Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Getting better all the time — recent progress in the development of CRISPR/Cas-based tools for plant genome engineering

Niklas Capdeville, Patrick Schindele and Holger Puchta

Since their first adaptation for plant genome editing, clustered regularly interspaced short palindromic repeats/CRISPR-associated system nucleases and tools have revolutionized the field. While early approaches focused on targeted mutagenesis that relies on mutagenic repair of induced double-strand breaks, newly developed tools now enable the precise induction of predefined modifications. Constant efforts to optimize these tools have led to the generation of more efficient base editors with enlarged editing windows and have enabled previously unachievable C–G transversions. Prime editors were also optimized for the application in plants and now allow to accurately induce substitutions, insertions, and deletions. Recently, great progress was made through precise restructuring of chromosomes, which enables not only the breakage or formation of genetic linkages but also the swapping of promoters.

Address
Karlsruhe Institute of Technology (KIT), Botanical Institute, Chair Molecular Biology and Biochemistry, Fritz-Haber-Weg 4, 76131 Karlsruhe, Germany

Corresponding author: Holger Puchta (holger.puchta@kit.edu)

Current Opinion in Biotechnology 2023, **79**:102854

This review comes from a themed issue on **Plant Biotechnology**

Edited by **Alisdair Fernie** and **Jianbing Yan**

https://doi.org/10.1016/j.copbio.2022.102854

Introduction

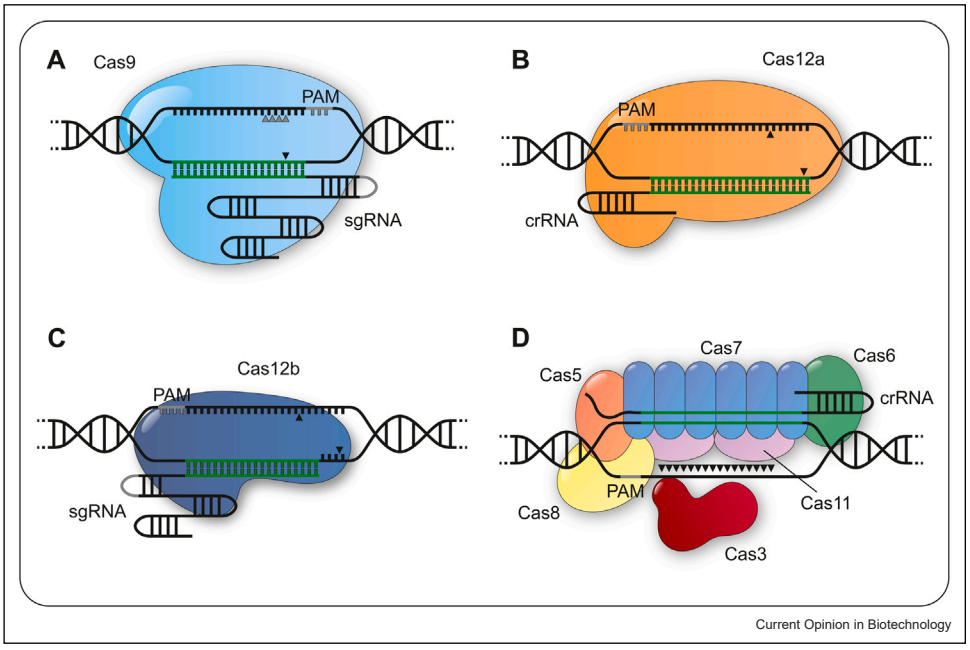
In view of the growing challenges in the cultivation of crops, the demand for the breeding of new cultivars by either optimizing existing plant lines or domesticating wild varieties is increasing. A central prerequisite is access to a wide range of different traits and trait expression, which requires a high number of different genotypes. The development of programmable nucleases and genome engineering tools has greatly accelerated the target-specific induction of such genetic variability. Especially, the adaptation of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system has revolutionized targeted mutagenesis through error-prone repair of induced double-strand breaks (DSB). The characterization of various natural CRISPR/Cas nucleases and their optimization through protein engineering have made almost any site of the plant genome accessible. Furthermore, tools for the modification of gene expression and chromatin status have been developed based on catalytically dead Cas enzymes (for review see [1]).

However, many agronomically important traits are conferred by only a few specific base changes that cannot be induced using the regular toolkit. Thus, new tools were developed enabling the induction of precise and predefined genomic alterations. Another bottleneck is the inheritability of traits. Recent progress in the restructuring of whole chromosomes promises the possibility to break and generate genetic linkages in a targeted matter as well as to influence local recombination patterns. In regard to the high significance of such sequence changes, this article will focus on technological advances in the manipulation of DNA sequences achieved since the publication of the last review on the same topic in this series [2].

