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1 Introduction

The genome editing field has changed rapidly since the development of the first site-directed nucleases (SDNs) and zinc finger nucleases (ZFNs) in 1996 (Kim et al., 1996). Since this time, many tools have been developed that allow for the targeted change of genetic sequences, the most broadly used being CRISPR/Cas9 (Jinek et al., 2012). SDNs have allowed researchers to easily target sequences within the genome and introduce changes in a very specific manner across a broad range of organisms, including plants (Feng et al., 2013). The use of SDNs has led to a diverse array of new phenotypes in plants in the short time since their introduction.

The focus of early genome editing was mostly on gene knockouts, which are easily achieved by targeted nucleases. SDNs form double-strand breaks (DSBs) that are repaired by the host's native repair machinery. This typically results in a return to the original genomic sequence, or an insertion or deletion

http://dx.doi.org/10.19103/AS.2020.0082.06

Chapter taken from: Willmann, M. R. (ed.), *Genome editing for precision crop breeding*,
Burleigh Dodds Science Publishing, Cambridge, UK, 2021, (ISBN: 978 1 78676 447 8; www.bdspublishing.com)

(indel) at the genomic break site – although other repairs such as in-frame substitutions are possible. The mechanism of DSB repair, reviewed in Schmidt et al. (2018), is not fully understood. Patterns can be observed, often thought to be a result of microhomology at each end of the cut site, which causes the final repair outcome to be nonrandom (van Overbeek et al., 2016). Researchers often rely on the repair pattern that is commonly associated with a given nuclease, that is, single-base insertions with Cas9 (Allen et al., 2019), to inform a target location that will result in their desired outcome. For knockouts, the target region is generally in the 5′ end of a gene with the hope that the repair results in a frameshift that produces a premature stop codon, and thus loss of the gene product. Computational tools have been developed to attempt to predict these editing outcomes, reviewed in Molla and Yang (2020); however, plants obtained after editing with SDNs will still contain an array of editing outcomes.

While SDNs can be very precisely targeted to chosen genomic regions, making specific changes, such as polymorphism conversions, is difficult due to the still developing understanding of repair outcomes. Previously, such targeted conversions have been attempted using homology directed repair (HDR) strategies; however, the editing frequency with HDR has been extremely low and thus extremely resource intensive. The advent of base editors addressed the need for targeted conversion by combining a fully or partially deactivated nuclease with a deaminase domain. The deaminase domain causes deamination of bases exposed by the deactivated nuclease which are then converted to alternative bases by the cells' native repair machinery. Base editors convert nucleotide bases within an editing window, defined by the structure of the complex at the target site, at a specific genomic location defined by the nuclease targeting component. Depending on the deaminase used, the most common conversions are cytosine to thymine (C>T) and adenine to guanine (A>G), with multiple conversions being possible within an editing window that varies with each deaminase and nuclease used. While base editors are relatively new in the field of plant genome editing, they represent a rapidly growing toolset for predictable genome editing outcomes.

The RNA-guided CRISPR/Cas9 nuclease broadened the toolkit of targeted genome editing. The Cas9 protein contains two nuclease domains, the RuvC domain that cleaves the PAM-containing strand, and the HNH domain that cleaves the PAM-complement DNA. Either domain can be inactivated by a single amino acid mutation. Nuclease dead Cas9 (dCas9) contains a D10A mutation in the RuvC domain and a H840A mutation in the HNH domain retains sequence-specific DNA-binding activity (Jinek et al., 2012). The first base editor, (BE), fused a cytidine deaminase domain to nuclease dead Cas9 to enable delivery of the cytidine deaminase to sequence-specific sites in the genome. The next design (BE2) included a uracil DNA glycosylase inhibitor (UGI) domain, and

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dCas9 was replaced by Cas9 (D10A) nickase in BE3, to enhance the desired editing outcome. With these improvements, BE3 achieved an average of 37% editing efficiency across six loci in human cells. Further base editor architecture optimization resulted in BE4, which contained altered protein linkers and an additional UGI domain, and BE4-Gam, which additionally is fused to a bacteriophage MU protein, to improve editing efficiency and product purity (Komor et al., 2017). Following cytosine base editor (CBE) success, an adenosine base editor (ABE) was developed by replacing the cytidine deaminase with an engineered DNA adenine deaminase domain derived from multiple rounds of directed evolution and protein engineering (Gaudelli et al., 2017). Both CBE and ABE function with limited editing windows, and editing efficiency varies from locus to locus. In general, BE3 and ABE7.10 yield the highest editing efficiency, with an editing window from positions 4–8 and 4–7, respectively (counting the PAM as position 21–23).

The mechanisms of the CBE and ABE are similar, with both editors producing a deamination event that is ultimately resolved into an alternate base. To use BE3 as an example (Fig. 1), once the editing complex is delivered to the Cas9 binding site, the cytidine deaminase converts any cytidine base in the editing window, on the PAM-containing strand to uracil, creating a U:G mismatch. Which cytidine bases are targeted is mostly determined by the amount of ssDNA exposed and accessible to the deaminase domain by the deactivated nuclease. This mismatch can be repaired back to C:G or converted to U:T. In the design of BE3, the UGI domain inhibits repair of U back to C, and the nick on the PAM-complement strand stimulates replacement of G with A, using the PAM-containing strand that now contains a U as the template, hence a C:G to T:A conversion (Komor et al., 2016). In the case of the ABE, any adenine base in the editing window is first converted to inosine which will be converted to G following repair (Gaudelli et al., 2017).

