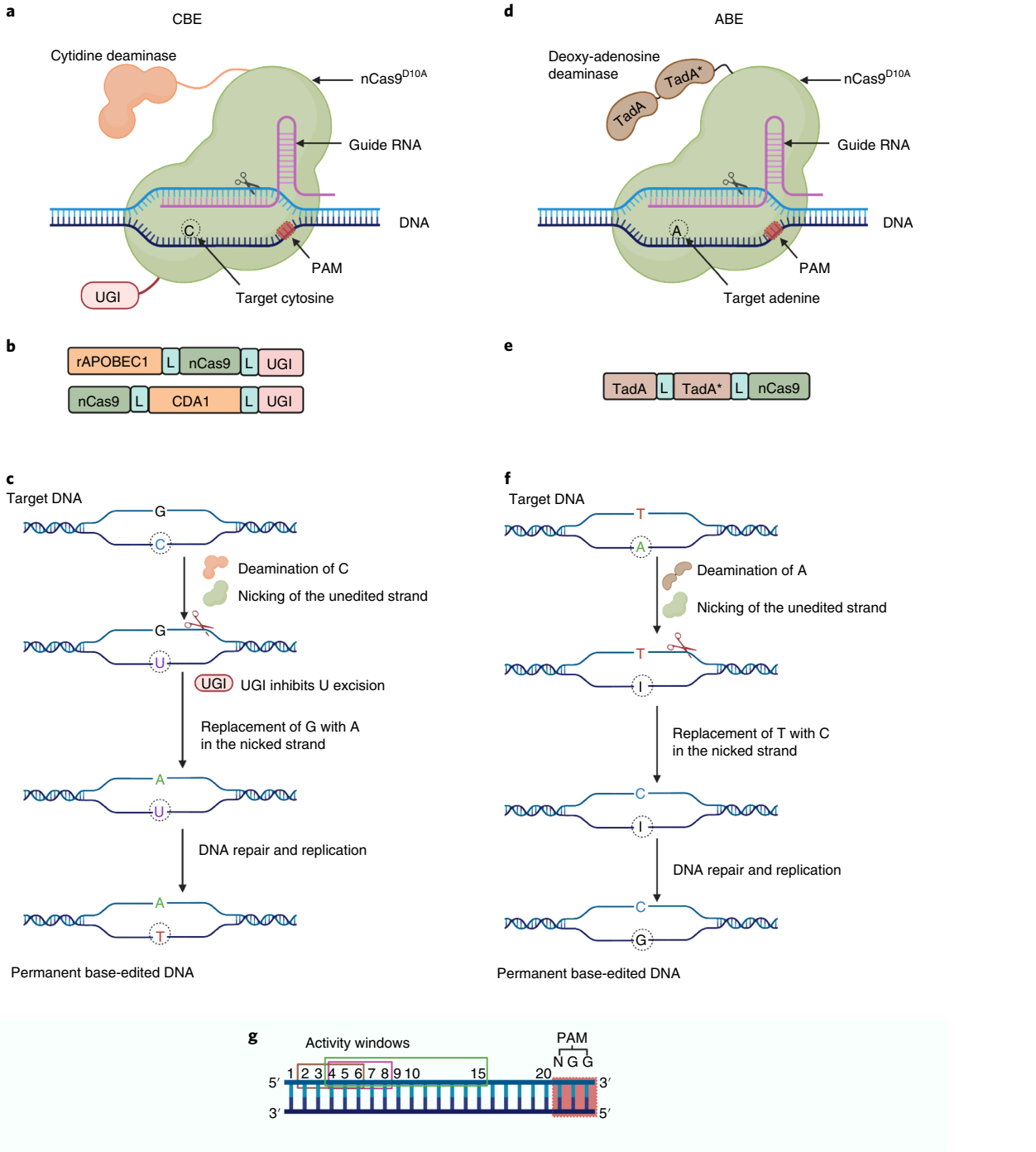

Fig. 1 | Cytosine and adenine base editing in nuclear DNA. a, A CBE in the DNA context. The target cytosine is indicated (dotted circle). **b**, CBE architectures (N to C terminal) containing cytidine deaminase (peach), nCas9 (green) and UGI (pink). CDA1, sea lamprey cytidine deaminase 1. **c**, Schematic of the mechanism of C-to-T editing by CBEs. The cytosine deaminase converts a target C to U. The generated U•G mismatch can be resolved either by cellular mismatch repair or BER machinery. The nCas9 would make a nick in the guanidine (G)-containing unedited DNA strand. The nick actuates the removal of G by the cellular mismatch repair pathway and the use of uracil as a template for repair leads to the desired T•A base pair formation. Cellular uracil DNA *N*-glycosylase (UDG or UNG) removes U from the DNA, ultimately leading to a reversion to the original C•G base pair. UGI protein increases the rate of T•A base-pair generation by safeguarding the U intermediate. **d**, ABE version 7.10 (ABE-7.10) in the DNA context. The target adenosine is indicated (dotted circle). **e**, The ABE-7.10 architecture (N to C terminal) containing a heterodimeric deaminase (brown) and nCas9 (green). ABE-7.10 contains one wild-type TadA monomer and one evolved TadA (TadA*) monomer. **f**, The mechanism of A-to-G editing by ABEs. Once the ABE is recruited to the desired genomic locus by gRNA, the target A base is deaminated to inosine (I) and, as a result, an A•T pair becomes an I•T base pair. Although inosine is known to pair with C, A or U during translational mRNA–tRNA pairing, it behaves as G and pairs with C during the replication. The I•T base pair is resolved by cellular mismatch repair or DNA replication. The nCas9 of the ABE nicks the T containing strand to induce the mismatch repair machinery to remove the T and incorporate C opposite to I to form an I•C base pair that eventually becomes a G•C pair as the outcome. **g**, Activity windows of CBEs and ABEs (coloured boxes) over a 20 bp protospacer sequence (the NGG PAM is counted as 21–23). The activity window varies depending on the Cas protein and deaminase variants. L, linker.

genomic region by a sgRNA. Once the sgRNA–CBE complex binds to the target DNA, it generates a single-stranded DNA R-loop[13]. This non-target single-stranded DNA becomes accessible to CBE cytidine deaminase, which catalyses the hydrolytic deamination of an exposed cytosine (C). Deamination and subsequent cellular mismatch repair result in C-to-T base editing outcomes. Uracil (U) base excision repair (BER) hinders this outcome and either regenerates the original base pair or gives rise to an indel[14]. UGI subverts BER and increases the likelihood of C-to-T editing[11,12] (Fig. 1c). Although antibiotics are generally used to select transformants, selecting base-edited cells in a population could be difficult. To overcome the limitation, a surrogate reporter system was established in plants based on the correction of a defective hygromycin-resistance gene[15].

Plant biologists have rapidly adopted CBEs into many plant species, such as *Arabidopsis*[16–19], rice[20–25], wheat[26,27], maize[26], tomato[22,28–30], potato[23,28,29,31], watermelon[32], cotton[33], soybean[34], apple[35], pear[35], strawberry[36], moss[37], poplar[38] and rapeseed[39,40].

Activity windows. Although the single-stranded DNA in the R loop is exposed during base editing, the whole region may not be equally accessible to the cytidine deaminase. As a result, many cytosine bases are not efficiently edited if they are not present within a particular BE activity window, which is the range of bases within the 20-nucleotide protospacer sequence that is optimally edited by a BE[10] (Fig. 1g). The activity window varies among different BEs and is ~4–10 nucleotides long for most of the Cas9-based BEs. The Cas12a-based CBE exhibited an activity window ranging from 8–13 in the protospacer when the base next to the protospacer-adjacent motif (PAM) 5′-TTTV-3′ (where V indicates A, C or G) is counted as 1 (in contrast to Cas9, Cas12 has a 5′ PAM)[10,41]. Several CBE platforms have been developed to extend or shorten the activity windows, which could be beneficial for specific editing requirements[10,14,42,43] (Table 1). In many cases, a single-nucleotide conversion is desired by base editing. When multiple C bases are present within the activity window, CBEs can convert non-target C nucleotides, a phenomenon known as the bystander editing effect. In the case of protein-coding gene editing, bystander editing can be problematic if it generates nonsynonymous or nonsense mutations. CBEs with minimized bystander editing have been developed by deaminase engineering[44–46]. Although CBEs with an extended activity window may increase bystander editing, they may be suitable in large-scale mutation applications such as directed evolution and editing of promoters or other *cis*-regulatory elements (CREs).

Cytidine deaminases. The natural diversity of cytidine deaminases could be harnessed to develop base editing tools for specific needs. Although the seminal studies used a rat cytidine deaminase (rAPOBEC1)[11] and a *Petromyzon marinus* cytidine deaminase 1 (*Pm*CDA1)[12], many other studies reported the successful use of diverse naturally occurring cytidine deaminases in constructing CBEs. For example, human-APOBEC3A-based CBE has been demonstrated in wheat[23], rice[23,47,48], strawberry[36], potato[23] and rapeseed[40], whereas APOBEC3B (hA3B) was used for base editing in rice[49]. Although human activation-induced deaminase (hAID) failed to exhibit detectable deaminase activity in vitro, an engineered version, hAID*Δ, lacking a nuclear export signal sequence, displayed substantial base editing efficiency in rice[50]. Other deaminases used in CBE include human APOBEC3G[46], *Rhinopithecus roxellana* A3F, *Alligator mississippiensis* APOBEC1, *Sus scrofa* APOBEC3B and *Pongo pygmaeus* APOBEC1[51].

Most CBEs are based on the BE3 configuration (Fig. 1a). However, not all CBEs work equally well in plants (Table 1). For example, *Pm*CDA1-CBE was found to outperform rAPOBEC1-CBE according to multiple studies[52–55]. *Pm*CDA1 was also found to be superior to hAID*Δ for base editing in rice[56]. rAPOBEC1 shows poor base editing efficiency in the GC context[10,11]. APOBEC3A (A3A), hAID*Δ and *Pm*CDA1 showed superiority in targeting a C immediately downstream of a G nucleotide[10,23,50]. To overcome sequence context limitations and increase editing efficiency at difficult loci, three deaminases—evoAPOBEC1, evoFERNY and evoCDA1—have been evolved[57]. Plant high-efficiency CBEs (PhieCBEs) based on the above three evolved deaminases showed high efficiency in rice, with evoFERNY exhibiting the best performance[48]. Ancestral sequence reconstruction of APOBEC homologues was performed to generate Anc689 with enhanced expression[58]. Anc689-CBE has been reported to enhance C-to-T editing in rice NRT1.1B and SLR1 loci[59]. A highly efficient A3A^{Y130F}-CBE was recently reported to reach up to 100% editing efficiency in poplar[38]. Base editing efficiency by high-activity CBEs is pretty much on par with the NHEJ mutagenesis efficiency mediated by a Cas nuclease in plants.

Orthologous and engineered Cas proteins for expanding editing scope. One constraint that limits base editing applications is the targeting scope of BEs. Cas9 from *Streptococcus pyogenes* (*Sp*Cas9) has been the most widely used nuclease in CBEs in plants[10,20–22,26]. *Sp*Cas9 requires an NGG PAM (where N indicates A, T, G or C), which substantially limits the number of targetable sites. A plethora of orthologous Cas proteins and engineered Cas9 variants with different PAM requirements have been adopted for CBEs, broadening the targeting scope in plants[43,60]. For example, Cas9 from *Staphylococcus aureus* (*Sa*Cas9) and from *Streptococcus canis* (*Sc*Cas9) were successfully incorporated into the CBE architecture to expand base editing to NNGRRT and NNG PAMs (where R indicates G or A), respectively, in rice[61,62]. VQR-Cas9 and *Sa*KKH-Cas9 were used in generating rAPOBEC1-based CBEs that can recognize NGA and NNNRRT PAMs, respectively[24]. VQR-CBE with *Pm*CDA1 was used in rice[63]. Similarly, other studies reported the use of *Sa*Cas9- and *Sa*KKH-Cas9-CBEs with rAPOBEC1 and *Pm*CDA1 in rice and potato[61,64]. By grafting the PAM-interacting domain of *Streptococcus macacae* Cas9 (*Smac*Cas9) to the *Sp*Cas9, a hybrid iSpymacCas9 was engineered to recognize 5′-NAAN-3′ PAMs[65], which are more frequent than NGG PAMs in rice, wheat and maize[56]. Two iSpymacCas9 CBE systems with *Pm*CDA1 and hAID*Δ deaminases were found to be effective in rice[56,66]. Three new *Sp*Cas9 variants have recently been evolved to recognize NRRH, NRTH and NRCH PAMs[67] (where H indicates A, C or T), and CBEs based on these variants were demonstrated in rice[47,68].

The *Sp*Cas9 variants xCas9 and *Sp*Cas9-NG can recognize non-canonical NG PAMs[69,70]. CBEs based on *Sp*Cas9-NG were demonstrated in rice[47,48,54,59,71–74], potato[29] and tomato[29] (Table 1). Similarly, CBEs with the xCas9 backbone were used for base editing at NGN PAM sites in rice, albeit with a low efficiency[71,73–75]. *Sp*Cas9-NG was also reported to recognize GAT, GAA, CAA, NAC, NTG, NTT and NCG PAMs in plants[72,73]. In general, CBEs based on *Sp*Cas9-NG are more efficient than CBEs based on xCas9 for editing NG and other non-canonical PAMs in plants. To further lessen the PAM constraint, two versions of *Sp*Cas9—namely *Sp*G (which recognizes NGN PAMs) and *Sp*RY (which recognizes NRN and, with lower efficiency, NYN PAMs (where Y indicates C or T))—were engineered[76]. *Sp*RY is therefore a near PAM-less variant[76]. Within a short time, *Sp*RY CBEs were demonstrated in rice with variable efficiencies at different PAMs[25,47,68,77]. Although Cas9 variants with relaxed PAMs greatly expand genome targetability, it is important to note that they are prone to generating additional off-target mutations. Moreover, the PAM-less variant *Sp*RY makes the system vulnerable to self-targeting when delivered into plants in the DNA format[25].