Expanding the CRISPR/Cas toolbox with new nucleases

In contrast to class-I CRISPR/Cas systems, class-II systems are characterized by a single effector protein and, thus, have long been the major systems applied for genome editing approaches. Many efforts were put into the optimization of especially the type-II Cas9 and the type-V Cas12a effectors. In order to bypass one of the main restrictions, the requirement of a protospacer-adjacent motif (PAM), a number of novel Cas9 and Cas12a variants were developed. Recently, the group of Yiping Qi generated SpMacCas9, as well as an improved variant (iSpMacCas9), by transferring the PAM-interacting domain of Cas9 from *Streptococcus maccacae* to the most commonly used Cas9 ortholog from *Streptococcus pyogenes* (SpCas9), enabling efficient editing of 5′-NAA-3′-flanked sequences in plants [3,4]. The broadest PAM recognition in plants was obtained by combining the two variants SpCas9-NG and xCas9, resulting in XNG-Cas9

Figure 1



Representative structures of CRISPR/Cas systems. **(A)** Class-II type-II CRISPR/Cas9 system. Cas9 uses either a CRISPR-RNA (crRNA) together with a transactivating crRNA (tracrRNA) or a synthetic single-guide RNA (sgRNA) to identify its target sequence. It cleaves the DNA 3 bp upstream of its G-rich PAM sequence on the target strand and 3–6 bp upstream on the nontarget strand, resulting in either a blunt-ended DSB or up to 3-nt-long 5′ overhangs. **(B)** Class-II type-V-A CRISPR/Cas12a system. Cas12a naturally only needs a crRNA to identify its target sequence and cleaves the DNA 18 bp and 23 bp downstream of the T-rich PAM sequence on the nontarget and target strand, respectively. **(C)** Class-II type-V-B CRISPR/Cas12b system. Cas12b is significantly smaller than Cas9 or Cas12a and combines characteristics of both. Like Cas9, it needs an additional tracrRNA that can be fused with the crRNA to form a sgRNA. However, similarly to Cas12a, it recognizes T-rich PAM sequences and induces a staggered DSB with 5–6-nt 5′ overhangs. **(D)** Class-I type-I-E CRISPR/Cas3 system. Unlike class-II systems, target binding and cleavage in class-I systems is mediated by a protein complex. The Cascade complex containing Cas5, Cas6, Cas7, Cas8, Cas11, and the crRNA binds to the target site that is then digested by Cas3.

[5]. However, relaxing PAM requirements can increase off-target effects. Therefore, the group of Lei Yin most recently developed a variant of Cas12a from *Lachnospiraceae bacterium* (LbCas12a) with more stringent PAM recognition and lower off-target frequencies in mammalian cells [6]. Another problem of Cas12a systems is their reduced cutting efficiency at typical plant cultivation temperatures. Thus, a temperature-tolerant LbCas12a version was developed, outperforming previously engineered Cas12a variants at 22°C and 28°C [7]. Most recently, editing efficiency could be further enhanced by introducing introns into the open reading frame of ttLbCas12a [64].

Besides the manipulation of existing systems, the ongoing search for new CRISPR/Cas systems has provided a wide variety of new nucleases. Over 70 new Cas9 orthologs with different PAM requirements could be identified [8] and six new Cas12a orthologs showed robust editing efficiencies in rice [9]. Furthermore, a number of new subtypes of type-V nucleases were identified. Similar to Cas12a, Cas12b induces staggered cuts with 6–8-nt-long 5′ overhangs at targets flanked by a 5′ T-rich PAM and was recently adapted for gene editing in both monocotyledons and dicotyledons [10,11]. Driven by the need for smaller effectors in the delivery of gene therapy agents, Cas12e and Cas12j (formerly CasX and CasΦ, respectively) were adapted as gene editors in human cells [12,13]. Recently, a new class of especially small Cas nucleases was characterized and assigned to the type-V-F family [14]. As the development of transgene-free edited plants relies on viral-based vectors with strict cargo size limitations, such small nucleases are also of great interest for plant breeders and researchers. Thus, the Cas12f1 nuclease from *Syntrophomonas palmitatica* was engineered for genome editing in both human cells and maize [15]. Another Cas12f system was used successfully to provide viral resistance through transient expression in *Nicotiana benthamiana* [16].

Class-I type-I CRISPR/Cas systems are promising candidates as well, as they are the most abundant systems in bacteria. So far, nucleases of type I-D and -E were successfully applied in both mammals and plants and shown to provide new mutation patterns [17,18] (Fig. 1).

Base editing

While targeted mutagenesis has been proven to be a valuable tool, the induction of predictable changes is of great interest for plant research and breeding. Base editors, which consist of an engineered Cas nuclease paired with a base deaminase, enable targeted substitutions without InDel formation. Therefore, constant efforts are being taken to improve their efficiency and possible applications. Original BE enabled transitions from cytidine (C) to thymidine (T) (cytidine base editors, CBE) based on uracil, which results from the deamination of cytidine, being recognized as thymidine by polymerases. The additional fusion with a uracil glycosylase inhibitor was able to further boost CBE efficiencies. Later, adenine base editors (ABE) were developed that enabled adenine (A) to guanine (G) transitions through adenine deamination, resulting in an inosine (I), which is treated as a G by polymerases (Fig. 2). As the deamination is the crucial step, different natural deaminases as well as engineered versions were used to improve the mutagenesis frequency. Most recently, Hua et al. achieved increased editing efficiencies at almost all tested targets in rice using a simplified version of an existing ABE [19]. Moreover, inhibition of histone deacetylation might be a promising approach to enhance editing efficiencies, as was shown in human cells [20]. Nevertheless, target availability is another limiting factor concerning the application of BE, as the targeted bases have to be located within a certain distance to the PAM. Thus, BE based on different Cas9 orthologs [21–23] or LbCas12a [24] were used to reduce PAM-dependent restrictions. Furthermore, several SpCas9 PAM variants were explored, increasing the amount of targetable sequences [25]. Most recently, a near-PAM-less ABE was applied successfully in rice [26,27]. However, it was shown that relaxation of PAM requirements leads to an increased off-target frequency [28]. Thus, engineered versions of BE were developed to increase their sequence specificity [29,30]. Another bottleneck is the relatively small editing window of most BE. To overcome this limitation, an additional fusion of a single-strand DNA-binding domain was used to expand the editing range [31]. Most recently, a CBE based on an engineered version of the human AID cytosine deaminase provided consistent editing efficiency within an increased activity window [32].