2 Progress in mammalian systems

Base editing was initially designed specifically with therapeutic intent. As such, significant effort has been applied to optimize base editors for this purpose – resulting in a plethora of design variety which can be informative to base editing in plants. Base editing application in the human genome toward therapeutic benefits requires high specificity of the editing tool to edit the intended C or A at high efficiency while avoiding changes in bystander targets or off-targets. Introduction of four point-mutations (N497A, R661A, Q695A and Q926A) to Cas9 was shown to improve Cas9 binding specificity by eliminating nonspecific interactions between Cas9 and the target stand (Kleinstiver et al., 2016). The same mutations are also demonstrated to improve base editor specificity, leading to development of high-fidelity base editor (HF-BE3) (Rees et al., 2017).

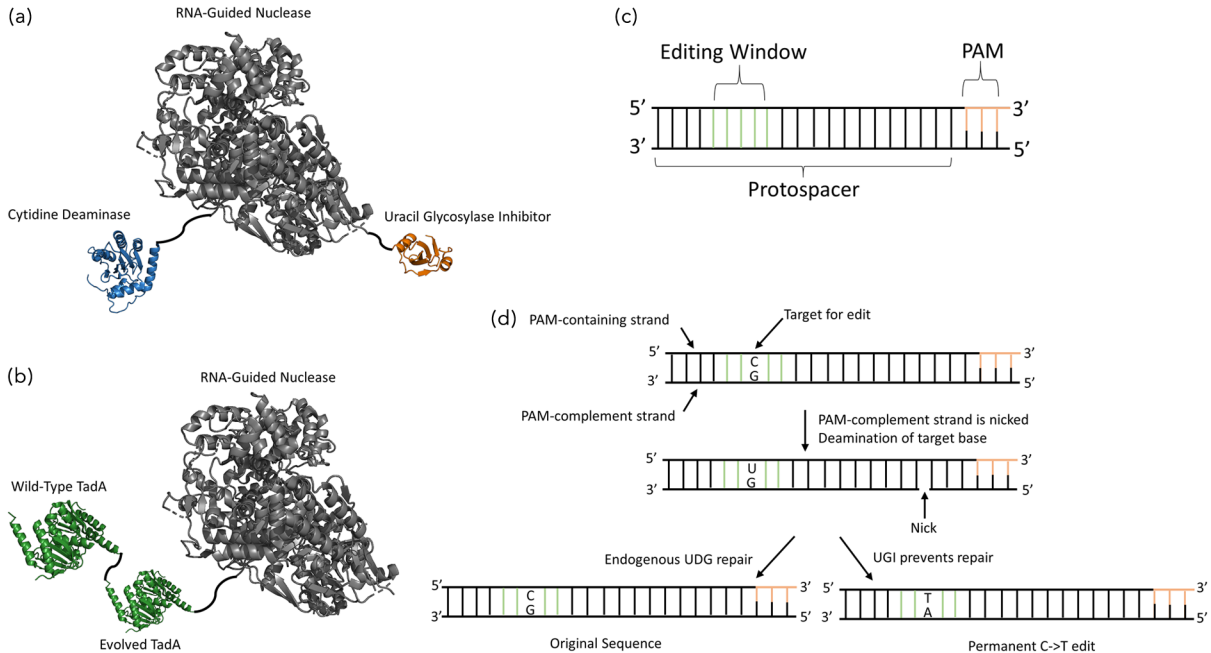

Figure 1 (a) An example of a C>T base editor composed of three parts. A cytidine deaminase (blue, PDB ID: 4XXO) and a uracil glycosylase inhibitor (UGI, orange, PDB ID: 1UGI) are fused to a deactivated RNA-guided nuclease such as SpCas9 (grey, PDB ID: 4CMP) (Bohn et al., 2015; Jinek et al., 2014). (b) An example of an A>G base editor composed of three parts. A wild-type TadA monomer and an engineered TadA monomer (green, PDB ID: 1Z3A) are fused to a deactivated RNA-guided nuclease such as SpCas9 (grey, PDB ID: 4CMP) (Jinek et al., 2014; Kim et al., 2006). (c) Example of a base editing window for a Cas9 cytosine base editor. The editing window is indicated in green, and the protospacer adjacent motif (PAM) is indicated in orange. (d) A simplified scheme of cytosine base editing. The PAM-complement strand of DNA is nicked by the nuclease, and the target cytosine is deaminated to uridine by the cytidine deaminase. Two options are then possible: (1) endogenous DNA repair machinery UDG can repair the DNA, resulting in the original sequence; (2) UGI prevents repair, resulting in a permanent C>T edit.

Cas9 variants that recognized different PAM sequences were also explored to expand target accessibility by base editors (Huang et al., 2019; Liu et al., 2019; Wang et al., 2019b; Zhang et al., 2017, 2020). In addition to the APOBEC1-based base editor, a variety of cytidine deaminases can be used to achieve flexible editing windows (Cheng et al., 2019). An engineered human APOBEC3A with higher sequence specificity is shown to effectively reduce bystander mutations when more than one C is present within the editing window (Gehrke et al., 2018). Continuous directed evolution has been applied to both the APOBEC domain and the Cas9 domain to achieve higher expression and activity (Wang et al., 2018a), as well as to broadened PAM compatibility and increased DNA specificity (Hu et al., 2018; Thuronyi et al., 2019). Editing window and editing efficiency details of various base editor versions is out of scope of this chapter; therefore, we direct interested readers to other papers (Kleinstiver et al., 2019; Rees and Liu, 2018).