Cas12-based CBEs could render editing at T-rich PAMs in the future. Cas12a (formerly known as Cpf1) nucleases generally recognize canonical 5′-TTTV-3′ PAMs in genome editing[78,79]. Developing Cas12a nickase seems to be complicated and, therefore, only catalytically inactive Cas12a (dCas12a)-based CBEs were generated for

Table 1 | Features of plant BEs and PEs

Type of genome editing	Tools	PAM	Activity windows	Editing efficiencies in the first generation of transgenic plants	Distinct features of the tools
Cytosine base editing	BE3-*Sp*Cas9	NGG	4–8	0.1–43.5% in rice[20,26] 10% in maize[26] 1.25% in wheat[26] 26.67–57.78% in cotton[33] 6–18.2% in soybean[34] 23% in watermelon[32] 1.8% in rapeseed[39]	Canonical
	BE3-*Sa*Cas9	NNGRRT	5–18	6.3–56.3% in rice[61]	Alternative PAM Extended editing window
	BE3-*Sa*KKH	NNNRRT	1–16	3.6–19.4% in rice[61]	
	BE3-VQR	NGA	8–15	61–71% in rice[24]	
	BE3-xCas9	NG	4–8	0–35.7% in rice[71]	Expanded targetability
	BE3-NG	NG	4–8	0–72% in rice[71]	
	BE4-NG	NG	4–8	0–50% in rice[48]	Expanded targetability High fidelity
	evoBE4max-NG	NG	2–10	0–66.7% in rice[48]	GC context editing Expanded targetability
	eBE3-*Sp*Cas9	NGG	4–8	53.5–88.9% in rice[212]	High fidelity
	eBE3-*Sp*RY	NR NY	3–7	0–52.1% in rice[47]	Expanded targetability High fidelity
	eBE3-*Sp*G	NG	2–8	37.5–87.5% in rice[47]	
	eBE3-NRRH	NRRH	3–9	33.3–75% in rice[47]	
	eBE3-NRCH	NRCH	3–9	0–100% in rice[47]	
	eBE3-NRTH	NRTH	3–9	0–75% in rice[47]	
	evoFERNY-NG	NG	2–10	40.6–86.3% in rice[48]	GC context editing Expanded targetability Small size
	Target-AID-*Sp*Cas9	NGG, NAG	1–9	38.9–68.8% in rice[53] 0–53.8% in rice[63] 7.7–91.6% in tomato[22,30] 0–100% in poplar[38] 1.6–55% in *P. patens*[37]	Canonical Shifted editing window
	Target-AID-NG	NG	1–14	30.4–45% in rice[73] 9–57% in potato[29] 64% in tomato[29]	Expanded targetability
	Target-AID-*Sp*RY	NR NY	4–8	10–33.3% in rice[25]	
	Target-AID-VQR	NGAG	1–5	0–90% in rice[63]	Alternative PAM
	Target-AID-*Sa*Cas9	NNGRRT	1–11	0–2.1% in rice[61]	
	Target-AID-*Sa*KKH	NNNRRT	4	0–6.3% in rice[61]	
	Target-AID-iSpyMacCas9	NAAA	2–8	22–31% in rice[56]	
	Evo-Target-AID-NG	NG	2–12	0–44% in rice[48]	GC context editing Expanded window Expanded targetability
	eTarget-AID	NGG	1–7	75–85.7% in rice[212]	High fidelity
	hAID*Δ-*Sp*Cas9 (rBE5)	NGG	3–7	30–57% in rice[50]	Without UGI GC context editing
	hAID*Δ-NG	NG	4–14	0–37.5% in rice[71]	GC context editing Expanded targetability
	hAID*Δ-*Sc*Cas9	NAG	3–7	37% in rice[62]	GC context editing Alternative PAM
	hAID*Δ-*Sp*RY	NR NY	3–14	0–34.15% in rice[77]	GC context editing Expanded targetability
	A3A-*Sp*Cas9	NGG	1–17	16.7–22.5% in wheat[23] 44.1–82.9% in rice[23] 6.5% in potato[23] 100% in strawberry[36] 3.17–31.25% in rapeseed[40]	Wide window GC context editing

Continued

Table 1 | Features of plant BEs and PEs (continued)

Type of genome editing	Tools	PAM	Activity windows	Editing efficiencies in the first generation of transgenic plants	Distinct features of the tools
	A3Amax-NG	NG	3–13	2.9–72.4% in rice[48]	Expanded targetability High fidelity
	eA3A-*Sp*RY	NR NY	3–12	2–15% in rice[47]	
	eA3A-*Sp*G	NG	1–18	29.2–77.1% in rice[47]	
	eA3A-NRRH	NRRH	1–12	25–43.8% in rice[47]	
	eA3A-NRCH	NRCH	2–15	4.2–35.4% in rice[47]	
	A3Bctd-BE3	NGG	3–10	High efficiency in rice[49]	Reduced gRNA-independent off-target effects
	Anc689BE4max-*Sp*Cas9	NGG	4–15	71.4–82.8% in rice[59]	High efficiency
	Anc689BE4max-NG	NG	4–15	17.2–57.1% in rice[59]	High efficiency Expanded targetability
	A3A-Y130F-CBE	NGG	5–18	19–95.5% in poplar[38] 43.8–72.7% in tomato[93]	High efficiency High fidelity
Adenine base editing	ABE7.10-*Sp*Cas9	NGG	4–12	2.81–61.3% in rice[101,102] 0.4–1.1% in wheat[103] 0.7–0.8% in *P. patens*[37]	Canonical
	ABE7.10-*Sa*Cas9	NNGRRT	6–14	17–63.2% in rice[61,102]	Alternative PAM
	ABE7.10-*Sa*KKH	NNNRRT	8–10	0–16.1% in rice[61]	
	ABE7.10-iSpyMacCas9	TAAA	9	12.5% in rice[56]	
	ABE7.10-*Sc*Cas9	NAG	5	47.5% in rice[62]	
	ABE7.10-VRER	NGCG	5	0–2.6% in rice[24]	
	ABE7.10-VQR	NGA	3–10	30–74.3% in rice[24]	
	ABE7.9-*Sp*Cas9	NGG	4–12	1.34–39% in rice[101]	
	ABE7.10-xCas9	NG	6	0–4.8% in rice[71]	Expanded targetability
	ABE7.10-NG	NG	4–8	2–11.9% in rice[71]	
	ABE7.10-S	NGG	1–12	11.1–96.3% in rice[71]	Mini ABE (smaller size)
	ABE7.10-NG-S	NG	5–11	2.9–7.7% in rice[71]	MiniABE Expanded targetability
	ABEmax	NGG	4–8	40.7–48.3% in rice[59] 0–95% in poplar[38]	Increased efficiency
	ABEmax-NG	NG	4––8	8.3–41.2% in rice[59]	Increased efficiency Expanded targetability
	ABE8e-*Sp*Cas9	NGG	4–8	[a]30–91.67% in rice[109] 30.95–60.87% in *N. benthamiana*[108]	High efficiency
	ABE8e-*Sc*Cas9	NAG	4–10	[a]27–97.92% in rice[109]	High efficiency Alternative PAM
	ABE8e-*Sp*RY	NR NY	3–10	0–93.75% in rice[25,77]	High efficiency Expanded targetability
	ABE8e-*Sp*G	NG	4–12	79.2–100% in rice[47]	
	ABE8e-NG	NG	4–8	[a]31.25–100% in rice[109]	
	ABE8e-NRTH	NRTH	3–12	100% in rice[47]	
	ABE8e-NRRH	NRRH	2–11	52–100% in rice[47]	
	ABE8e-NRCH	NRCH	3–11	87.5–100% in rice[47]	
	ABE8.17-*Sp*Cas9	NGG	6–8	[a]2–31.25% in rice[109]	Increased efficiency
	ABE8.17-*Sc*Cas9	NAG	6–8	[a]0–72.92% in rice[109]	Increased efficiency Alternative PAM
	ABE8.17-NG	NG	6–8	[a]0–89.36% in rice[109]	Increased efficiency Expanded targetability

Continued

Table 1 | Features of plant BEs and PEs (continued)

Type of genome editing	Tools	PAM	Activity windows	Editing efficiencies in the first generation of transgenic plants	Distinct features of the tools
	ABE8.20-*Sp*Cas9	NGG	4–6	[a]0–58.33% in rice[109]	High efficiency
	ABE8.20-*Sc*Cas9	NAG	6–8	[a]0–79.17% in rice[109]	High efficiency Alternative PAM
	ABE8.20-NG	NG	4–6	[a]0–97.87% in rice[109]	High efficiency Expanded targetability
	ABE9-*Sp*Cas9	NGG	1–12	56–93.75% in rice[109]	High efficiency
	ABE9-*Sc*Cas9	NAG	4–12	[a]41.67–95.83% in rice[109]	High efficiency Alternative PAM
	ABE9-NG	NG	4–10	[a]77–100% in rice[109]	High efficiency Expanded targetability
Dual-base editing	STEME-*Sp*Cas9 STEME-NG	NGG NG	1–17 (for C) 4–8 (for A)	13.18% in rice[125]	Simultaneous C-to-T and A-to-G editing
Organellar editing	mtDdCBE	NA	C (3–13) in the TC motif	[a]1.9–24.9% in rapeseed[137]	TALEN-based C-to-T editing in organellar DNA
	cpDdCBE	NA		[a]0.5–38.4% in lettuce[137] [a]0.1–0.84% in rapesssed[137] 64% in rice[140] 40–100% in *Arabidopsis*[139]	
Prime editing	PE2-*Sp*Cas9	NGG	+1 to +33 Insertion/deletion between +1 to +6 is more efficient.	1.0–31.3% in rice[150] [a]0.3–2.0% in rice[151] 0.0–2.6 in rice[155] [b]50% in potato[157]	Canonical PE2 strategy
	PE3-*Sp*Cas9	NGG		2.6–21.8% in rice[149] 10.4–18.8% in rice[150] [a]2.2% in rice[151] 2.2–9.4% in rice[152] 1.1–1.4% in rice[153] 4.8–53.2% in maize[156] 3.4–6.7% in tomato[158]	Canonical PE3 strategy
	PE3b-*Sp*Cas9	NGG		6.3% in rice[150] 4.8–6.5% in maize[156]	Canonical PE3b strategy
	Surrogate pPE2-*Sp*Cas9	NGG		7.3–16.7% in rice[150]	Enhanced screening efficiency of edited plants HPT-ATG selection recovery
	PE3-*Sp*Cas9-HPT	NGG		1.7–26.0% in rice[153]	Enhanced screening efficiency of edited plants
	PE3-*Sp*Cas9-paired pegRNAs	NGG		0.12–24.5% in rice[159]	Enhanced efficiency
	PE3-*Sp*G-paired pegRNAs	NG		0.62–2.88% in rice[159]	Enhanced efficiency

For consistency in naming the base editing tools, sometimes we diverged from the nomenclature of original articles. For BEs, deaminases are shown first followed by the Cas proteins used. Activity windows shown here are based on the reported editing events at genomic loci tested. Windows may sometimes vary from target to target. ABEs were named according to the mutated version of TadA used. For base editing activity windows, the PAM (NGG) is counted as 21, 22 and 23 with the first nucleotide in the protospacer as 1. For prime editing windows, the NGG PAM is counted as +4, +5 and +6, with the first nucleotide adjacent to the cleavage site as +1. A3A, human APOBEC3A; A3Bctd, truncated human APOBEC3B; ABE9, ABE8e with the V82S and Q154R mutations in the Tad domain; Anc689BE4max, ancestral sequence reconstructed and codon-optimized deaminase; BE3, BE with rat APOBEC1; BE4, BE3 plus one additional UGI; canonical, BEs adopted from the first reports in mammalian system; evo, artificially evolved; hA3A^{Y130F}, human APOBEC3A with the Y130F mutation; Target-AID, BE with sea lamprey *Pm*CDA; hAID*Δ, human activation-induced deaminase devoid of nuclear export signal. [a]Efficiencies were measured as transgenic calli. [b]Only two plants were assayed.

editing in mammalian cells[41,80]. Although Cas12a and Cas12b have been reported for plant genome editing[79,81], Cas12-derived BEs have not yet been reported in plants.

Efforts to decrease gRNA-dependent and -independent off-target editing and other unintended editing. Both the protospacer and the corresponding PAM sequence collectively determine the targeting specificity of a CRISPR–Cas system[82]. However, off-target editing could still occur at potential genomic sequences with close homology to the protospacer in addition to the PAM. These off-target edits are known as guide RNA (gRNA)-dependent off-target effects, which are common to all CRISPR–Cas mediated editing ventures. Base editing is no exception. Potential genome-wide gRNA-dependent off-target sites can be predicted using many gRNA designing web tools. However, BEs seem to have far fewer gRNA-dependent off-target effects compared with wild-type Cas9 with the same gRNAs[10,14,42]. This might be partially due to the absence of an editable cytosine in the activity window of the potential genomic off-target sites.