In order to enable simultaneous cytidine and adenine base editing, several *saturated targeted endogenous mutagenesis editors* were developed [33]. On the other hand, aptamer-based *CRISPR simultaneous and wide-editing induced by a single system* enabled multiplexed base editing as well as InDel generation in a target-dependent manner [34]. Most recently, the CRISPR-Combo system was developed, which consists of a BE that simultaneously enables gene activation. The desired effect at the specific target is mediated by the gRNA. In order to prevent break induction and, thus, base editing at gene activation targets, a shortened gRNA is used. In its stem loops, it contains aptamers that recruit corresponding coat proteins, fused to a SunTag GCN4 epitope chain. Thus, up to forty copies of an activation domain can be recruited to a single target [35] (Figure 2B).

While the beforementioned systems can only generate transition mutations, recently, BEs were developed which enabled C–G editing in several plants by recruiting a uracil glycosylase, leading to an abasic site. In plants, such sites are repaired by the translesion polymerase REV1 that preferably incorporates a C opposite to an abasic site [36–38] (Fig. 2C). In order to enable transversions from A to Y (C or T), the group of Qinlong Zhu attempted to recruit enzymes of the base excision repair and alternative excision repair pathways, as both of these pathways are involved in I:T mismatch repair. However, both approaches yielded no A–Y conversions, and partly lead to InDel formation [39].

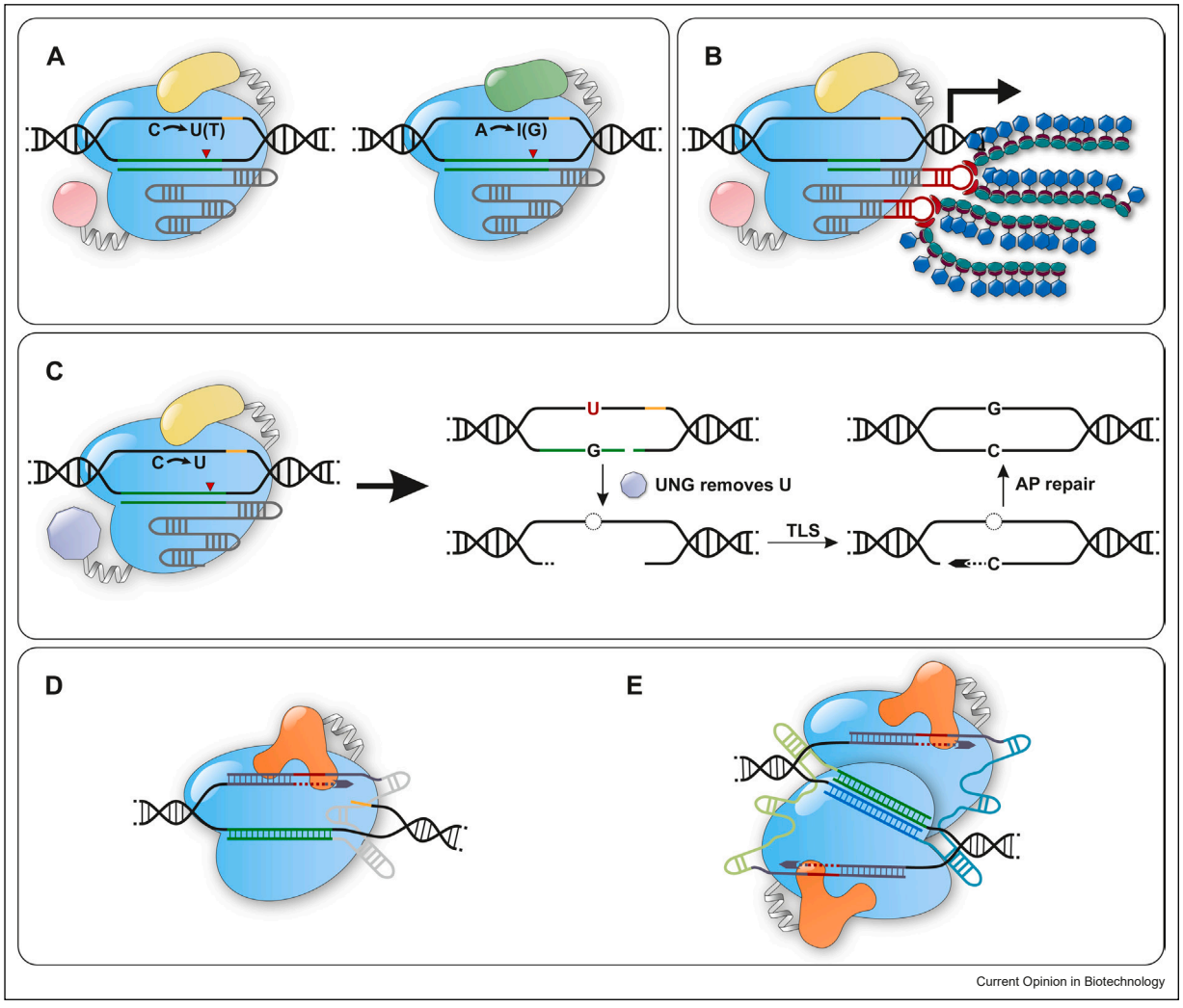
Although altered Cas nucleases and deaminases now possess greatly improved editing efficiency and target availability, heritable base editing remains challenging. By viral delivery of the gRNA in Cas-expressing plants, Liu et al. achieved heritable base editing in transgenic Arabidopsis plants [40]. Most recently, transgene-free base editing was achieved through viral vector-based delivery of an intein-splitted ABE [41].