In addition to engineering protein components of base editors, delivery methods and protein recruitment strategies are also effective ways to improve editing activity and specificity. For example, delivery of the base editor and sgRNA as ribonucleoprotein (RNP) complexes reduced off-target activity compared to plasmid delivery, while maintaining the same on-target activity level (Rees et al., 2017). Additionally, free UGI co-transfection with BE3 increased the ratio of desired base conversions over undesired indels (Wang et al., 2017). Further, instead of a direct fusion between Cas9 and the deaminase, GCN4 peptides fused to nCas9 allow recruitment of multiple copies of scFv-fused APOBEC-UGI-GB1 to achieve a broadened editing window (Jiang et al., 2018). Similar multi-copy recruitment of deaminases can also be achieved using RNA aptamers such as MS2 stem loops (Hess et al., 2016).

Base editing success in human cell laboratory lines, such as commonly used HEK293T cells, inspired application of these tools in other cell types and organisms. Nuclear localization signal and codon optimization (Koblan et al., 2018), linker optimization (Tan et al., 2019) and protein engineering (Liang et al., 2017) have proven to be effective ways of tuning editing efficiency and fidelity to the cell type of interest. The body of work in various human and animal cells provides useful guidance for adapting base editing tool to plant systems.

3 Cytosine base editing in plants

Different versions of CBEs have been applied to, and optimized for, targeted base modifications in plants. Table 1 provides an editing efficiency overview of base editor variants applied in plant gene editing to date. In contrast to the focus on specificity in therapeutic applications, plant base editing work has to date focused on increasing CBE activity.

Table 1 Examples of base editing in plant systems. Less accurate analysis methods, such as Sanger sequencing, are specifically noted

Species	Editor	Architecture	Frequency	Notes	Citation
Arabidopsis	Cytosine	BE3	2%	Analysis of T1 plants	(Chen et al., 2017)
Arabidopsis	Cytosine	BE3	2-8%	Sanger sequencing of T1 plants	(Xue et al., 2018)
Arabidopsis	Cytosine	Target-AID	50%	Measured in T1 plants	(Bastet et al., 2019)
Cotton	Cytosine	BE3	26–58%		(Qin et al., 2020a)
Maize	Cytosine	BE3	14%		(Li et al., 2019)
Maize	Cytosine	BE3	10%		(Zong et al., 2017)
Potato	Cytosine	PmCDA1	25%		(Veillet et al., 2019b)
Potato	Cytosine	A3A	7%		(Zong et al., 2018)
Rice	Cytosine	BE1	9%	Frequency estimated from Sanger sequencing	(Lu and Zhu, 2017)
Rice	Cytosine	BE3	0–80%	Tested with multiple Cas9 variants	(Hua et al., 2019)
Rice	Cytosine	BE3	25–89%	Includes efficiency increase due to sgRNA modifications	(Qin et al., 2020b)
Rice	Cytosine	BE3	5–72%	Tested with multiple Cas9 variants	(Qin et al., 2020a)
Rice	Cytosine	BE3	26%	Tested with Cas9-NG variant	(Endo et al., 2019)
Rice	Cytosine	BE3	40–65%		(Jin et al., 2019)
Rice	Cytosine	BE3	43%		(Zong et al., 2017)
Rice	Cytosine	BE3	2–44%	Measured with restriction enzyme test, not sequencing	(Li et al., 2017)
Rice	Cytosine	BE3	0–20%	Frequency estimated from Sanger sequencing	(Xu et al., 2019)
Rice	Cytosine	BE4	0–55%	Tested with multiple Cas9 variants	(Zeng et al., 2020)
Rice	Cytosine	BE4max	17–83%	Includes Anc689 as deaminase domain, tested with multiple Cas9 variants	(Wang et al., 2019a)
Rice	Cytosine	rBE5	8–73%	Uses hyperactive hAID mutant	(Ren et al., 2018)
Rice	Cytosine	PmCDA	2–9%	Tested with multiple Cas9 variants	(Qin et al., 2020b)
Rice	Cytosine	PmCDA1	38–50%		(Shimatani et al., 2017)
Rice	Cytosine	PmCDA1	0–90%	Tested with multiple Cas9 variants	(Wu et al., 2019)