One possibility to reduce the gRNA-dependent off-target effects is to replace wild-type nCas9 with high-fidelity nCas9 variants[60,83,84]. Recently, three high-fidelity Cas9 variants—*Sp*Cas9-HF2 (ref. [84]), e*Sp*Cas9(1.1) (ref. [83]) and HypaCas9 (ref. [85])—have been used in CBEs for base editing in rice[52,74]. Another strategy for reducing off-target editing is to deliver BEs as ribonucleoprotein complexes (RNPs) or RNA reagents instead of DNA reagents[10]. As DNA-based reagents integrate into the genome and show long-term expression, they are likely to increase off-target editing compared with RNP

and RNA reagents, which confer only transient editing activity. The delivery of RNPs and RNA reagents led to efficient on-target base editing with reduced off-target modifications in mammalian cells[9,86,87]. Although RNPs and RNA reagents have been successfully used for editing with the Cas9 nuclease systems in plants in earlier studies[88,89], only a single study reported DNA-free base editing[23]. It may help to avoid regulatory hurdles and address public concerns about genetically modified organisms[10]. Even if protoplast transfection and biolistic delivery are viable options for delivering RNP- and RNA-based CBEs, regenerating and screening edited plants remain a major bottleneck in these experiments.

Editing by BEs at genomic sites that do not have any sequence similarities with the gRNA is known as gRNA-independent off-target editing. Interestingly, whole-genome sequencing studies showed that CBEs could generate genome-wide gRNA-independent off-target mutations in mouse[90] and rice[91]. These off-target effects are attributed to the function of the deaminase rAPOBEC1. Cytosine deaminases used in CBEs bind to single-stranded DNA[11,12]. It is indicative that these undesired off-target effects result from the intrinsic affinity of cytidine deaminase to single-stranded DNAs, which exist in genomic regions undergoing replication or transcription. Engineering the deaminase domain or using an alternative deaminase to rAPOBEC1 therefore represents an effective strategy to reduce the off-target effects. This has recently been demonstrated by examining alternate deaminase domains (CDA, AID, A3A, A3B, A3G) and engineered deaminase variants in mammalian cells[92]. The study also confirmed that the gRNA-independent off-target editing activity of CBEs greatly varies with different deaminase domains[92]. Similarly, eight new CBEs have been developed using alternate deaminases (*Rr*A3F, *Am*APOBEC1, *Ss*APEBEC3B and *Pp*APOBEC1) and their engineered variants in mammalian cells[51]. Those next-generation CBEs exhibit up to a 45-fold overall reduction in gRNA-independent off-target editing compared with rAPOBEC1 (ref. [51]). Interestingly, an assessment of ten CBE platforms with different deaminases revealed that *Pm*CDA1-CBE does not cause detectable genome-wide gRNA-independent off-target editing in rice[66]. In a recent study in tomato, A3A^{Y130F}-BE3 was found to generate slightly higher gRNA-independent single-nucleotide variations and indels compared with the control, although the difference was not statistically significant[93]. The same study also reported that A3A^{Y130F}-BE3 does not create transcriptome-wide off-target effects[93]. Note that such off-target effects, if generated, are dwarfed by the plant tissue-culture-induced somaclonal variation.

Notably, two studies used a method named the orthogonal R-loop assay to rapidly evaluate the propensity of a BE to trigger gRNA-independent off-target deamination[51,92]. Using this method, Jin et al. screened 25 truncated variants of human APOBEC3B (hA3Bctd) and developed two new CBE variants—A3Bctd-VHM-BE3 and A3Bctd-KKR-BE3—that exhibit markedly reduced gRNA-independent off-target editing in rice cells[49]. As whole-genome sequencing and subsequent analysis are expensive and time consuming, the orthogonal R-loop assay could be advantageous for evaluating the gRNA-independent off-target effects of new CBEs.

Few earlier studies reported CBE-induced unintended editing in the protospacer sequence and indel formations in plants, ranging from 0–10% (refs. [10,20]). Recent studies showed up to 38% and 16% indel formation in tomato and rice, respectively, by A3A^{Y130F}-BE3 at different protospacers[66,93]. Uracil in the genome, induced by the CBE, is treated as an error, and removed by uracil DNA glycosylase (UDG), forming an abasic site. Subsequent repair by error-prone polymerase incorporates random bases causing unintended conversions, such as C-to-G and C-to-A[10]. High deaminase activity, active cellular UDG and nicking by nCas9 may sometimes cause DSBs, resulting in indel formation. Although a canonical CBE contains a single UGI to subvert UDG, increasing the UGI number improved editing efficiency and reduced indel formation[94]. Plant CBEs were designed with four or five copies of UGI to improve editing efficiency and purity[47,61,66,95].

Recently, a method called Detect-seq was applied to capture out-of-spacer editing and target-strand editing by CBEs in human cells[96]. Such off-target effects have not been detected or documented in plants, suggesting that they are at most rare events. Nevertheless, the off-target effects and other unintended edits, if they occur, are not so problematic for crop improvement applications. If any of such off-target mutations are deleterious, they can be segregated away through conventional breeding[10].

AFIDs. Although Cas9-nuclease-generated indels can be predicted using machine learning[4], reliably deleting a larger genomic fragment is difficult. APOBEC–Cas9 fusion-induced deletion systems (AFIDs) were recently developed to achieve predictable large deletions within the protospacer sequence in rice and wheat genomes[97]. AFIDs consist of a fusion of cytidine deaminase, Cas9, uracil DNA glycosylase (UNG) and apurinic/apyrimidinic site lyase (AP lyase) in N-to-C-terminal sequence. AFIDs can induce deletions from the Cas9 cleavage sites to the 5′ deaminated C bases in the protospacer sequence[97]. Once a C base is converted to U by cytidine deaminase, UNG removes the U and forms an AP site. AP lyase then removes the AP site and generates a nick. Cas9 generates a DSB, usually at 3 bp upstream of the PAM sequence[4]. The AP-lyase-mediated nick and Cas9-induced DSB give rise to predictable deletions of the intervening region. The preliminary AFID system (AFID-3) was designed with APOBEC3A, and it was further improved with an engineered APOBEC3B (A3Bctd)[97]. The enhanced version (eAFID-3) works better when the target C is preceded by T (lies in a TC motif)[97]. AFIDs have been applied to generate predictable deletion in effector-binding elements of SWEET promoters, miRNA genes and *cis*-acting elements[97]. Even though strategies such as paired gRNAs and nickases and TevCas9 dual nuclease have previously been reported to induce large deletions[98,99], AFIDs is potentially a robust deletion tool for plant biology and crop improvement. It could be assumed that a deaminase reported having a 5′ shifted editing window would be suitable for generating even larger deletions.

ABEs. Theoretically, inferred from CBEs, a combination of adenine deaminase and nCas9 would give rise to ABEs for converting an A•T base pair to a G•C base pair. However, none of the reported naturally occurring adenine deaminases work on DNA[100]. By directed evolution and protein engineering, Gaudelli et al. evolved a transfer RNA (tRNA) adenosine deaminase (TadA) variant that works on a single-stranded DNA substrate[100]. A total of 14 mutations were installed in TadA to develop the engineered version (TadA*)[100]. As TadA acts as a dimer to catalyse deamination, a heterodimeric protein with a wild-type TadA non-catalytic monomer and an engineered catalytic monomer (TadA*) was devised[42,100]. The fusion of this heterodimer (TadA–TadA*) with nCas9 generated ABEs that efficiently converted A to G in mammalian cells with high purity[10,100] (Fig. 1d,e). In contrast to uracil excision repair, cellular inosine excision repair is weak and was not found to impede the A•T to G•C conversion[10,100]. Thus, no additional glycosylase inhibitor protein was used to develop ABEs[100] (Fig. 1f).

Like CBEs, ABEs were swiftly adopted and demonstrated in various plants, including rice[101–106], wheat[103], *Arabidopsis*[107], *Brassica napus*[107], *Nicotiana benthamiana*[108], poplar[38] and moss[37].

ABEs based on orthologous and engineered Cas9 variants. Similar to CBEs, ABEs were developed using Cas9 variants with alternative PAM compatibility[10,42,43] (Table 1). *Sa*Cas9-ABE, which can recognize NNGRRT PAM sequences, was reported in rice[61,102]. *Sc*Cas9-ABE has been shown to be effective in NNG genomic sites in rice[62,109].

*Sa*KKH-Cas9, VRER-Cas9 and VQR-Cas9, which can recognize NNNRRT, NGCG and NGA PAM sequences, respectively, were also used to develop ABEs for rice[24,61,110]. Alternative ABEs have been generated by adopting NRRH-, NRCH- and NRTH-Cas9 variants[47]. *Sp*Cas9-NG has been adopted to create NG PAM-targeting ABEs in plants[59,69,71,72,74,111]. Moreover, ABEs have recently been developed using *Sp*RY for nearly PAM-less A-to-G editing in rice[25,47,68,77,112]. There have been no reports on Cas12a-based ABE for editing T-rich PAMs until recently an evolved deaminase was found to be compatible with Cas12a in mammalian cells[113]. However, the same has not yet been reported in plants. Nevertheless, iSpyMacCas9 was recently integrated into the ABE architecture to offer adenine base editing at A-rich PAM sites in rice[56].

Adenosine deaminases in ABEs. Naturally occurring single-stranded-DNA-editing adenosine deaminases do not seem to exist[10,42]. Gaudelli et al. initially reported four different highly active ABEs-ABE6.3, ABE7.8, ABE7.9 and ABE7.10 (ref. [100]). Although ABE7.10 was used most commonly in plants[101–104,107], base editing with ABE6.3 (ref. [107]), ABE7.8 (ref. [107]) and ABE7.9 (ref. [101]) was also reported. For increasing expression levels of the ABE-deaminase domain (TadA–TadA* dimer), codon optimization and addition of a bipartite nuclear localization signal were effective in mammalian cells[58]. This variant is known as ABEmax and has outperformed ABE7.10 in a recent study in rice[59]. Although earlier reports on ABEs in mammalian and plant cells described the use of the TadA–TadA* heterodimer[10,42], the latest studies demonstrated that the wild-type TadA monomer is not required for ABE activity in mammalian cells[113–115] and rice[109,116,117]. This miniABE, comprising TadA* monomer fused to nCas9, even showed improved editing efficiency compared with the heterodimer ABE7.10 (refs. [109,116]). These studies indicate that intraconstruct TadA–TadA* heterodimerization may not be required for ABE activity[113–116]. However, those studies do not rule out the possibilities of in *trans* TadA*8–TadA*8 dimer formation[115]. Indeed, a high-resolution cryo-electron microscopy structure of substrate-bound miniABE (with a single TadA domain) confirmed that the TadA*8 domain dimerizes in *trans* during deamination[118].

In two recent studies, ABE7.10 was further evolved to generate eighth-generation ABEs, ABE8.20 and ABE8e, with substantially increased deamination kinetics[113,115]. ABE8e has been quickly adapted for high-efficiency base editing (up to 100%) in rice[25,47,77,109,117] and *N. benthamiana*[108]. ABE8e was found to outperform ABE8.20 in rice[109]. ABE8e was combined with two additional mutations, V82S and Q154R, to construct ABE9 for high efficiency base editing in rice[109].

Activity windows of ABEs. Among the originally reported ABE variants, ABE7.10 favourably edits the target A located at the protospacer position 4–9 (counting the NGG PAM as 21–23), whereas ABE6.3, ABE7.8 and ABE7.9 may offer higher efficiency if the target A is at position 8–10 (Fig. 1g)[100]. In rice, ABE7.9 was shown to perform better than ABE7.10 for the target A at position 7 of the protospacer[101]. The same study reported ABE7.10-mediated editing in an extended window at position 12 of the protospacer[101]. Similarly, another report showed editing at position 10 of the protospacer by ABE7.10 (ref. [102]). In rice, *Sa*Cas9-ABE7.10 showed a favourable activity window ranging from position 4–14 of protospacer, whereas VQR-Cas9-ABE7.10 exhibited activity window at protospacer position 3–10 (ref. [24]). In mammalian cells, activity windows for ABE8e varied with Cas proteins: position 4–8 for *Sp*Cas9; 3–14 for *Sa*Cas9; and 4–14 for LbCas12a[113]. However, in rice protoplasts, Cas9-ABE8e exhibited editing ability at the third to fourteenth base, whereas *Sp*RY-ABE8e showed variable activity at protospacer position 3–10 (ref. [25]). Another study with *Sp*RY-ABE8e reported that the activity window ranges from protospacer position 4–9 (ref. [77]). It is evident that activity windows depend on the Cas proteins and deaminase variants used (Table 1).

gRNA-dependent and -independent off-target and other unintended editing. Compared with CBEs, ABEs generate cleaner products, probably due to a weaker cellular inosine excision repair than uracil excision repair[10]. ABE-treated rice and wheat plants did not exhibit any undesired edits at both on- and off-target genomic sites[102,103]. However, a recent study reported unintended proximal base editing (G to A and A to T) in rice plants treated with ABE[101]. Similarly, another study showed ABE-mediated cytosine conversion[119]. No off-target effects were detected in rice plants edited with eCas9–ABE[74]. The development of more high-fidelity Cas protein-based ABEs is warranted to minimize gRNA-dependent off-target editing. Although ABEs have higher fidelity than CBEs, they are similar in causing bystander editing if additional targetable A is present in the activity window. For example, an earlier report in rice showed the generation of nonsynonymous mutations due to ABE-induced bystander editing[101]. In contrast to CBEs, ABEs do not generate genome-wide gRNA independent off-target editing in rice[91]. The rate of ABE-induced genome-wide single-nucleotide variants is insignificant compared with the rate of spontaneous mutations[91].