Gene targeting and prime editing

While base editing is a promising tool for the targeted induction of base substitutions, it is not suitable for the induction of predefined modifications on a larger scale. A suitable tool for the achievement of such changes is gene targeting (GT), which relies on homologous-directed repair (HDR) of induced DSB [42]. However, since HDR is not the main repair pathway in somatic plant cells, GT efficiencies are still relatively low, despite constant efforts to optimize the system. As one of the limiting factors is the efficiency of DSB induction, the choice of the nuclease plays a key role for successful GT. By using ttLbCas12a, heritable GT events could be generated in tobacco with improved efficiencies in comparison with Cas9 [43]. Through overexpression of the two morphogenic genes *WUSCHEL* and *BABYBOOM*, not only higher transformation rates but also increased GT efficiencies were achieved in maize and sorghum [44,45].

However, GT relies on the induction of DSB that harbors the potential for unwanted InDel formation. The recently developed prime editing method on the other hand enables precise sequence modifications without requiring a DSB. Prime editors (PE) are based on a reverse transcriptase (RT) fused to a Cas9 nickase. Using a 3′-elongated prime editing

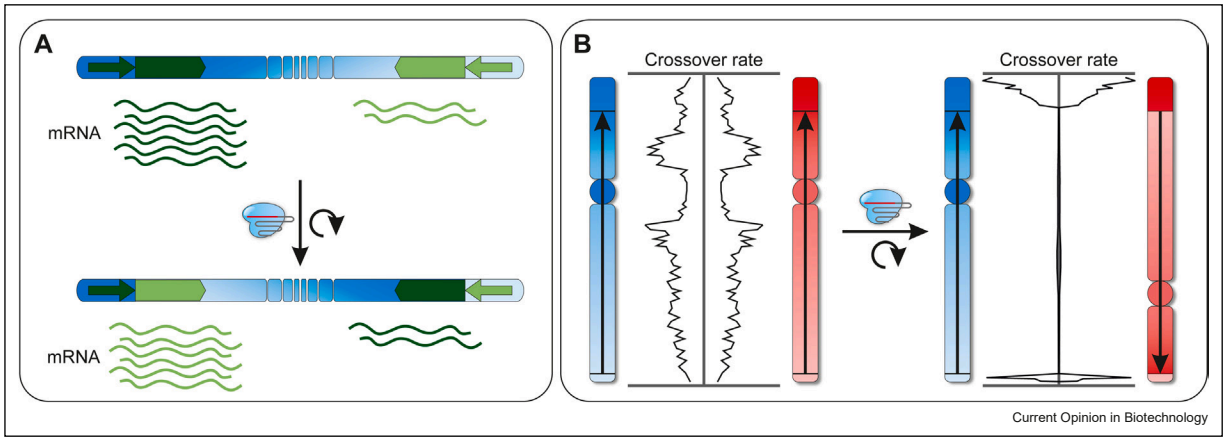
Figure 2



Representative structures of base and PE. **(A)** Cytidine and ABE. Using a respective deaminase domain, both enable the induction of transition mutations. Deamination of cytidine results in a uracil that is recognized as a thymidine by polymerases. Removal of uracil is inhibited through additional fusion of a uracil N-glycosylase (UNG) inhibitor. Deamination of adenine yields an inosine that is recognized as guanine by polymerases. In both cases, Cas9 nickase-mediated cleavage of the nonedited strand increases the frequency of stable base editing. **(B)** CRISPR-Combo system. In order to enable base editing at one target and activation of gene expression at another, the CRISPR-Combo system uses aptamer-based recruiting of GCN4 epitope chains that are recognized by a specific antibody fused to an activator domain. Thus, up to forty copies of the activator domain can be recruited to a specific target. Cas9 cleavage activity is inhibited by using a shortened guide RNA. **(C)** C–G base editor. By replacing the UNG inhibitor domain of a CBE with a UNG, uracil removal is promoted. During repair of the nicked strand, translesion synthesis opposite of the apurinic (AP) site preferably integrates a cytidine. Subsequent AP repair effectively results in a C–G transversion in the edited strand. **(D)** PE consist of a Cas9 nickase fused to a RT. The elongated pegRNA includes a part that is complementary to the nicked DNA strand and serves as PBS for the RT. Following the PBS, the pegRNA encodes desired edits that the RT transfers to the DNA. **(E)** By combining two PE that induce the same edits in opposite DNA strands, editing efficiencies can be significantly increased.