Rice	Cytosine	PmCDA1	5-100%	Frequency estimated from Sanger sequencing	(Xu et al., 2019)
Rice	Cytosine	PmCDA1	10–50%	Single transcript of CBE coding sequence and sgRNA	(Tang et al., 2019)
Rice	Cytosine	PmCDA1	30–45%	Tested with Cas9NG variant	(Zhong et al., 2019)
Rice	Cytosine	Target-AID	19–85%	Includes efficiency increase due to sgRNA modifications	(Qin et al., 2020b)
Rice	Cytosine	A3A	44%		(Zong et al., 2018)
Tomato	Cytosine	PmCDA1	12–81%		(Veillet et al., 2019b)
Watermelon	Cytosine	BE3	23%		(Tian et al., 2018)
Wheat	Cytosine	BE3	3%		(Zhang et al., 2019)
Wheat	Cytosine	BE3	1%		(Zong et al., 2017)
Wheat	Cytosine	A3A	16–82%		(Zong et al., 2018)
Arabidopsis	Adenine	ABE 7.10	85%	Measured in T1 plants	(Kang et al., 2018)
Rice	Adenine	ABE 7.10	0–66%	Tested with multiple Cas9 variants	(Hua et al., 2019)
Rice	Adenine	ABE 7.10	0–62 %		(Yan et al., 2018)
Rice	Adenine	ABE 7.10	5–61%	Tested with multiple Cas9 variants	(Hua et al., 2018)
Rice	Adenine	ABE 7.10	7–63%	Tested with multiple Cas9 variants	(Qin et al., 2020b)
Rice	Adenine	ABE 7.10	3–46%		(Li et al., 2018)
Rice	Adenine	ABE 7.10	21–60%		(Jin et al., 2019)
Rice	Adenine	ABE 7.10	0–6.5%	Tested with multiple Cas9 variants	(Zeng et al., 2020)
Rice	Adenine	ABE 7.10	7–16%		(Hao et al., 2019)
Rice	Adenine	ABEmax	8–48%		(Wang et al., 2019a)
Rice	Adenine	Simplified ABE 7.10	6–96%	Modified to contain only single TadA domain	(Hua et al., 2020b)
Wheat	Adenine	ABE 7.10	0.4–1%		(Li et al., 2018)
Rice	Cytosine/Adenine	STEME-1	(0.49–15.10%) C>T + A>G		(Li et al., 2020)
Rice	Cytosine/Adenine	hAID*Δ/TadA*	24–28%	Mixed CBE and ABE vectors with guide libraries	(Kuang et al., 2020)

A major approach to increasing activity has been the substitution of deaminase variants, rAPOBEC1, hAPOBEC3A, PmCDA1 and hAID. The substitution of the rat ABOBEC1 subunit with human ABOBEC3A (A3A-PBE) (Zong et al., 2018), demonstrated in rice, wheat and potato, exemplifies successful CBE activity and editing window enhancement in plants. This optimized version comprises a codon-optimized N-terminal APOBEC3A fused to a SpCas9 D10A nickase and a C-terminal UGI flanked by two nuclear localization sequences (NLSs). Direct comparison of this optimized CBE to a plant-optimized BE3-version at multiple target sites in wheat, rice and potato showed a 11–13-fold higher C>T conversion efficiency. Moreover, the new A3A-PBE (plant base editor) had an extended 17-nt editing window compared to the limited 7-nt editing range of BE3. Through A3A-PBE application at different target sites in rice, wheat and potato, edited plants could be regenerated with editing frequencies ranging from 6.5% to 82.9%. Only very low indel frequency could be detected in protoplast assays and regenerated rice and potato mutants, demonstrating high A3A-PBE specificity. Another beneficial A3A-PBE feature is efficient C>T conversion independent of genomic context. In contrast to BE3, which showed no activity at three target sites in rice and wheat in a high CG context, editing efficiencies of up to 41.2% could be detected for A3A-PBE (Zong et al., 2018). Similarly, rAPOBEC1 substitution by PmCDA1 revealed higher editing efficiency at three out of four target sites in rice protoplast assays (Tang et al., 2019). While C>T conversions were below 10% using rAPOBEC1-nCas9-UGI, the nCas9-PmCDA1-UGI variant showed 10–50% editing efficiencies. However, the authors emphasized that the rAPOBEC1 sequence had not been codon optimized for this study; thus protein levels could contribute a substantial effect on editing efficacies (Tang et al., 2019). Similar results were obtained in transgenic rice calli with codon-optimized expression cassettes where rAPOBEC1 substitution by PmCDA1 led to 3–10-fold higher activity at five out of seven target sites (Xu et al., 2019).

To increase CBE efficiency on GC targets, a new variant was developed that carried a human AID triple mutant lacking a nuclear export signal (AID*Δ) (Ren et al., 2018). The deaminase domain was codon optimized for application in rice and fused to a nCas9 with no UGI domain (rBE5). This CBE variant functioned well on GC, AC, TC and CC target sites in rice protoplasts. Moreover, the AID*Δ-based CBE was able to efficiently catalyze C>T conversions at a genomic locus in transgenic rice lines that had been inaccessible for rAPOBEC1-CBE. It is worth emphasizing that 71.9% of transgenic lines also carried indel mutations in the target region. Further enzyme modification via UGI domain fusion reduced the indel ratio; however, frequencies were still higher than with rAPOBEC1-CBE (Ren et al., 2018).

Besides exchanging different CBE subunits, major improvements in C>T base conversion efficiencies were achieved by protein biosynthesis and nuclear

transport optimization, both of which had been identified as key base editing bottlenecks in human cells (Koblan et al., 2018). In rice, CBE efficiency could be increased through codon optimization, integration of bipartite nuclear localization signals and substitution of the deaminase domain by Anc689, an ancestor of rAPOBEC1 previously identified through ancestral sequence reconstruction using APOBEC homologs (Koblan et al., 2018; Wang et al., 2019a).