C-to-G and C-to-A transversion BEs. CBEs and ABEs can install C-to-T (G-to-A in the complementary strand) and A-to-G (T-to-C in the complementary strand) mutations, respectively. Collectively, they empower us to achieve 4 out of 12 possible base substitutions and catalyse only base transitions (pyrimidine to pyrimidine and purine to purine). To further expand the base editing toolkit for base transversion, four independent groups recently described the development of new base-editing platforms that are capable of C-to-G editing in mammalian cells and C-to-A editing in bacterial cells[120–123] (Fig. 2a–c). These studies have recently been reviewed[124]. The CGBEs consist of a UNG fused to nCas9 (nCas9^{D10A}) and cytidine deaminase rAPOBEC1 (ref. [121]) or rAPOBEC1 (R33A)[120]. In bacterial cells, a similar platform containing hAID in place of rAPOBEC1 yielded C-to-A conversion[121]. C is deaminated to U by a cytidine deaminase. UNG generates an apurinic/apyrimidinic (AP) site by removing U. Subsequent error-prone polymerase activity probably incorporates G and A at the AP site in mammalian and bacterial cells, respectively, resulting in base transversion editing. Further investigation is warranted to discover why mammalian cells prefer G and bacterial cells prefer A insertion at the AP site. Another CGBE was developed by fusing X-ray repair cross-complementing protein 1 (XRCC1) to APOBEC1–nCas9 (ref. [122]). In this case, a cellular UNG removes the U generated by APOBEC1 and crenates the AP site. Next, XRCC1, a BER protein, aids the preferred incorporation of a G at the AP site resulting in a C-to-G outcome. Recently, ten CGBE platforms with different DNA repair proteins have been extensively characterized in a library of >10,000 target sites in the mammalian genome[123].

CGBE editing efficiency was highly target dependent, ranging from 3% to 70% (refs. [120–122]). Current CGBE platforms have an exceptionally narrow activity window with a high preference for protospacer position 6, while weaker editing efficiency for cytosines at positions 5 and 7 was observed[120–123] (Fig. 2d). However, at one target site, C at position 9 was substantially edited by CGBEs[123]. As rAPOBEC1 inherently does not prefer a GC sequence, the CGBE platforms exhibited very low to negligible editing if a G precedes the target C. When the target C is flanked by A and/or T bases, it is highly likely that C will be edited by CGBEs[120,123]. To overcome these limitations, varying linker length, engineered or alternative cytidine deaminase and Cas9 could be beneficial[124]. Although CGBEs are yet to be demonstrated in plant systems, they represent a useful tool for installing precise transversion mutations in plant genomes with reduced bystander effect. Despite that the UNG–nCas9–AID BE showed more than 80% C-to-A editing efficiency in *Escherichia coli*[121], a C-to-A base editor remains elusive in eukaryotes.

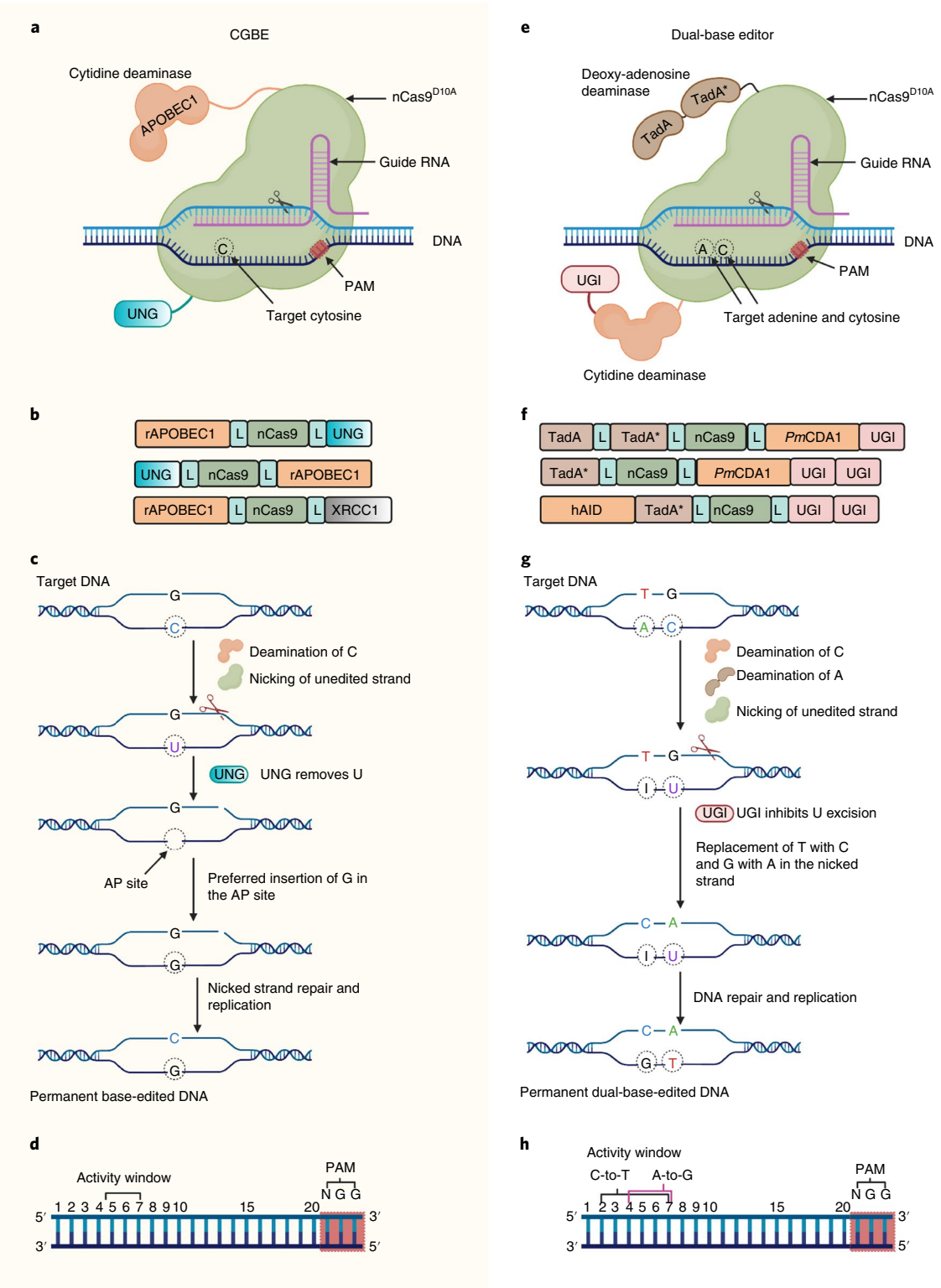

Fig. 2 | C-to-G and dual (simultaneous C-to-T and A-to-G) base editing in nuclear DNA. a, A CGBE in the DNA context. The target cytosine is indicated (dotted circle). **b**, CGBE architectures (N to C terminal) containing cytidine deaminase (peach), nCas9 (green) and uracil DNA *N*-glycosylase (UNG) (sea green). **c**, The mechanism of CGBE is shown. Once the target C is deaminated to U by cytidine deaminase, UNG removes the U to generate an AP site. Nicking the non-edited strand by nCas9, preferred insertion of G at the AP site and subsequent repair/replication events ultimately convert a C•G base pair to a G•C. **d**, CGBE optimally edits at position 6 of the protospacer (counting the NGG PAM as 21–23). C bases at positions 5 and 7 are edited with reduced efficiency. **e**, A dual-base editor for concurrent installation of C-to-T and A-to-G mutations. The target C and A are indicated (dotted circles). **f**, Schematic of dual-base editor architectures composed of adenosine deaminase (brown), nCas9 (green), cytidine deaminase (peach) and UGI. **g**, The mode of action of dual-base editors is similar to individual CBE and ABE operation. Simultaneous deamination of C and A is carried out by cytidine and adenosine deaminase, respectively. nCas9 nicks in the non-edited strand and UGI protects from U removal. **h**, Activity windows for C-to-T and A-to-G editing overlap. The PAM is highlighted (red). TadA, wild-type TadA from *E. coli*; TadA*, evolved TadA.

Dual-base editors. None of the above-mentioned BEs can reliably perform simultaneous conversions of two different types of nucleotides in the same protospacer sequence. To this end, recent studies have reported dual-base editors by fusing both cytidine and adenosine deaminases to a single Cas protein[125–129] (Fig. 2e,f). Dual-base editors concurrently introduce C-to-T and A-to-G substitutions into the target genomic region (Fig. 2g,h). The platforms such as synchronous programmable adenine and cytosine editor (SPACE), A&C-BEmax, Target-ACEmax, and adenine and cytosine BE (ACBE) were generated for mammalian cells, whereas saturated targeted endogenous mutagenesis editors (STEMEs) were developed for plants[125–129]. The adenosine deaminase component of SPACE consists of monomeric TadA*, whereas other four platforms contained TadA–TadA* heterodimer. Out of four different architectures tested, STEME1 with the APOBEC3A–TadA–TadA*(7.10)–nCas9–UGI configuration exhibited the highest dual-base editing efficiency[125]. STEME1 showed C-to-T editing activity within protospacer position 1–17 and A-to-G conversion within position 4–8. STEMEs were successfully used for the directed evolution of the *ACC* gene for herbicide tolerance in rice[125]. Note that the indel percentage was not found to be increased compared with the single-base editors[125–127,129]. Interestingly, the SWISS platform has been developed to simultaneously induce adenine base editing, cytosine base editing and indel formation at different targets in plant genomes[130]. Undeniably, these dual-base editors would be beneficial for installing multinucleotide variants, protein engineering, CRE engineering, cell lineage tracing, directed evolution and saturation mutagenesis.

Organellar BEs. Two organelles, mitochondria and plastids, contain their own genomes, which are vital to organelle biogenesis, energy production and cellular metabolism. Tools for editing organellar DNA have long been sought for organellar genetics. Although the CRISPR–Cas-derived BEs described above are highly effective for nuclear genome manipulation, it is difficult to repurpose them for organellar DNA editing due to the lack of effective means to transport sgRNAs through the double-layered membranes of mitochondria or plastids. However, an all-protein-based system would work as an organellar genome editor using the existing protein import machinery of chloroplast and mitochondria. Programable DNA-binding proteins such as zinc fingers (ZFs) and transcription-activator-like effectors (TALEs) are suitable for this purpose.

mtDNA base editing. An interesting recent study reported a first-of-its-kind BE—DddA-derived cytosine BE (DdCBE)—for C-to-T conversion in human mitochondrial DNA (mtDNA)[131]. The DdCBE architecture consists of a mitochondrial targeting signal (MTS), a TALE array, a DddA cytidine deaminase and a UGI (Fig. 3a). Cytidine deaminases in nuclear CBEs usually act on single-stranded DNA, transiently generated in the Cas9-mediated R-loop structure. Although a TALE array fused to an MTS sequence can be targeted to mtDNA, TALEs are incapable of unwinding double-stranded DNA and cannot provide single-stranded DNA substrates for cytidine deaminases. Remarkably, Mok et al. discovered a deaminase from Gram-negative bacterium *Burkholderia cenocepacia*, DddA, that deaminates cytosines in double-stranded DNA[131]. As DddA is cytotoxic to mammalian cells, the DddA domain was cleaved into two inactive halves. Similar to the Fok1 monomer assembly in transcription activator-like effector nucleases (TALENs)[132], the left TALE array and right TALE array were fused with an inactive DddA half (Fig. 3a). DddA would be functional only once both halves reconstitute adjacently to the target DNA (Fig. 3b). Other than mitigating cytotoxicity, splitting DddA has another advantage of minimizing off-target editing effects.

Optimized DdCBE displayed 4.6–49% editing efficiency across five different human mitochondrial genes[131]. Factors that potentially impact the efficiency include the following: TALE design, spacing regions between two Split-DdCBE halves, the position of the target C from the TALE-binding site and the sequence context of the target C[131,133]. Note that DdCBE has a substrate preference for 5′-TC-3′ in its current form. To be edited by DdCBE, a T must precede the target C. To further expand the scope of mtDNA editing, exploring the natural diversity of double-stranded DNA cytidine deaminases or engineering DdCBE to alter its sequence preference would be useful. In principle, such DdCBE systems should also work for mitochondrial DNA base editing in plants. Compared with animals, plant mitochondrial genomes are complex, dynamic and larger in size[134]. They consist of repeats, large introns and non-coding regions of which the function remains unclear. Variants of plant mtDNA were associated with abnormal growth phenotypes and cytoplasmic male sterility[134], which are of high value in crop breeding. Previously, mtDNA editing has been achieved by mitochondria-targeted TALENs in rice and rapeseeds[135], as well as in *Arabidopsis*[136]. It is exciting to note that a recent study reported successful DdCBE-mediated base editing in the mitochondrial genome with up to 25% efficiency in rapeseed calli[137].