guide RNA (pegRNA) as a template, this tool precisely introduces insertions, deletions, and substitutions into the nicked DNA strand. One part of the pegRNA is complementary to the DNA sequence adjacent to the induced single-strand break and serves as a primer for the RT (primer-binding site, PBS). A second part contains the desired edits and is referred to as RT template (RTT). Subsequent DNA repair can then result in stably edited dsDNA (Fig. 2D). The first systems were based on the RT from Moloney murine leukemia virus (M-MLV). By inducing eight amino acid changes, this RT was optimized, resulting in PE2 with increased temperature tolerance and efficiency in mammalian cells. Further amelioration of editing frequencies could be obtained by inducing a second nick on the nontargeted DNA strand either several bp downstream of or within the modified sequence (PE3/PE3b)

Figure 3



CRISPR/Cas-mediated change of chromosomal structures. **(A)** By inducing two DSB, a 911-kb inversion was incited in rice. As the break sites were chosen between two genes and their respective promoter, the inversion effectively resulted in a promoter swap of those genes, which consequently leads to exchange of their expression rate. **(B)** On a larger scale, almost a whole chromosome was successfully inverted in Arabidopsis, which resulted in massive crossover suppression within the inverted sequence.

[46]. Nevertheless, constant efforts are being made to further improve the system. In human cells, transient inhibition of mismatch repair was shown to increase the efficiency of PE2 and PE3 (PE4/PE5) [47]. By replacing the nickase with a nuclease, Adikusuma et al. achieved enhanced prime editing initiation in mammalian cells. However, intended edits were often accompanied by additional InDels [48]. Interestingly, PE2 did not yield efficient editing in plants and, in contrast to animals, frequencies were not increased using PE3 systems. While Xu et al. were able to enhance screening efficiency using a surrogate plant PE2 system in rice to enrich edited cells [49], further improvements are required before PE can be widely used in plants. An attempt to exchange the RT domain with the RT of the plant-specific Cauliflower mosaic virus or a retron-derived RT from *E. coli* unfortunately even reduced editing efficiencies [50]. However, N-terminal fusion of the PE2 M-MLV RT was shown to perform better in rice and maize [51]. Most recently, Zong et al. designed an engineered plant PE by removing the ribonuclease H domain of M-MLV RT and adding a virus-derived protein with nucleic acid chaperone activity [52]. However, not only the RT domain is subject to constant efforts for improvement, the expression system as well as the length of PBS and RTT of pegRNAs were shown to have a major impact on editing efficiencies as well [53,54]. Lin et al. found that by optimizing the melting temperature of the PBS, PE efficiency can be improved. Additionally, it was shown that using two pegRNAs that encode the same edits but target complementary DNA strands highly promotes the desired outcome (Fig. 2E). The resulting increased PAM restrictions for the target design could be overcome using SpCas9 PAM variants [55]. In human cells, a similar approach with two pegRNAs encoding complementary DNA flaps was used successfully to precisely delete the sequence in- between [56].

Taken together, the induction of predefined sequence changes is achievable in plants, although further efforts are necessary to increase the efficiency of this promising system.

Restructuring genomes

CRISPR/Cas-based gene editing tools, such as base and PE, have been proven to be valuable instruments for the precise induction of single base pair changes, thus allowing the generation of alleles with improved agronomically important traits. However, to combine such traits in a single cultivar, breeders rely on meiotic recombination that can be hindered by naturally occurring inversions and genetic linkages. Thus, the targeted induction of recombination as well as the creation and breakage of genetic linkages is of great interest for plant breeders. Recently, it was proven that it is possible to induce predictable chromosomal inversions or translocations through the simultaneous induction of two DSB on either the same or on two different chromosomes, respectively [57,58]. Chromosomal inversions can be utilized for a promotor swap, as was shown in rice, resulting in a strong upregulation of gene expression without the need to insert regulatory elements [59] (Fig. 3). On a larger scale, Schwartz et al. were able to revert a 75.5-Mbp pericentric natural inversion in maize, unlocking this region for recombination [60]. More recently, Rönspies et al. achieved massive crossover suppression by inverting almost an entire chromosome in Arabidopsis [61] (Fig. 3 B). Thus, chromosomal engineering can be a valuable tool for breeders, as it facilitates the generation of improved varieties of existing crops [62]. However, CRISPR/Cas applications also open new doors for synthetic plant biology: by inducing multiple DSB in functional repeats, which resulted in

whole-genome elimination, tissue-specific Cas9 expression was used for the controlled elimination of distinct organs, such as petals and side roots [63].