The fidelity of rAPOBEC1 (BE3) and PmCDA1 (CDA) CBEs was further improved by increasing UGI concentration *in planta* (Qin et al., 2020a). A DNA sequence encoding three UGI-NLS copies each preceded by the self-cleaving peptide, 2A, was attached to the 3′ end of codon-optimized BE3 and CDA sequences (eBE3 and eCDA, respectively) leading to a polycistronic transcript and increased UGI amounts after translation. C>T conversion and indel formation was analyzed at five different target sites in transgenic rice plants. While editing efficiencies for both CBE variants did not differ significantly at most target sites (25–58.8%), indel frequency was drastically reduced from 12.5% to 25.0% for BE3 to 0% for the optimized variant. In addition, unwanted base changes were considerably lower with the optimized enzyme design (0–3.5%, eBE3) than for conventional BE3 (2.5–14.7%). This result could be reproduced in rice plants transformed with eCDA (Qin et al., 2020b).

Interestingly, a study using PmCDA1 in rice and tomato in a configuration that did not contain a UGI domain observed not only C>T conversions but also C>G changes (Shimatani et al., 2017), suggesting that a larger array of changes may be possible with CBEs when the UGI domain is removed. Indeed, similar results have been observed in subsequent studies in which a larger diversity of outcomes are observed. For instance, a study in *Arabidopsis* observed C>T, C>G and C>A changes when a PmCDA1 editor was utilized without a UGI domain (Bastet et al., 2019). While this configuration results in a larger array of editing outcomes, undesired changes can be removed through subsequent segregation or backcrossing, thus providing further utility to CBEs beyond C>T changes.

It is very challenging to define the best CBE design for every plant species. Due to the different experimental designs and analyses of each individual study, no direct conclusions can be drawn. However, researchers have identified some key factors that are generally important for successful application of CBEs in plants. As can be seen above, the choice of deaminase can have a dramatic effect on editing efficiency. While the A3A and PmCDA1 deaminases appear to be more efficient than the original rAPOBEC1, further studies directly comparing each design are required to determine the optimal deaminase in plants. One major advancement of CBE designs for plants includes the optimization of coding sequences to increase CBE concentrations in vivo (Koblan et al., 2018). Moreover, nuclear import can be enhanced by integration

of two bipartite NLSs (Wang et al., 2019a). Finally, co-localization of additional UGI domains can increase CBE fidelity (Qin et al., 2020b).

4 Adenine base editing in plants

The recently developed adenine base editors have so far been applied in monocots (Hua et al., 2018, 2019, 2020b; Li et al., 2018; Wang et al., 2019a; Yan et al., 2018) as well as dicots (Kang et al., 2018). As with CBEs, various optimizations have been employed to achieve high gene expression and increase editing efficiencies in plants. Several ABE variants have been generated by fusion of evolved adenine deaminase heterodimer TadA-TadA*7.10 or TadA-TadA*7.8 to a SpCas9 nickase or a dead SpCas9 (Yan et al., 2018). However, only TadA-TadA*7.10 fused to nSpCas9 catalyzed desired A>G conversions in rice calli. Through substitution of nSpCas9 by its ortholog from *S. aureus* a new ABE version was created that efficiently generated desired base edits in rice and showed a broader base editing window (Hua et al., 2018; Qin et al., 2019). Moreover, replacement of SpCas9 by VQR-SpCas9 or Cas9-NG variants allowed efficient editing and multiplexing at NGA PAMs, which were not accessible with SpCas9-ABE (Hua et al., 2019; Zeng et al., 2020). Other optimizations of ABEs in plants focused on N- and C-terminal fusions of the ABE7.10 heterodimer to nSpCas9 and the number and position of nuclear localization signals (Li et al., 2018). Remarkably, integration of three NLSs into ABE led to significantly increased editing efficiencies in rice protoplasts compared to ABEs with only one or two NLSs. However, ABE variants in which TadA-TadA*7.10 was fused to the C-terminus of nCas9 were ineffective (Li et al., 2018). Further optimization of ABEs for application in plants was achieved through substitution of the TadA-TadA*7.10 heterodimer by the TadA*7.10 mutant, a strategy used for adenine base editing in bacteria (Gaudelli et al., 2017; Hua et al., 2020b). This simplified ABE version showed higher protein levels in rice calli and protoplasts, which was associated with an up to 1.9-fold increase of A>G edits at six out of seven target sites in two rice varieties (Hua et al., 2020b). Moreover, editing efficiencies of ABEs harboring different nCas9 variants (nSaCas9 and nSaKKHCas9) could be increased by adopting these enzymes to the simplified design (Hua et al., 2020b). Two major advantages of ABEs over CBEs are very low indel frequency of about 0.1% and precise base exchange in plants, respectively (Hua et al., 2018; Kang et al., 2018; Li et al., 2018).

5 Broadening protospacer adjacent motif (PAM) accessibility

One major limitation of targeted base modification with SpCas9-based tools is the prerequisite of an NGG PAM adjacent to the target site. To increase the number of accessible genomic sequences in plants, *S. pyogenes* Cas9 can be substituted by different enzyme variants (Qin et al., 2019). Extensive

expansion of Cas9 PAM recognition has been achieved via rational design as well as directed evolution (Hu et al., 2018; Kleinstiver et al., 2015; Nishimasu et al., 2018). In addition to Cas9-based editors, Cas12a-based editors have been developed that recognize T-rich PAM sequences (Kleinstiver et al., 2016; Li et al., 2018; Sanson et al., 2019). While not all PAM variants of Cas9 and Cas12a have been adapted and optimized for base editing, the diversity of available Cas DNA-binding platforms indicates that a base editor should be able to access nearly any base in a genome (Fig. 2).