Plastid DNA base editing. Following a similar strategy, plastid BEs, especially chloroplast BEs, could be prepared using chloroplast transit peptide (Fig. 3c,d). Alternatively, DNA could be directly delivered into plastids using a gene gun[138]. Three independent groups swiftly adopted Split-DddA to generate chloroplast BEs (cp-DdCBEs) for editing plastomes of lettuce[137], rapeseed[137], *Arabidopsis*[139] and rice[140]. Plastomes were base-edited in lettuce and rapeseed protoplasts with 30% and 15% efficiency, respectively[137]. The same report described DNA-free base editing with DdCBE mRNA in lettuce chloroplasts. Interestingly, a cp-DdCBE-induced streptomycin-resistant mutation in the 16S rRNA gene in lettuce protoplasts was maintained in regenerated calli after cell division[137]. A single plant cell may contain many chloroplasts and it is therefore not easy to obtain homoplasmic substitutions. Interestingly, cp-DdCBE showed 64% editing efficiency with near homoplasmic substitutions in T_0 regenerated rice plants[140]. Similarly, cp-DdCBE has induced high efficiency of homoplasmic mutations in T_1 transgenic *Arabidopsis* plants[139]. The study in *Arabidopsis* showed that the cp-DdCBE-induced mutations were inheritable[139]. The plastid BEs are poised to stimulate plastid genetics research and engineering to improve photosynthesis, yield, nutritional quality, and herbicide or stress tolerance.

RBEs. Editing individual nucleobases in the RNA transcriptome enables modifications in protein sequences without permanent changes in the genome. Once modifications are installed in the RNA sequence, they are not further processed by the cell[14]. Two types of RNA BEs (RBEs) have been reported to date for transcriptome modifications.

RBE for A-to-I editing. Taking advantage of the RNA targeting ability of Cas13 without any PAM requirements, the RNA editing for programmable A-to-I replacement (REPAIR) system has been recently developed[141]. A catalytically dead Cas13b enzyme from *Prevotella* sp. was tethered to the deaminase domain of adenosine deaminase acting on RNA type 2 (ADAR2) containing a hyperactivating mutation E488Q to construct the REPAIR version 1 (REPAIRv1) (Fig. 4a). During the cellular splicing and translation, inosine is read as guanosine and, therefore, many existing G-to-A mutations could be corrected using REPAIR[10,141]. REPAIRv1 displayed on average 28% editing efficiency at 33 sites in human cells[141]. The study also revealed that the REPAIR system achieves higher editing with 50-nucleotide gRNA compared with 30-nucleotide gRNA. The binding of the gRNA with the target RNA results in a double-stranded RNA,

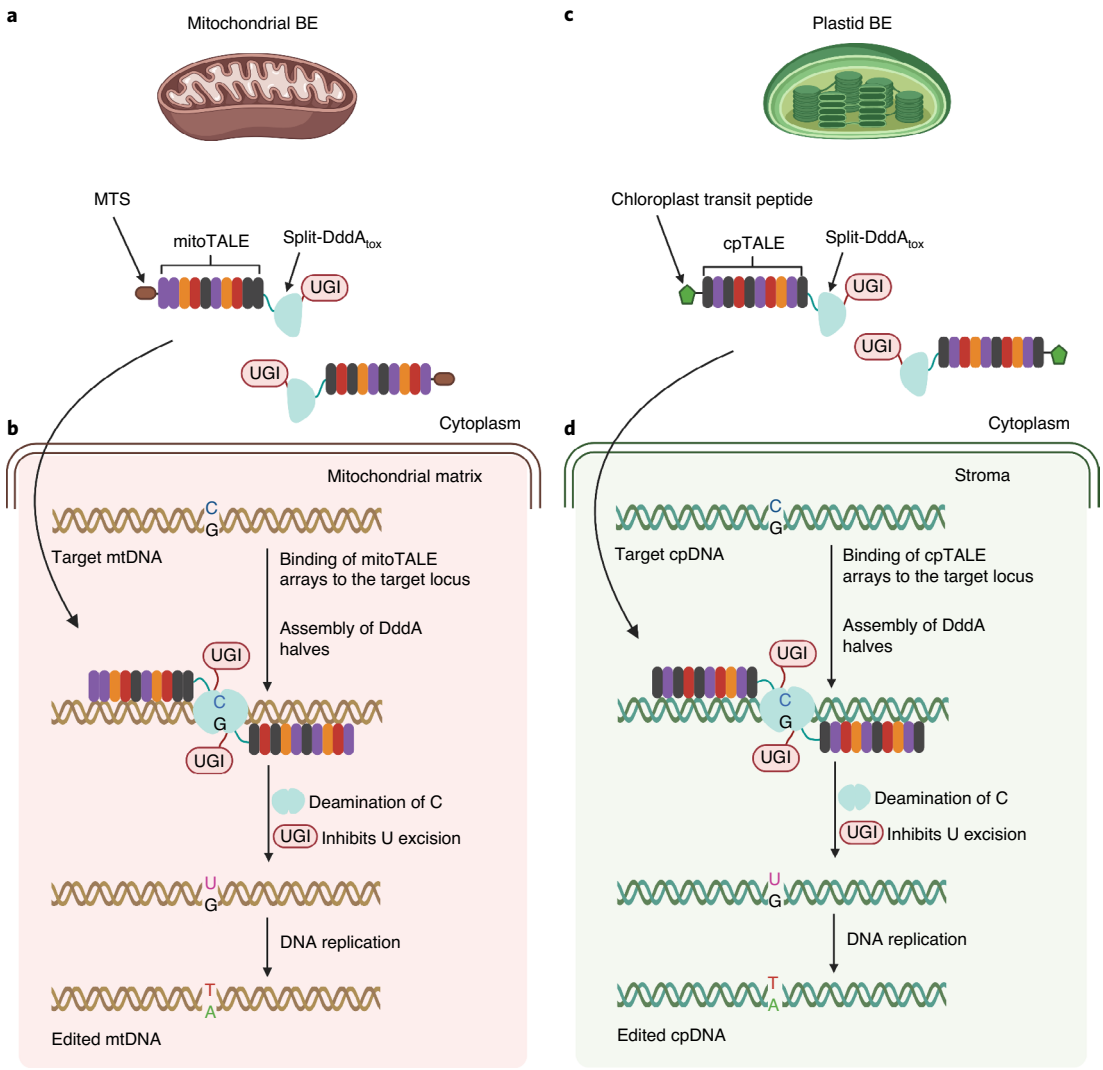

Fig. 3 | Base editing in organellar DNA. a, The architecture of the mitochondrial BE DdCBE. DdCBE is split into two parts, each of which consists of an MTS, a TALE array for binding to specific mtDNA, an inactive half (Split-DddA$_{tox}$) of bacterial cytidine deaminase that works on double-stranded DNA and a UGI. **b**, DdCBE acts similarly to a CBE. The MTS transports the two halves into the mitochondrial matrix. Two TALE arrays bind to the target DNA sequence and bring the two inactive halves into proximity. After reconstitution of active DddA$_{tox}$, it performs deamination of C in double-stranded DNA. The rest of the mechanism is similar to CBE acting on nuclear DNA. **c**, Plastid (chloroplast in this example) BE is split into two segments. Each segment contains a chloroplast transit peptide, a TALE array that binds to target chloroplast DNA (cpDNA), an inactive Split-DddA$_{tox}$ and a UGI. **d**, The mechanism of chloroplast BE is similar to mitochondrial BE. The assembly of DddA halves is mediated by the binding of two separate TALE arrays specific to the adjacent chloroplast DNA sequence. DddA, *B. cenocepacia* double-stranded DNA deaminase A; mitoTALE, TALE array targeting mitochondrial DNA; cpTALE, TALE array targeting chloroplast DNA.

fulfilling the substrate requirement of ADAR2. A mismatched cytidine was incorporated into the gRNA opposite to the target adenine to be edited for increasing A-to-I editing efficiency[141]. In contrast to most of the BEs discussed in the sections above, REPAIRv1 had no sequence context preference surrounding the targeted A and could theoretically edit every A in the RNA.

However, REPAIRv1 exhibited substantial transcriptome-wide off-target editing, which was independent of dCas13b and attributed to the activity of the deaminase domain (ADAR2$_{DD}$)[141]. The fusion of a double mutant ADAR2$_{DD}$(E488Q/T375G) to dCas13b resulted in an improved version, REPAIRv2, which reduced the off-target editing by 900-fold relative to REPAIRv1 (ref. [141]). Mutations that increase specificity sometimes compromise on-target efficiency. The REPAIRx platform has recently been developed for A-to-I editing in RNA, which has the specificity level of REPAIRv2 and the efficiency level of REPAIRv1 (ref. [142]).

RBE for C-to-U editing. Learning from the REPAIR system, a fusion of naturally occurring RNA cytosine deaminase to dCas13b would theoretically give rise to an RBE platform for C-to-U editing. However, the high affinity of RNA cytosine deaminase for every cytosine present in single-stranded RNA is problematic and could generate a high level of transcriptome-wide off-target editing. Abudayyeh et al. therefore used directed evolution to engineer ADAR2$_{DD,}$ which behaves like a cytosine deaminase acting on double-stranded RNA[143]. The fusion of this evolved enzyme to dCas13 resulted in the platform called RNA editing for specific C-to-U exchange (RESCUE) (Fig. 4b). Interestingly, RESCUE is capable of both adenosine and cytidine deamination. Thus, RESCUE could be used for multiplex A-to-I and C-to-U editing in RNA by supplying a pre-crRNA guide array as Cas13 can self-process the pre-crRNA. RESCUE was optimally active with a 30-nucleotide gRNA harbouring a C or U mismatch opposite to the

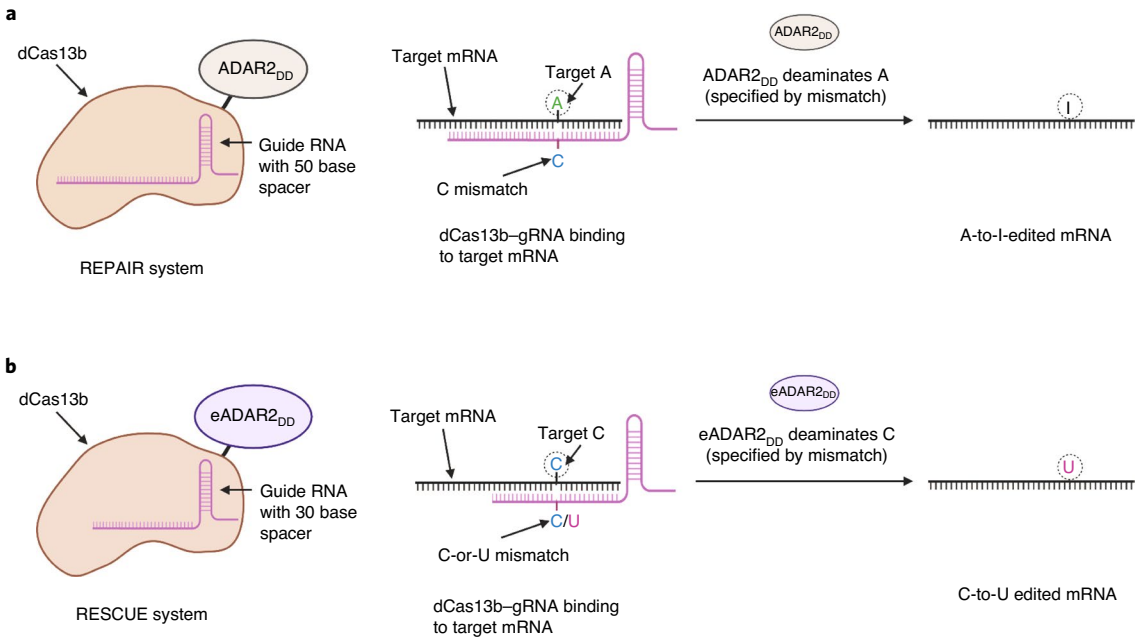

Fig. 4 | Base editing in RNA. a, A-to-I editing in RNA by the REPAIR system. The REPAIR architecture contains a deactivated Cas13b fused to ADAR2. REPAIR is targeted to mRNA (black) by a gRNA with a 50-nucleotide spacer (purple). The target A is specified by an induced A–C mismatch in the mRNA–gRNA duplex. **b**, C-to-U editing in RNA using the RESCUE platform. RESCUE is composed of a deactivated Cas13b fused to an engineered ADAR2 variant (eADAR2). The RESCUE platform works optimally with a gRNA with 30-nucleotide spacer. The target C is specified by an induced C–C or C–U mismatch in the mRNA–gRNA duplex.

target C[143]. The additional incorporation of a S375A mutation in RESCUE architecture yielded a highly specific version, RESCUE-S, which maintained ~76% on-target C-to-U editing with reduced off-target conversion.

Although organellar RNA editing is a known phenomenon in plants, the nuclear RNA editing system is unknown or poorly explored[144]. While none of the RBEs has been experimentally demonstrated yet in plant cells, these tools could open a new avenue in plant RNA biology and crop engineering.