Conclusion

The ongoing discovery of new CRISPR/Cas systems and efforts to further optimize existing as well as to develop new tools, have made almost the entire plant genome accessible for precise editing. Thus, we were able to overcome the need for hoping for random favorable mutations. Instead, we are now able to not only induce modifications ranging from single bases up to several Mbp with high specificity but to also influence recombination on a chromosomal level. One can be optimistic that, if we will be able to keep up the high pace of innovation in the field, the efficient generation of new crop varieties, better suited to address current and future challenges of our ever-changing environment, will soon come into reach.

CRediT authorship contribution statement

Niklas Capdeville: Writing – original draft, Visualization; **Patrick Schindele:** Writing – review & editing; **Holger Puchta:** Writing – review & editing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

Acknowledgements

Our apologies to all those colleagues whose exciting work we were not able to cite. Owing to space limitations, we could only discuss a fraction of the work relevant to the topic. We thank Michelle Rönspies for critical reading of the paper. This work was funded by the European Research Council (Advanced grant ERC-2016-AdG_741306 CHRISBREED to H.P.).

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Gardiner J, Ghoshal B, Wang M, Jacobsen SE: **CRISPR-Cas-mediated transcriptional control and epi-mutagenesis**. *Plant Physiol* 2022, **188**:1811-1824.

2. Schindele A, Dorn A, Puchta H: **CRISPR/Cas brings plant biology and breeding into the fast lane**. *Curr Opin Biotechnol* 2020, **61**:7-14.

3. Chatterjee P, Lee J, Nip L, Koseki SRT, Tysinger E, Sontheimer EJ, Jacobson JM, Jakimo N: **A Cas9 with PAM recognition for adenine dinucleotides**. *Nat Commun* 2020, **11**:2474.

4. Sretenovic S, Yin D, Levav A, Selengut JD, Mount SM, Qi Y: **Expanding plant genome-editing scope by an engineered iSpyMacCas9 system that targets A-rich PAM sequences**. *Plant Commun* 2021, **2**:100101.

5. Niu Q, Wu S, Li Y, Yang X, Liu P, Xu Y, Lang Z: **Expanding the scope of CRISPR/Cas9-mediated genome editing in plants using an xCas9 and Cas9-NG hybrid**. *J Integr Plant Biol* 2020, **62**:398-402.

6. Zhou J, Chen P, Wang H, Liu H, Li Y, Zhang Y, Wu Y, Paek C, Sun Z, Lei J, Yin L: **Cas12a variants designed for lower genome-wide off-target effect through stringent PAM recognition**. *Mol Ther* 2022, **30**:244-255.

7. • Schindele P, Puchta H: **Engineering CRISPR/LbCas12a for highly efficient, temperature-tolerant plant gene editing**. *Plant Biotechnol J* 2020, **18**:1118-1120.
Development of a LbCas12a variant with improved efficiency at typical plant cultivation temperatures.

8. Gasiunas G, Young JK, Karvelis T, Kazlauskas D, Urbaitis T, Jasnauskaite M, Grusyte MM, Paulraj S, Wang P-H, Hou Z, *et al.*: **A catalogue of biochemically diverse CRISPR-Cas9 orthologs**. *Nat Commun* 2020, **11**:5512.

9. Zhang Y, Ren Q, Tang X, Liu S, Malzahn AA, Zhou J, Wang J, Yin D, Pan C, Yuan M, *et al.*: **Expanding the scope of plant genome engineering with Cas12a orthologs and highly multiplexable editing systems**. *Nat Commun* 2021, **12**:1944.

10. Ming M, Ren Q, Pan C, He Y, Zhang Y, Liu S, Zhong Z, Wang J, Malzahn AA, Wu J, *et al.*: **CRISPR-Cas12b enables efficient plant genome engineering**. *Nat Plants* 2020, **6**:202-208.

11. Wu F, Qiao X, Zhao Y, Zhang Z, Gao Y, Shi L, Du H, Wang L, Zhang Y-J, Zhang Y, *et al.*: **Targeted mutagenesis in Arabidopsis thaliana using CRISPR-Cas12b/C2c1**. *J Integr Plant Biol* 2020, **62**:1653-1658.

12. Liu J-J, Orlova N, Oakes BL, Ma E, Spinner HB, Baney KLM, Chuck J, Tan D, Knott GJ, Harrington LB, *et al.*: **CasX enzymes comprise a distinct family of RNA-guided genome editors**. *Nature* 2019, **566**:218-223.

13. Pausch P, Al-Shayeb B, Bisom-Rapp E, Tsuchida CA, Li Z, Cress BF, Knott GJ, Jacobsen SE, Banfield JF, Doudna JA: **CRISPR-CasΦ from huge phages is a hypercompact genome editor**. *Science* 2020, **369**:333-337.

14. Karvelis T, Bigelyte G, Young JK, Hou Z, Zedaveinyte R, Budre K, Paulraj S, Djukanovic V, Gasior S, Silanskas A, *et al.*: **PAM recognition by miniature CRISPR-Cas12f nucleases triggers programmable double-stranded DNA target cleavage**. *Nucleic Acids Res* 2020, **48**:5016-5023.