Several Cas9 variants have been tested in plants and demonstrated to function in base editing configurations. For instance, VQR-SpCas9n using PmCDA1 as the cytosine deaminase broadened the accessibility of SpCas9 CBEs to reach some NGA PAMs, most notably NGAG in rice (Wu et al., 2019). The same study also found that SpCas9n using PmCDA1 as the cytosine

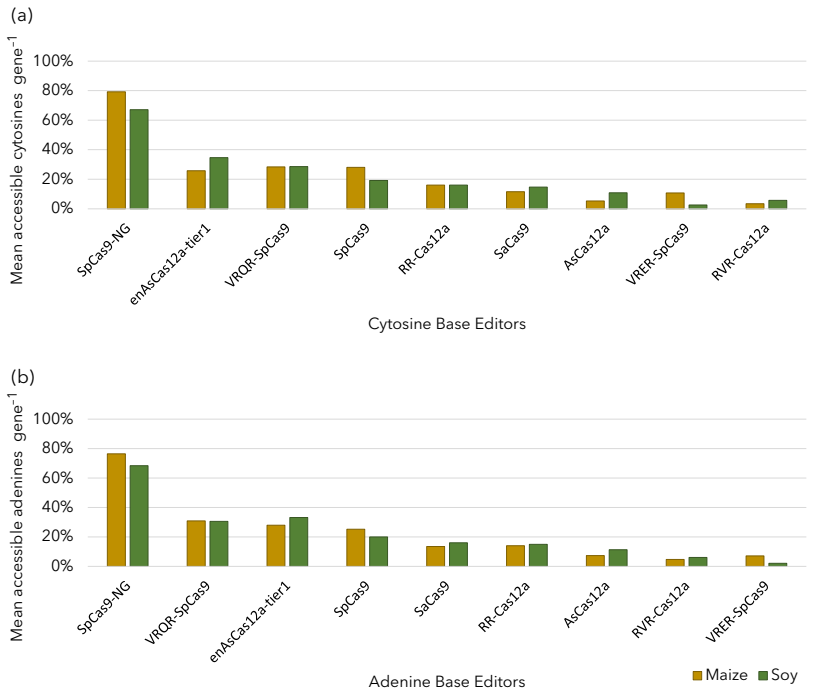

Figure 2 Base accessibility to CRISPR/Cas base editors in corn and soy genomes. (a) Mean cytosines accessible by various CRISPR/Cas cytosine base editors in maize and soy genes. (b) Mean adenines accessible by various CRISPR/Cas adenine base editors in maize and soy genes. Base editing window positions are as follows: SpCas9 and variants, 4 through 8; Cas12a and variants, 8 through 13; SaCas9, 2 through 12. The enAsCas12a-tier1 PAMs are defined in Kleinstiver et al. (2019). Base editing windows assume the use of rAPOBEC1 and ABE7.10 as the cytosine deaminase and adenine deaminase domains, respectively.

deaminase could effectively edit with an NAG PAM (Wu et al., 2019). Among the most promiscuous Cas9 variants is Cas9-NG, which recognizes noncanonical NG PAMs. In rice protoplasts, nCas9-NG-PmCDA-UGI showed high editing efficiency at most noncanonical PAMs, but low efficiency at NGG PAMs (Zhong et al., 2019). The strong deamination activity of PmCDA deaminase further complemented this broad PAM recognition with a wide editing window of 14 nt. The nickase xCas9 variant reported to have a NG PAM recognition fused to rAPOBEC1 demonstrated high editing efficiency at NGG PAMs comparable to nCas9-CBE, while a fusion to PmCDA1 increased C>T changes at C2 and C4 when compared in rice protoplasts (Zhong et al., 2019). However, other reports have shown xCas9 CBEs to be ineffective in rice (Zeng et al., 2020). In addition to Cas9-NG-CBE, an eCas9-NG variant CBE was developed to reduced off-target effects (Zeng et al., 2020). Both Cas9-NG-CBE and eCas9-NG-CBE enabled C-to-T changes at noncanonical PAM sites and increased the editing window width relative to Cas9-CBE.

Cas9 from other species also provide DNA-binding scaffolds to build base editors such as nSaCas9 and nSaKKHCas9 that recognize NNNRRT PAMs (Kim et al., 2017; Komor et al., 2017). Fusions were made with these Cas9 variants to rAPOBEC1 and either one or three copies of UGI. Three copies of UGI provided higher editing efficiency and increased the editing window at most target sites compared to the CBE with a single UGI moiety in rice (Qin et al., 2020b). However, replacing rAPOBEC1 with PmCDA1 in the nSaCas9 and nSaKKH CBEs resulted in lower editing efficiencies on average (Qin et al., 2020b).

6 Sequence diversification in crops

Crop improvements have long benefited from the introduction of genetic diversity and selective breeding. Chemical mutagens and radiation treatments have historically generated this diversity randomly in the genome. More targeted, semi-random indel diversity generation has been demonstrated in crops using CRISPR methods with dramatic phenotypic results from creating diversity in a known fruit size QTL and in promoters controlling inflorescence and plant architecture (Rodriguez-Leal et al., 2019). Similar spacer-dependent approaches in plant breeding with base editing would seem attractive. Base editing technologies also lend themselves to spacer-targeted sequence diversification within an editing window. Recently, simultaneous C and A base editing have been achieved in plants with effectors that incorporate both cytosine and adenine deamination activities in a single protein (Li et al., 2020). Pairing dual deaminases with NGG targeting Cas9 and with Cas9NG allowed access to 64% of all bases in a 1200-bp editing window and resulted in an overall 13% editing efficiency in rice. Moreover, C>G and C>A base changes

were observed in addition to the expected C>T and A>G changes, generating additional sequence diversity (Li et al., 2020).