Prime editing

The mechanism of prime editing. Although BEs are powerful in introducing point mutations with high efficiency, they cannot generate precise indels and barely avoid bystander mutations. By contrast, PEs can introduce all 12 possible transition and transversion mutations and small indels, as well as combinations thereof with favourable intended editing to byproduct indel ratios[145]. They are versatile, precise genome editing tools that directly write new genetic information into a specified DNA target site using a Cas9 nickase (nCas9; H840A) fused to an engineered reverse transcriptase (RT). The RT is programmed with a prime editing gRNA (pegRNA) that specifies the target site and encodes the desired edit[145]. PegRNA is a modified sgRNA with 3′ extension of the RT template and primer-binding site (PBS) sequences (Fig. 5a). The nCas9 (catalytically impaired Cas9 harbouring a H840A mutation) is used to nick the editing strand of the double-stranded DNA target. Next, the nicked strand is used for priming the reverse transcription of an edit-encoding extension (RT template) on the pegRNA directly into the target site[145] (Fig. 5b). This results in a branched intermediate consisting of two competing single-stranded DNA flaps. The 3′ flap contains the edited sequence, whereas the 5′ flap contains the unedited sequence. The 5′ flap is preferentially cleaved by structure-specific endonucleases such as FEN1 (ref. [146]) or 5′ exonucleases such as Exo1 (ref. [147]) in mammalian cells. Ligation of the 3′ flap incorporates the edited DNA strand into the heteroduplex DNA containing one edited strand and one unedited strand. Finally, to resolve the heteroduplex, DNA repair machinery permanently installs the desired edit by copying the information from the edited strand to the complementary strand (Fig. 5c).

PEs. Anzalone et al., in their seminal paper, introduced three versions of prime editing systems[145]. Prime editor 1 (PE1) harbours Cas9^{H840A} nickase with a C-terminal fusion of a wild-type Moloney murine leukaemia virus RT (M-MLV-RT). Prime editor 2 (PE2) incorporates the engineered M-MLV-RT pentamutant (D200N/L603W/T330P/T306K/W313F) with increased thermostability, processivity, DNA–RNA substrate affinity and inactivated RNase H activity (Fig. 5b). Compared with PE1, PE2 has about threefold improved editing efficiency in human cell lines[145]. Prime editor 3 (PE3) involves nicking the non-edited strand to stimulate DNA repair machinery and therefore further increases the editing efficiency in human cells by twofold to fourfold compared with PE2. The PE3 strategy involves the double nicking of two pairing DNA strands, which could generate a DSB, leading to indel formation due to NHEJ repair. To reduce this outcome, a variant of the PE3 system, prime editor 3b (PE3b), has been introduced. PE3b uses a nicking gRNA with a protospacer that matches the edited strand but not the original allele. PE3b resulted in a thirteenfold decrease in the average number of indels compared with PE3 in human cell lines without compromising editing efficiency[145]. Prime editing has been demonstrated in multiple mammalian cell lines with varying efficiencies[145]. For example, PE3 installed point mutations at positions 12–33 (relative to the start of the protospacer) with ~40% efficiency at the human *HEK3* locus. Furthermore, PE3 created 1 bp and 3 bp deletions at ~30% efficiency and generated 5–80 bp deletions at even higher efficiencies (52–78%)[145]. Prime editing has been demonstrated in rice[148–155], wheat[149], maize[156], potato[157], *Arabidopsis*[154], *N. benthamiana*[154] and tomato[158]. Surprisingly, in plants, PE3 or PE3b generally do not yield higher editing efficiencies than PE2 (refs. [148–151,157]). Overall, PE2- and PE3-mediated editing efficiencies reported in plants

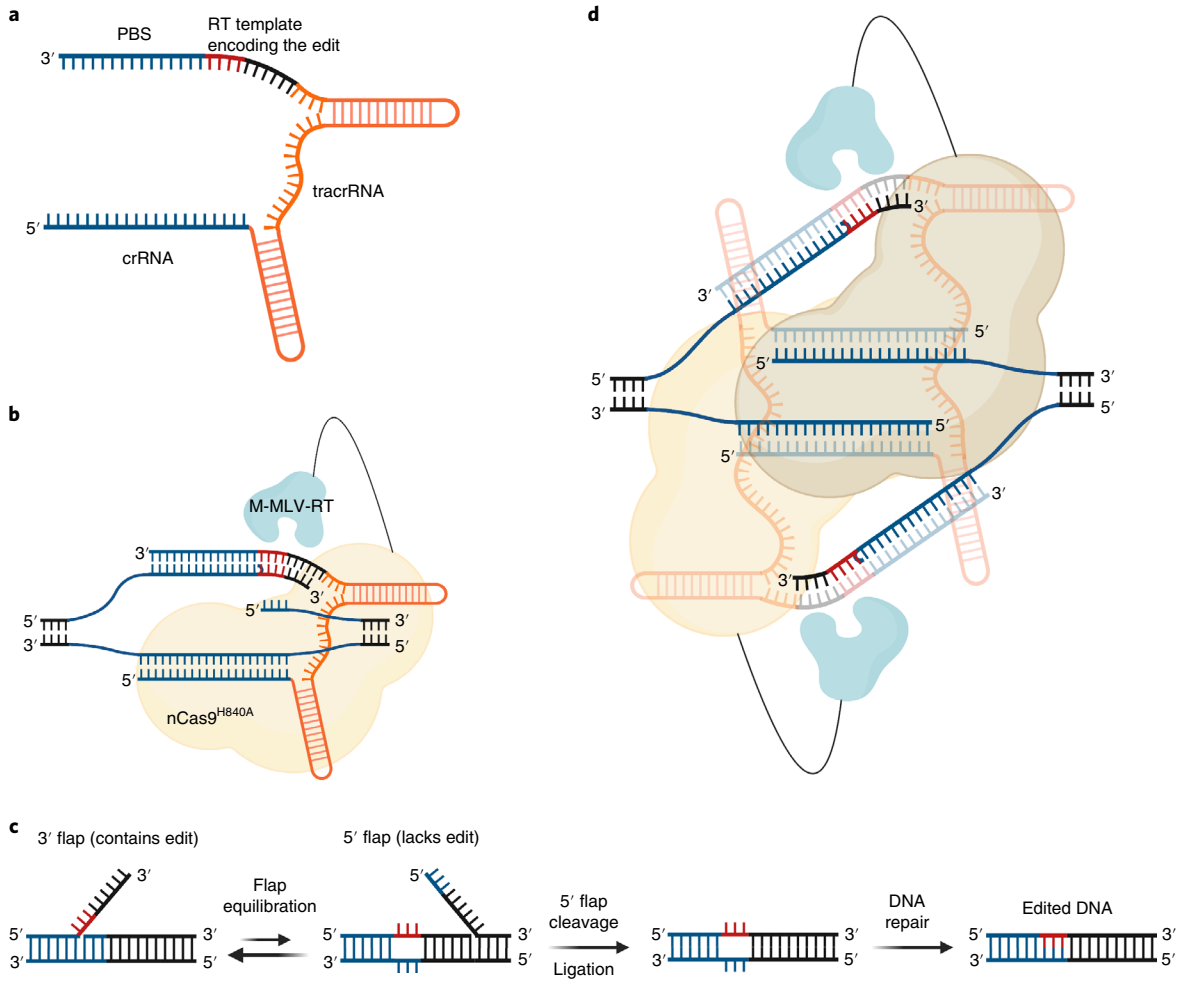

Fig. 5 | Prime editing. a, pegRNA consists of CRISPR RNA (crRNA), transactivating crRNA (tracrRNA), a RT template and a PBS. crRNA guides the PE to the target site, tracrRNA renders nCas9^{H840A} nickase active, the RT template encodes the desired edit and the PBS primes the reverse transcription. **b**, PE, a ribonucleoprotein consisting of pegRNA, nCas9^{H840A} nickase and C-terminally fused M-MLV-RT located at the target site within the genomic DNA. nCas9-mediated R-loop enables the nicked exposed single DNA strand to be primed and extended with M-MLV-RT. **c**, The mechanism that leads to the permanent incorporation of the desired edit into targeted region of the double-stranded DNA. Reverse transcription produces a branched intermediate consisting of two competing single-stranded DNA flaps. The 5′ flap containing the non-edited sequence is preferentially cleaved and the 3′ flap containing the desired edit is ligated and forms heteroduplex DNA. DNA repair mechanisms enable the permanent installation of the edit into double-stranded DNA. **d**, The dual pegRNA strategy used at the same target site and encoding the same edit in *trans* for prime editing with the aim of enhancing the prime editing efficiency.

ranged from almost zero to a few per cent, far lower than the efficiencies reported in the original human study[145]. A surrogate PE2 system has been developed in rice to enrich prime-edited cells to enhance screening efficiency[150].

Before being widely used by plant biologists, prime editing efficiency needs further improvement. It seems that the design of pegRNAs should be tailored for each target site independently. The RT template and PBS within a pegRNA present many possible design choices to optimize the efficiency of a desired edit. Typically, only a few RT template and PBS combinations would support optimal prime editing efficiencies. In general, RT templates should be between 10–20 nucleotides long, while the PBS should be approximately 13 nucleotides long but can span from about 7–17 nucleotides in length[145]. The melting temperature (T_m) is important for the stability of DNA–RNA hybrids. A recent study showed that the PBS T_m strongly influenced prime editing efficiency and recommended to use a PBS T_m of ~30 °C for obtaining maximal efficiency[159]. Although an earlier attempt was not successful[149], exploring more RTs and optimizing their expression may still represent a sound strategy to improve prime editing in plants. Furthermore, prime editing efficiency may be improved if we gain further understanding of the plant DNA repair mechanisms favouring incorporation of the desired prime editing outcomes in plants. Although PEs also have relatively narrow editing windows centred around the PAM site[145], this limitation on targeting scope could be overcome with the use of orthogonal and engineered Cas9 proteins with altered PAM requirements, as demonstrated with CBEs and ABEs. Thus, low prime editing efficiency is the major bottleneck for its wide applications in plants. Interestingly, a recent study developed strategies to boost PE efficiency in rice ranging from 2.9-fold to 17.4-fold[159], including using paired pegRNAs encoding the same edits in *trans* (Fig. 5d) and designing a PBS with a T_m of 30 °C (ref. [159]). At first glance, having two pegRNAs in proximity might present a steric hindrance problem. However, the paired pegRNA approach yielded higher prime editing efficiency compared with that of a single pegRNA approach, which could be due to the dual-pegRNA-mediated simultaneous editing in both DNA strands[159].

The targeting specificity of prime editing. As PEs need three tiers of specific DNA hybridization—namely pegRNA spacer-target DNA for binding to the genomic target, pegRNA PBS-target DNA for initiating reverse transcription and RT-product-target DNA for flap resolution—the off-target effects of PEs are theoretically highly limited[145]. A recent comprehensive study showed that PEs generate low frequencies (0.00–0.23%) of pegRNA-dependent off-target editing in rice[160]. The authors analysed whole-genome sequences of 29 PE-edited plants and confirmed that PEs do not induce genome-wide pegRNA-independent off-target edits[160]. As PEs ectopically express the RT enzyme, it is imaginable that PEs might lead to RT-mediated undesirable alteration. The same study revealed that prime editing does not alter the copy number of retrotransposons and telomere structure and does not cause pegRNA insertion into the rice genome[160].

Computational tools for base editing and prime editing

Conventional CRISPR–Cas-mediated knockout experiments require a relatively straightforward gRNA design. Predicting the efficiency and mutation outcome for a gRNA has been possible using machine learning and deep learning[4,161]. However, designing gRNAs for base editing and pegRNAs for prime editing seems to be more complicated. The activity windows for BEs and PEs are distinct; BEs generally favour a PAM distal activity window, whereas PEs prefer a PAM proximal activity window. Owing to its built-in complexity, pegRNA design can be especially tricky.

Tools for base editing. In recent years, several web tools have been developed to assist in gRNA design[162–164], selecting guides[164–166] and BEs[167], and predicting editing efficiencies[165,166] and outcomes[165,166] of CBEs and ABEs (Table 2). Interestingly, a recent study has developed a data-driven model for predicting editing outcomes of CGBEs[123]. Similar to knockout experiments, in silico prediction of Cas-dependent off-targets for base editing gRNAs can be performed using any plant-specific web tools, such as CRISPR-PLANT v2 (ref. [168]) and CRISPR-P 2.0 (ref. [169]). Targeted deep sequencing is considered to be a reliable method for measuring base editing efficiency in a population of cells. Web tools have been developed to analyse the base conversion ratio from deep-sequencing of PCR amplicons[162,170], which is more expensive and time-consuming than Sanger sequencing. As a consequence, the EditR and BEAT programs were generated to quantify base editing from Sanger sequencing[171,172], albeit with lower sensitivity. Note that most of the currently available tools for base editing gRNA design, efficiency and outcome prediction mainly support mammalian genomes. Plant species-specific base editing gRNAs can be designed using BE-designer[162]. Ideally, more plant genome-specific base editing tools need to be developed to assist plant investigators.

Tools for prime editing. Similarly, several online pegRNA design tools have been developed to accommodate the PE2, PE3 and PE3b versions of PEs, including PlantPegDesigner[159], pegFinder[173], PrimeDesign[174], Primeedit[175], PINE-CONE[176] and multicrispr[177] (Table 2). The availability of these tools with a user-friendly interface greatly simplified the intricate design of pegRNAs and therefore contributed to a broader accessibility of the prime editing technology. Although all of the listed pegRNA design tools except for PlantPegDesigner were developed for the mammalian systems, they can also be used for plants. However, plant researchers and breeders could benefit tremendously from plant-specific tools such as PlantPegDesigner, especially if specific pegRNA design features are essential for improved editing efficiency in plants.