15. Bigelyte G, Young JK, Karvelis T, Budre K, Zedaveinyte R, Djukanovic V, van Ginkel E, Paulraj S, Gasior S, Jones S, *et al.*: **Miniature type V-F CRISPR-Cas nucleases enable targeted DNA modification in cells**. *Nat Commun* 2021, **12**:6191.

16. Haider S, Faiq A, Khan MZ, Mansoor S, Amin I: **Fully transient CRISPR/Cas12f system in plants capable of broad-spectrum resistance against Begomovirus**. *bioRxiv* 2022,495110.

17. • Osakabe K, Wada N, Miyaji T, Murakami E, Marui K, Ueta R, Hashimoto R, Abe-Hara C, Kong B, Yano K, Osakabe Y: **Genome editing in plants using CRISPR type I-D nuclease**. *Commun Biol* 2020, **3**:648.
Adaptation of type I-D CRISPR/Cas system for genome editing in plants.

18. Osakabe K, Wada N, Murakami E, Miyashita N, Osakabe Y: **Genome editing in mammalian cells using the CRISPR type I-D nuclease**. *Nucleic Acids Res* 2021, **49**:6347-6363.

19. Hua K, Tao X, Liang W, Zhang Z, Gou R, Zhu J-K: **Simplified adenine base editors improve adenine base editing efficiency in rice**. *Plant Biotechnol J* 2020, **18**:770-778.

20. Liu N, Zhou L, Lin G, Hu Y, Jiao Y, Wang Y, Liu J, Yang S, Yao S: **HDAC inhibitors improve CRISPR-Cas9 mediated prime editing and base editing**. *Mol Ther Nucleic Acids* 2022, **29**:36-46.

21. Liu T, Zeng D, Zheng Z, Lin Z, Xue Y, Li T, Xie X, Ma G, Liu Y-G, Zhu Q: **The ScCas9++ variant expands the CRISPR toolbox for genome editing in plants**. *J Integr Plant Biol* 2021, **63**:1611-1619.

22. Wang M, Xu Z, Gosavi G, Ren B, Cao Y, Kuang Y, Zhou C, Spetz C, Yan F, Zhou X, Zhou H: **Targeted base editing in rice with CRISPR/ScCas9 system**. *Plant Biotechnol J* 2020, **18**:1645-1647.

23. Xu R, Qin R, Xie H, Li J, Liu X, Zhu M, Sun Y, Yu Y, Lu P, Wei P: **Genome editing with type II-C CRISPR-Cas9 systems from Neisseria meningitidis in rice**. *Plant Biotechnol J* 2022, **20**:350-359.

24. Wang G, Xu Z, Wang F, Huang Y, Xin Y, Liang S, Li B, Si H, Sun L, Wang Q, *et al.*: **Development of an efficient and precise adenine base editor (ABE) with expanded target range in allotetraploid cotton (*Gossypium hirsutum*)**. *BMC Biol* 2022, **20**:45.

25. Li J, Xu R, Qin R, Liu X, Kong F, Wei P: **Genome editing mediated by SpCas9 variants with broad non-canonical PAM compatibility in plants**. *Mol Plant* 2021, **14**:352-360.

26. Tan J, Zeng D, Zhao Y, Wang Y, Liu T, Li S, Xue Y, Luo Y, Xie X, Chen L, *et al.*: **PhieABEs: a PAM-less/free high-efficiency adenine base editor toolbox with wide target scope in plants**. *Plant Biotechnol J* 2022, **20**:934-943.

27. Ren Q, Sretenovic S, Liu S, Tang X, Huang L, He Y, Liu L, Guo Y, Zhong Z, Liu G, *et al.*: **PAM-less plant genome editing using a CRISPR-SpRY toolbox**. *Nat Plants* 2021, **7**:25-33.

28. Wu Y, Ren Q, Zhong Z, Liu G, Han Y, Bao Y, Liu L, Xiang S, Liu S, Tang X, *et al.*: **Genome-wide analyses of PAM-relaxed Cas9 genome editors reveal substantial off-target effects by ABE8e in rice**. *Plant Biotechnol J* 2022, **20**:1670-1682.

29. Jin S, Fei H, Zhu Z, Luo Y, Liu J, Gao S, Zhang F, Chen Y-H, Wang Y, Gao C: **Rationally designed APOBEC3B cytosine base editors with improved specificity**. *Mol Cell* 2020, **79**:728-740.e6.

30. Jeong YK, Lee S, Hwang G-H, Hong S-A, Park S-E, Kim J-S, Woo J-S, Bae S: **Adenine base editor engineering reduces editing of bystander cytosines**. *Nat Biotechnol* 2021, **39**:1426-1433.

31. Wei C, Liu H, Wang W, Luo P, Chen Q, Li R, Wang C, Botella JR, Zhang H: **Expanding the editing window of cytidine base editors with the Rad51 DNA-binding domain in rice**. *Front Plant Sci* 2022, **13**:865848.

32. Xiong X, Li Z, Liang J, Liu K, Li C, Li J-F: **A cytosine base editor toolkit with varying activity windows and target scopes for versatile gene manipulation in plants**. *Nucleic Acids Res* 2022, **50**:3565-3580.