7 Off-target base editing

Despite a frequent desire to increase diversity in plants, there has been increased interest in whether additional variation beyond the designed edit (off-target editing) has occurred. The primary focus has been on guide-RNA dependent effects which occur when a base editor binds to a DNA site with similar, but not identical, sequence to the target site. Several strategies have been developed to reduce guide RNA-dependent off-target editing with nucleases, which are also effective with base editors. These strategies include (1) careful spacer design and off-target analysis to limit similarity to repeat sequences, (2) utilizing CRISPR effectors with mutations that increase DNA specificity, (3) reducing duration of editing activity by delivering the editor as an RNP complex and (4) modifying the guide RNA (Kim et al., 2019; Rees et al., 2017; Yeh et al., 2018).

Findings to date demonstrate that the off-target variation induced by base editors is several orders of magnitude less than the variation found in standard breeding programs. Some alternative deaminase domains have been shown (Doman et al., 2020) to reduce off-target editing, as well as reduce bystander base editing because of a smaller base editing window, likely both caused by lower catalytic efficiency. To assess deaminase effects on guide RNA-independent off-target effects, the Gao Lab (Jin et al., 2019) analyzed genome-wide indels and single-nucleotide variants (SNVs) induced by cytosine (rAPOBEC1 deaminase) and adenine (ABE 7.10) base editors in stably transformed rice. The finding was that the CBEs do increase C>T SNVs with an average <2-fold higher than the transformation control (means of ~500–630 SNVs vs. ~350 SNVs, respectively), but that no other genome wide variations increased with treatment by either editor class. However, even the highest detected whole genome C>T variation (~700 SNVs) is similar to previously observed somaclonal mutation frequency range (440–2600 mutations per plant in rice) (Li et al., 2016) and well below another similar study (~20 000) (Zhang et al., 2014). Thus, while increased background variation results from the presence of deaminase domains, it is less than variation typically introduced through the transformation process used to introduce editors to plants and vastly dwarfed by the millions of variants in breeding pools (Darracq et al., 2018; Li et al., 2014; Springer et al., 2009; Wang et al., 2018b; Zhao et al., 2018).

In their unaltered states, even when fused to a CRISPR effector, some deaminases can demonstrate affinity for RNA. The Joung Lab (Grünewald et al., 2019) performed an extensive study in human cell lines showing that both BE3 (rAPOBEC1) and ABEmax (TadA::TadA*) edit tens of thousands of individual

RNA base positions. We are not aware of any published studies in plants examining potential off-target editing of RNA by base editors. Nonetheless, as the same base editing machinery is used in plants as in human cell lines it is likely that the RNA-editing phenomenon is likely occurring in plants while CRISPR/Cas base editors are expressed. However, as the effect would be limited to short-lived RNA transcripts and the editing distributed amongst the whole transcriptome, there is little risk associated with this phenomenon in plants.

The ability to selectively breed many agricultural crops allows for base editor gene segregation after a heritable edit has been generated, which could additionally segregate away unintended off-target DNA edits and would stop the RNA editing process. For crops that are vegetatively propagated, transient delivery of the gene editing reagents or other alternatives to removal through breeding-cycle segregation will likely be preferred. Secondly, selecting for phenotypically normal plants, which is central to agricultural breeding programs, is expected to automatically select against any potential ill effects to plant health. Identifying and eliminating off-types has always been central to plant breeding programs, and regulatory agencies consider this a reliable process for ensuring food safety (Tieman et al., 2017).

8 Current applications of base editors in crops

While conventional breeding relies on the random mutagenesis of the plant genome through application of mutagenic agents or radiation, SDNs and base editor development has, for the first time, facilitated breeders to precisely modify plant genomes. The added precision of base editors allows breeders to fine-tune the genome like-never-before. As a result, in the short time since their introduction, there are several examples of successful trait improvement through base editing.

Herbicide tolerance is a powerful trait for crop improvement. One challenge of generating transgene-free edited plants is the selection of edited plants from the pool of non-edited regenerants. Herbicide-resistant gene base editing enables regeneration of edited but transgene-free plants on herbicide selection medium while simultaneously introducing additional traits through multiplexing (Veillet et al., 2019b). In this regard, one prominent target is the acetolactate synthase gene (ALS), which encodes the first enzyme in the biosynthetic pathway of branch chained amino acids (McCourt and Duggleby, 2006). Specific amino acid substitution can create resistance to sulfonylurea and imidazolinone herbicides and was introduced into crops including rice, wheat, tomato, potato and watermelon (Zhang et al., 2019). Competing with crops for essential resources in the field, weeds are a major risk in modern agriculture (Oerke and Dehne, 2004). In this regard, herbicide treatment has become an essential agricultural technique and herbicide resistance