Table 2 | Web tools developed for base editing and prime editing experiments

Tools	Functions	Reference
BE-Hive	Base editing (with CBEs and ABEs) genotypic outcomes and efficiency	165
CGBE-Hive	CGBE editing efficiency and purity prediction	123
CBE_efficiency ABE_efficiency DeepCBE DeepABE	Cytosine base editing efficiency, adenine base editing efficiency, frequency of CBE editing outcomes and frequency of ABE editing outcomes	166
EditR	Quantification of base editing from Sanger sequencing	171
CRISPR-CBE1	Designing of gRNAs for CBE-mediated gene inactivation	163
BE-FF	Identifying suitable BEs to correct wrong amino acids introduced by point mutations in human	167
beditor	Designing gRNA libraries and selecting the best sets of gRNAs	164
BE-Designer BE-Analyzer	sgRNA designing tool for CRISPR BEs and analysing base editing outcomes from next-generation sequencing data	162
BEAT	Base-editing event quantification from Sanger sequencing data in batch	172
CRISPResso2	Analysing base editing outcomes from next-generation sequencing data	170
pegFinder	Design of pegRNAs	173
PrimeDesign	Design of pegRNAs	174
Primeedit	Design of pegRNAs for human pathogenic variants	175
PINE-CONE	Design of pegRNAs	176
multicrispr	Design of pegRNAs	177
PnB Designer	Design of prime and BE gRNAs	213
PlantPegDesigner	Design of pegRNA and paired pegRNA	159

Applications of base editing and prime editing in plants

BE- and PE-based genetic modifications. Base editing and prime editing technologies have tremendous potential to assist plant biologists in various basic investigations (Fig. 6). As each target locus might require a unique activity window and PAM compatibility, an enriched toolbox of BEs and PEs offers researchers exceptional opportunities to apply these powerful technologies for diverse needs. We highlight below a few of the distinct types of genomic alterations that could be achieved with BEs and PEs in plant research.

Installing nonsynonymous mutations. The currently available BE tools empower us to execute 6 types of base swapping (4 transitions and 2 transversions) out of 12 possible substitutions (Fig. 6a). Out of 61 codons for amino acids, 49 are targetable by CBEs, ABEs and CGBEs for nonsynonymous substitutions. By contrast, PE tools enable all 12 types of substitutions and small indels in the genome (Fig. 6a,b), although with lower efficiency. Together base editing and prime editing represent a unique opportunity for research communities to precisely alter amino acid residues in targeted protein sequences for the study of functional genomics. They could also facilitate in identifying conserved amino acids of biological significance[10].

Enabling molecular diversity and directed evolution. Plant genetics and breeding is fundamentally centred on linking genotypes to phenotypes. By increasing molecular diversity, proteins with novel

chemical and physical properties could be generated. Directed evolution is one such powerful approach for increasing genetic or molecular diversity[178]. Although the conventional CRISPR–Cas9 approach has been used for directed evolution in plants[179], CRISPR–Cas9-induced frameshift mutations are mostly useless, especially when they are not viable and heritable[180]. This problem is addressed by BE-mediated directed evolution as they, in principle, generate in-frame mutations (Fig. 6c). CBE architectures devoid of UGI were reported to yield diverse outcomes such as C to A, C to T and C to G, and have been used for directed evolution in mammalian cells[181,182]. Similarly, both CBEs and ABEs with gRNA libraries covering the coding sequence of rice acetolactate synthase (Os*ALS*) gene have been applied for directed evolution[25,66,183]. CBE-mediated targeted evolution of rice *EPSPS* for herbicide tolerance was reported[66]. Recently developed dual-base editors[125–129] (discussed in an earlier section), which can generate simultaneous A-to-G and C-to-G mutations, are better suited for directed evolution. For example, the STEME platform has been used for directed evolution of the acetyl coenzyme A carboxylase (Os*ACC*) gene to gain herbicide resistance in rice[125]. With 20 gRNAs spanning a DNA region encoding 56 amino acid residues of the ACC protein, STEME achieved near-saturated mutagenesis, which helped to create de novo Os*ACC* herbicide-resistant variants[125].

As BEs cannot currently perform many transversion mutations, BE-mediated random mutagenesis may not be all-inclusive and may overlook many mutations endowing the desired trait. Recently, a prime-editing-library-mediated saturation mutagenesis (PLSM) method identified 16 types of herbicide-resistance-conferring mutations, including several new ones[155]. PegRNA libraries with all possible combinations of substitutions at six different target residues in Os*ACC1* enabled a more comprehensive screening by PLSM than that which is achievable with BEs[155]. Alternatively, variants of a target gene could be made and evaluated in *E. coli* by random mutagenesis and then the best variant could be reproduced in plants by BE or PE tools.

Editing CREs. CREs are non-coding DNA sequences that regulate gene expression. Editing CREs will not only offer insights into their function but also provide an effective approach to fine-tune gene expression. CRISPR–Cas9 has been used for promoter editing to introduce quantitative trait variation in tomato[184,185], rice[186] and maize[187]. It was also recently applied to edit intronic enhancers in *Arabidopsis*[188]. Interestingly, editing upstream open reading frames (uORFs) using CRISPR–Cas9 could improve protein translation in *Arabidopsis* and lettuce[189]. Editing the uORF of FvebZIPs1.1 in strawberry using a CBE resulted in a continuum of sugar content[36]. With high precision, BEs and PEs will open tremendous opportunities to discover new CREs and manipulate known CREs to alter gene expression (Fig. 6d).

Generating and correcting premature stop codons. Canonical CRISPR–Cas9-mediated gene knockout depends on the generation of premature stop codons from frameshift-inducing indels, which does not represent the most straightforward way for introducing stop codons. Interestingly, CBEs can generate premature stop codons at predefined triplets to knock out gene function (Fig. 6e). They can precisely convert four codons—CAG, CGA, CAA and TGG—into stop codons (TAA, TAG and TGA)[10]. Similarly, CGBEs can generate two premature stop codons from TCA and TAC codons[124] (Fig. 6e). In total, six codons can be altered by currently available BEs to generate stop codons. CBEs were used to generate stop codons in many plant species, such as tomato[30], wheat[23], rice[22,190] and rapeseed[191]. Another interesting strategy to silence a gene could be to mutate the start codon (ATG) to ACG or GTG by ABE, as demonstrated recently[192]. In principle, introducing stop codons into early exons should be more reliable than introducing start codon mutations for knocking out protein-coding genes as some genes may have alternative start sites.

By contrast, ABEs can correct mutations that cause premature stop codons (Fig. 6e). Stop codons could be reverted to tryptophan (TGG), glutamine (CAA, CAG) and arginine (CGA) codons[10]. In rice, an early stop codon in the GFP coding sequence has been corrected by an ABE to restore GFP fluorescence[103]. Theoretically, CGBEs can perform the reversal of TAG and TGA stop codons by targeting the coding strand (Fig. 6e).

Modulating RNA splicing. For canonical eukaryotic mRNA splicing, splice donor (GT) and splice acceptor (AG) sites are required, and both can be disrupted by base editing (Fig. 6f). The donor site is editable with CBEs, ABEs and CGBEs by targeting the coding strand, whereas the acceptor site can be altered with ABEs (targeting non-coding strand), as well as CBEs and CGBEs (targeting coding strand). BEs can therefore induce mis-splicing, eventually leading to exon skipping, alternative splicing or intron retention[10]. Null mutants were generated in *Arabidopsis* and rice using this approach[18,19]. BE-mediated modulation of mRNA splicing is another way to disrupt the function of intron-containing genes. Similarly, PEs could also enable gene silencing by interfering with mRNA splicing. A similar approach could help in the study of the function of long non-coding RNAs[193].

Altering miRNAs or miRNA target sites. MicroRNAs (miRNAs) bind to untranslated regions or coding sequences in target mRNAs. PEs can modify the miRNA-encoding gene sequence to generate customized miRNAs to match new target mRNAs. The tailored miRNAs could be repurposed to silence new targets, including the plant's endogenous genes or even genes in pests and pathogens[194] (Fig. 6g). PEs can also be used for introducing multiple synonymous mutations at miRNA-binding sites to destroy the homology with the miRNA and make the gene resistant to miRNA-mediated negative regulation (Fig. 6h). This strategy would result in enhanced gene expression. A recent study showed that BE-induced mutations in miRNA target sites in rice *SPL14* and *SPL16* genes elevated their expression twofold to fivefold[66]. miRNA–mRNA binding can also occur with a few mismatches. PEs are better suited in this regard compared with BEs for making an mRNA resistant to the miRNA by introducing multiple silent mutations.

BEs and PEs in crop improvement. Single-nucleotide variants and small indels have often been associated with mono- and polygenic agronomic traits and are crucial for crop improvements. With the availability of BE and PE tools, it became feasible to install those variants for rapidly developing superior crop varieties. Even though the base editing and prime editing technologies have numerous potential applications in improving yields, stress resistance, herbicide tolerance, resource use efficiency and quality of a diverse range of crops, here we discuss the applications that have been mostly experimentally demonstrated (Fig. 7).

Engineering herbicide resistance. Herbicide-resistant crops are highly desirable in modern agriculture for easily and effectively managing weeds. Until recently, the development of herbicide-resistant crops relied on screening a large population of randomly mutagenized seeds or making transgenic plants expressing foreign genes. However, BEs and PEs offer a more effective and attractive way to develop herbicide resistance by precisely editing the endogenous genes coding for enzymes targeted by the herbicides. Naturally occurring point mutations in the target genes are known to confer resistance to the herbicides. BEs and PEs have been used to precisely install those naturally occurring point mutations (Fig. 7a). For example, BE-mediated acetolactate synthase (*ALS*) editing was achieved in rice[22,66,183,195], *Arabidopsis*[196], maize[197], wheat[27], watermelon[32] and rapeseed[39] to confer resistance to imidazolinone and sulfonylurea herbicides without a fitness penalty. Similar to *ALS*,

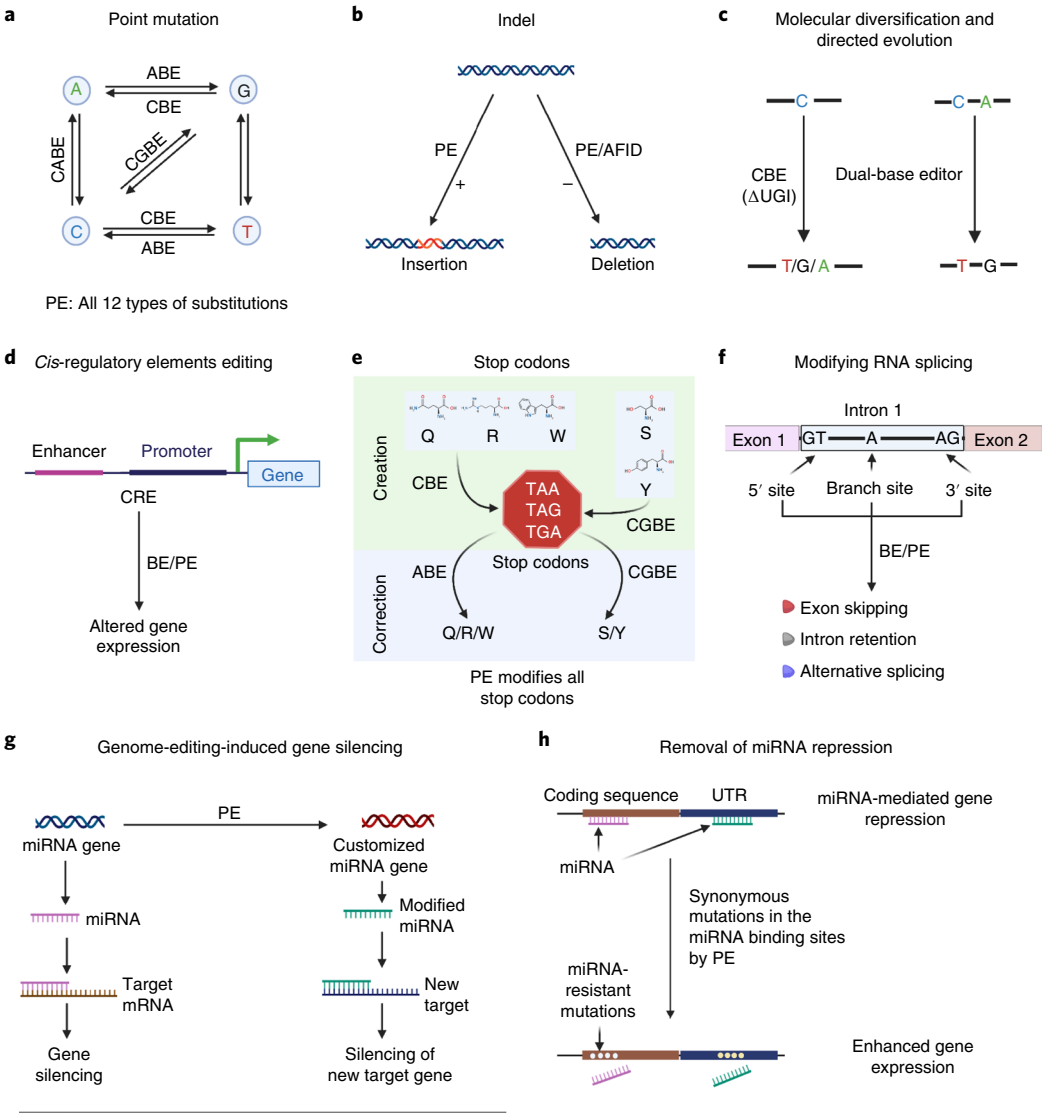

Fig. 6 | Base editing and prime editing applications for different kinds of genetic modifications. a, Four types of transition and two types of transversion mutations are possible with the currently available BE tools. The C-to-A BE (CABE) is applicable only in bacterial cells. PEs can perform all 12 possible types of base alterations. **b**, PE tools perform precise small insertions and deletions. AFID generates predictable deletions. **c**, A CBE architecture that lacks UGI generates diversifying edits from target C bases. A dual-base editor is better for generating molecular diversity and directed evolution. **d**, BEs and PEs targeted to enhancer and promoter regions modify CREs to alter gene expression. **e**, BEs and PEs can be used for creating and correcting early stop codons—CBEs and CGBEs together can artificially generate early stop codons by modifying codons for five amino acids. ABEs and CGBEs transmute premature stop codons to sense codons. PEs are applicable to execute all possible correction and creation of stop codons. **f**, The splice donor, acceptor and branch point can be edited using BE and PE tools for modifying splicing. **g**, miRNA genes could be modified by prime editing to customize miRNA and repurpose it to silence any endogenous target gene. **h**, PEs can be used to introduce multiple mutations to the miRNA-binding sites of a target gene sequence. For a protein-coding sequence, synonymous mutations need to be installed. The resultant mRNA from the edited gene will be resistant to miRNA-mediated negative regulation. This would enhance gene expression. Q, glutamine; R, arginine; S, serine; W, tryptophan; Y, tyrosine; UTR, untranslated region.

several other crucial metabolic genes are targeted by herbicides. Base editing of rice ACCase for haloxyfop-*R*-methyl[103,198], wheat ACCase for quizalofop[27], rice TubA2 for trifluralin[109,199], rice EPSPS for glyphosate[66], rice GS2 for glufosinate[109] and *Arabidopsis* CESA3 for C17 (ref. [200]) resistance have been reported.