introduction into crops is an important commercial agronomic trait that has been demonstrated recently in rice through base editing for directed evolution of ALS (Kuang et al., 2020). In addition, herbicide-resistant rice plants could be generated through amino acid substitution via adenine base editing in the acetyl-coenzyme A carboxylase gene, a key enzyme in fatty acid biosynthesis (Li et al., 2018). Another example is the introduction of virus resistance by base editor application which has recently been shown in *A. thaliana* (Bastet et al., 2019). An amino acid substitution (N176K; caused by a C>G conversion), naturally occurring in pea (*Pisum sativum*), was introduced into *A. thaliana* translation initiation factor 4E by creating SNPs in the respective *eIF4E1* gene using a CBE lacking a UGI domain. Transgene-free T4 plants homozygous for desired edits were no longer susceptible to clover yellow vein virus (ClYVV). At the same time modified plants did not show any pleiotropic effects – in contrast to *eIF4E1* knockout mutants. The introduction of naturally occurring amino acid variations via base editing of susceptibility factors like *eIF4E* in relevant agricultural species offers great opportunities for crop improvement and control of economically devastating pests (Bastet et al., 2019). Manipulation and characterization of proteins involved in other traits, including quality, can be and have been performed (Veillet et al., 2019a).

Besides targeted amino acid residue modification of proteins, base editing tools can be deployed to modify mature mRNA architecture. In eukaryotes, precursor mRNA is processed to mature mRNA by spliceosomes that connect exons through removal of introns (Reddy et al., 2013). In plants, alternative splicing events occur in more than 60% of intron-containing genes and play an important role in increasing protein diversity (Reddy et al., 2013). Manipulation of mRNA splicing outcomes through introduction of SNPs via base editing enables functional investigation of specific protein variations while gene expression is still regulated by the endogenous promoter. Splicing site modification has been achieved in *Arabidopsis* and rice by application of cytosine and adenine base editors (Kang et al., 2018; Li et al., 2017; Xue et al., 2018). In *Arabidopsis*, specific splicing outcomes were enhanced while others could be inhibited through mutation of splicing sites by CBEs (Kang et al., 2018; Li et al., 2017; Xue et al., 2018). In this way, functions of HAB1 and RS31A protein variants contributing to ABA hypersensitive and genotoxic tolerant phenotypes, respectively, could be analyzed in planta (Kang et al., 2018; Li et al., 2017; Xue et al., 2018).

9 Conclusion

In a short period, base editors have been widely adopted as gene editing tools in plants. While early development of base editors occurred in non-plant systems, in planta editing has been shown to often be efficient (Table 1). Refinements

for plant systems, such as codon optimization and plant-specific subcellular targeting sequences, have increased efficiency. To date, both adenine and CBEs have been demonstrated in plants with efficiencies over 80% and continue to be optimized. High-efficiency editing has been demonstrated in both monocot and dicot species showing that base editing should be possible in any transformable plant system. As can be seen in Fig. 2, accessibility varies depending on a given species genetic context, so base editor utility in plants is further increased by combining deaminase domains with CRISPR/Cas variants recognizing diverse PAM profiles.

A discussion on unintended, or off-target, editing often accompanies any conversation about genome editing; however, it is important to consider the use of editing tool in the context of its application. In plants, where increasing variation is central to the breeding process, background variation introduction is of little concern – especially in comparison to therapeutic uses in mammalian applications. Additionally, as base editors rely on the same RNA-guided machinery as traditional CRISPR/Cas nucleases, they maintain the same high-DNA specificity as SDNs, and off-target modification should be similarly low. The deaminase domain addition, which is specific to base editors, does add additional complexity as these domains have been shown to increase variation in both DNA and RNA sequences after delivery (Jin et al., 2019). While this would be a major concern in therapeutic applications, the variation extent observed is in line with traditional plant breeding practices and any off-types would be removed by standard agricultural breeding programs.

Recently, the development of prime editors (Anzalone et al., 2019) has shown promise as an additional tool that can result in predictable editing outcomes. While base editors are limited to changes of specific bases within a defined editing window, prime editors can replace entire portions of sequence in a base-independent manner, and with editing windows that are potentially dozens of bases long. While prime editors have the potential to be extremely powerful tools for gene editing, their efficiency in plants has thus far been low (Hua et al., 2020a; Lin et al., 2020). While some improvement in efficiency can be observed through the use of a surrogate selection system conferring herbicide resistance following a successful edit (Xu et al., 2020), it is clear that much more work needs to be done to increase the efficiency of this system to be widely applicable to plant applications.

The application of base editors to plant-specific applications has, to date, been limited. However, many aspects of the tools give base editors a great potential in plant gene editing. First, unlike traditional SDNs where the editing result is often a frame shift, base editors make nucleotide changes while maintaining the native reading frame. The result is a tool that is much better suited to sequence diversification for amino acid alterations. Additionally, the change that occurs as a base editing result is predictable, meaning that

precisely targeted alterations can be recovered. By combining adenine and CBEs together, sequence diversification can be attempted – in a targeted and predictable manner. Looking forward, base editing has the potential to be a powerful tool in the plant breeding toolbox.

10 Where to look for further information

For an introduction to genome editing in plants Wada et al. (2020) give a comprehensive overview of the current state of the field. Rees and Liu (2018) provide a comprehensive review of base editing technology and is an excellent starting resource on this specific topic. The papers listed in Table 1 are the best starting points for base editing papers on a specific plant species.

Conferences on genome editing or DNA repair are often the best sources to find the latest developments in the field, some recurring meetings of note:

- International Conference on Base Editing (Deaminet).
- CRISPR and Plant Genome Editing Conference.
- Plant Genomics and Gene Editing Congress.

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