Similarly, crucial amino acid residues in the ALS and ACC1 enzymes have been altered using prime editing in rice to develop resistance to bispyribac sodium and haloxyfop-*R*-methyl herbicide, respectively[151,155]. The ability of BE and PE tools to generate transgene-free herbicide tolerance traits by modifying one or a few bases in endogenous genes makes them enormously appealing to researchers and breeders.

Modulating nutritional composition. The nutritional attribute is a vital factor that determines consumer preference for food crops. The eating and cooking quality of rice largely depends on its amylose content. The waxy gene controls the biosynthesis of amylose. CBE-induced base editing was used to fine-tune amylose content in rice by altering three crucial amino acid residues of WAXY protein[201] (Fig. 7b). Similarly, CBE-mediated editing of the amylose biosynthesis gene in potato was reported[31]. For fruit, the taste is the key factor for consumer choice, although it is neglected in modern yield-directed breeding programs. Sugar content largely contributes to the taste of fruits. Interestingly, CBE-mediated editing at the uORF of a transcription factor gene, *ZIPs1.1*, generated strawberry

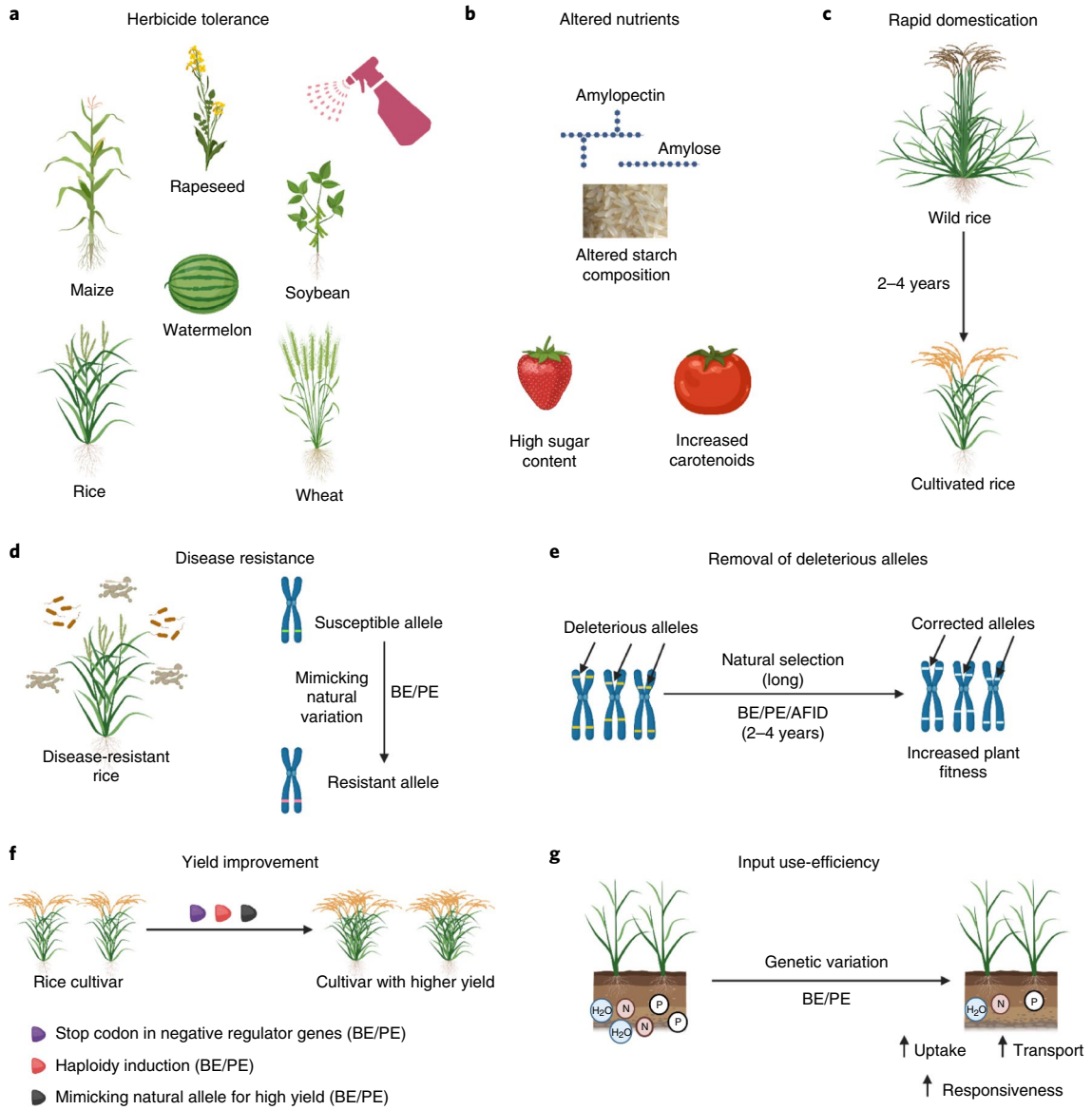

Fig. 7 | Examples of crop improvement by base editing and prime editing. Although BEs and PEs are applicable to numerous crop improvement strategies, only a few are highlighted here based on the already demonstrated examples. **a**, BEs and PEs can be used to modify amino acids in enzymes that are targeted by herbicides. **b**, Nutrient composition is altered by modifying biosynthetic pathway genes, splice sites or uORFs. **c**, Domestication genes are edited with BEs and PEs in wild relatives of crop species. **d**, BEs and PEs can be used to modify a susceptible allele into a resistant allele. **e**, The BE, PE and AFID systems can substantially reduce the time required for the removal of deleterious alleles. **f**, Endogenous genes are targeted by BEs and PEs for yield increment. **g**, BEs and PEs can be used to introduce variation into genes that are responsible for nutrient uptake, transport and utilization.

lines with higher sugar content[36]. Tomato mutants generated by a CBE displayed enhanced accumulation of total carotenoids in fruits and pericarp[30]. Thus, BEs and PEs hold great promise in modulating the nutritional compositions of food crops by fine adjustment in their DNA sequence.

Aiding rapid de novo crop domestication. Historically, it takes hundreds or even thousands of years to transform a wild plant species into domesticated crops. Domestication has been mostly productivity oriented and has caused severe loss of genetic diversity. In the wake of climate change and the increasing demand for food, harnessing that lost diversity could have a profound role in climate resilience and global food security. Remarkably, the application of CRISPR–Cas9 has accelerated the domestication of wild tomato (*Solanum pimpinellifolium*) and ground cherry (*Physalis pruinosa*) through the editing of multiple known domestication genes[202–204]. In a recent effort to domesticate *Oryza alata*, a tetraploid wild rice species, a BE has been used to obtain a gain-of-function mutation in the Oa*IPA1* gene[205] (Fig. 7c). Note that any genome editing application requires genomic information of the plant and established genetic transformation methods. When it comes to wild species, these two factors become a major bottleneck. Yu et al. generated utilizable genome information and standardized genetic transformation protocol of *O. alata* before attempting any editing[205]. Nevertheless, it gives much hope that we would be able to substantially reduce the time required for domestication of a crop by using precise genome editing technologies such as BEs and PEs with the help of whole-genome sequencing and genome annotation.

Engineering disease resistance. Breeding for disease resistance usually requires introgression of a resistance gene from a resistant source to a susceptible one. Plant susceptibility (*S*) genes help in the establishment of pathogens and could negatively modulate host defences[206]. Disruption of those genes should enhance resistance. The host eukaryotic translation initiation factors (eIFs) such as eIF4E and eIF4G are vital to virus infections[207]. Previously, CRISPR–Cas9-mediated genetic knockout of *eIF4E* or *eIF4G* was applied to create resistance to turnip mosaic virus in *Arabidopsis* and tungro spherical virus in rice, as well as broad-spectrum virus resistance in cucumber[207]. Similarly, *S*-gene knockout strategies can be used for developing resistance against fungal and bacterial pathogens[208]. However, the creation of null alleles of *S* genes may not be most desirable due to the potential fitness cost of losing a functional gene in the plant genome. Learning from a naturally occurring eIF4E virus-resistance allele in *Pisum sativum*, Bastet et al. introduced a C-to-T point mutation into the sequence encoding *Ate*IF4E1 for a N176K substitution, which successfully resulted in the generation of potyvirus (CIYVV)-resistant *Arabidopsis* lines[17]. Interestingly, recessive Pi36 and Pid2 (blast R genes) alleles were converted to dominant resistant alleles in rice with a CBE[47,50]. Os*FLS2*, a flagellin-sensing receptor kinase gene, was base-edited to convert a phosphorylation-blocking residue into a phosphorylation-mimicking residue[50] (Fig. 7d).

Removing deleterious mutations. Conventional breeding is lengthy and may incorporate undesirable traits along with the desired trait gene, a phenomenon known as linkage drag. Many plant genes are allelic in nature and some variants contain deleterious mutations, which differ in single or a few bases. Those deleterious point mutations and small variations could be precisely removed using BEs and PEs to rapidly alter a harmful allele into a beneficial one without any linkage drag (Fig. 7e).

Increasing yield and nutrient use efficiency. Improving crop yield with a minimal eco-footprint is the need of the hour to meet the global food demand sustainably. CBE-mediated introduction of early stop codons in three rice genes (*GS3*, *GW2* and *GN1a*), which are known to regulate grain parameters negatively, resulted in an increased grain length and grain width[66]. Similarly, a causal point mutation in the Os*SPL14* gene, which is responsible for defining ideal plant architecture and improving rice grain yield, was installed by ABEs[24,102]. Haploid induction is highly valuable in plant breeding applications for varietal improvement. For developing haploid inducer lines, wheat *MTL* loss-of-function mutants were generated using a CBE[23]. Crops with increased input use-efficiency could substantially reduce the ecological impact of agriculture. A single-nucleotide variation in Os*NRT1.1B*, which is associated with high nitrogen use-efficiency, has been installed by a CBE[59]. It is indicative that both BEs and PEs could be beneficial in breeding applications focused on improving yield and input use-efficiency (Fig. 7f,g).

Conclusions

The plant genome editing revolution has provided numerous opportunities for functional genetic studies and crop breeding that have not been seen before[209,210]. The precise genome editing technologies such as BEs and PEs described in this Review are still under rapid evolution for improved efficiency, enhanced specificity and capability, and refined editing purity. These emerging technologies have been demonstrated in a growing number of plant species with diverse applications. BEs and PEs would certainly help to develop superior varieties with enhanced yields, improved nutritional composition, wide adaptability across environments and increased agricultural input use-efficiencies.

Tissue-culture-based genetic transformation techniques are predominantly used to deliver the editing reagents and regenerate edited plants. Tissue culture is efficient for only a limited number of species and genotypes[211]. Overcoming the bottlenecks of reagent transformation and regeneration would ensure a broader application of BEs and PEs for crop improvement. For realizing the full potential of BEs and PEs, social acceptance and ease of regulations of crops generated through these new breeding tools are vital. The fruits of these technologies would be visualized quicker in the countries that apply product-based regulatory approaches compared with countries that are proponents of process-based regulations. Undoubtedly, the development and deployment of these technologies will continue to open unprecedented research fronts in plant science and crop breeding.

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Author contributions

Y.Q. led the project planning. K.A.M., S.S., K.C.B. and Y.Q. wrote the manuscript. K.A.M. and S.S. prepared the figures and tables. All of the authors read and approved the final manuscript.

Competing interests

Y.Q. is a consultant for Inari Agriculture and CTC Genomics, companies that use genome editing tools in plants. The other authors declare no competing interests.

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