33. Li C, Zhang R, Meng X, Chen S, Zong Y, Lu C, Qiu J-L, Chen Y-H, Li J, Gao C: **Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors**. *Nat Biotechnol* 2020, **38**:875-882.

34. Li C, Zong Y, Jin S, Zhu H, Lin D, Li S, Qiu J-L, Wang Y, Gao C: **SWISS: multiplexed orthogonal genome editing in plants with a Cas9 nickase and engineered CRISPR RNA scaffolds**. *Genome Biol* 2020, **21**:141.

35. • Pan C, Li G, Malzahn AA, Cheng Y, Leyson B, Sretenovic S, Gurel F, Coleman GD, Qi Y: **Boosting plant genome editing with a versatile CRISPR-Combo system**. *Nat Plants* 2022, **8**:513-525.
Development of new tool enabling simultaneous target dependent gene editing and gene activation based on a single Cas9 protein.

36. •• Sretenovic S, Liu S, Li G, Cheng Y, Fan T, Xu Y, Zhou J, Zheng X, Coleman G, Zhang Y, Qi Y: **Exploring C-To-G base editing in rice, tomato, and poplar**. *Front Genome Ed* 2021, **3**:756766.
Demonstration that C to G base editing is possible in plants by recruiting an uracil glycosylase.

37. Tian Y, Shen R, Li Z, Yao Q, Zhang X, Zhong D, Tan X, Song M, Han H, Zhu J-K, Lu Y: **Efficient C-to-G editing in rice using an optimized base editor**. *Plant Biotechnol J* 2022, **20**:1238-1240.

38. Takahashi S, Sakamoto AN, Tanaka A, Shimizu K: **AtREV1, a Y-family DNA polymerase in Arabidopsis, has deoxynucleotidyl transferase activity *in vitro***. *Plant Physiol* 2007, **145**:1052-1060.

39. Zeng D, Zheng Z, Liu Y, Liu T, Li T, Liu J, Luo Q, Xue Y, Li S, Chai N, *et al.*: **Exploring C-to-G and A-to-Y base editing in rice by using new vector tools**. *Int J Mol Sci* 2022, **23**:7990.

40. Liu D, Xuan S, Prichard LE, Donahue LI, Pan C, Nagalakshmi U, Ellison EE, Starker CG, Dinesh-Kumar SP, Qi Y, Voytas DF: **Heritable base-editing in Arabidopsis using RNA viral vectors**. *Plant Physiol* 2022, **189**:1920-1924.

41. Yuan G, Lu H, De K, Hassan MM, Liu Y, Li Y, Muchero W, Abraham PE, Tuskan GA, Yang X: **An intein-mediated Split-nCas9 system for base editing in plants**. *ACS Synth Biol* 2022, **11**:2513-2517.

42. Puchta H, Dujon B, Hohn B: **Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination**. *Proc Natl Acad Sci USA* 1996, **93**:5055-5060.

43. Huang T-K, Armstrong B, Schindele P, Puchta H: **Efficient gene targeting in *Nicotiana tabacum* using CRISPR/SaCas9 and temperature tolerant LbCas12a**. *Plant Biotechnol J* 2021, **19**:1314-1324.

44. • Barone P, Wu E, Lenderts B, Anand A, Gordon-Kamm W, Svitashev S, Kumar S: **Efficient gene targeting in maize using inducible CRISPR-Cas9 and marker-free donor template**. *Mol Plant* 2020, **13**:1219-1227.
Improvement of GT by overexpressing the morphogenic genes *WUSCHEL* and *BABYBOOM.*

45. Che P, Wu E, Simon MK, Anand A, Lowe K, Gao H, Sigmund AL, Yang M, Albertsen MC, Gordon-Kamm W, Jones TJ: **Wuschel2 enables highly efficient CRISPR/Cas-targeted genome editing during rapid *de novo* shoot regeneration in sorghum**. *Commun Biol* 2022, **5**:344.

46. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR: **Search-and-replace genome editing without double-strand breaks or donor DNA**. *Nature* 2019, **576**:149-157.

47. Chen PJ, Hussmann JA, Yan J, Knipping F, Ravisankar P, Chen P-F, Chen C, Nelson JW, Newby GA, Sahin M, *et al.*: **Enhanced prime editing systems by manipulating cellular determinants of editing outcomes**. *Cell* 2021, **184**:5635-5652.e29.

48. Adikusuma F, Lushington C, Arudkumar J, Godahewa GI, Chey YCJ, Gierus L, Piltz S, Geiger A, Jain Y, Reti D, *et al.*: **Optimized nickase- and nuclease-based prime editing in human and mouse cells**. *Nucleic Acids Res* 2021, **49**:10785-10795.

49. Xu R, Li J, Liu X, Shan T, Qin R, Wei P: **Development of plant prime-editing systems for precise genome editing**. *Plant Commun* 2020, **1**:100043.

50. Lin Q, Zong Y, Xue C, Wang S, Jin S, Zhu Z, Wang Y, Anzalone AV, Raguram A, Doman JL, *et al.*: **Prime genome editing in rice and wheat**. *Nat Biotechnol* 2020, **38**:582-585.

51. Xu W, Yang Y, Yang B, Krueger CJ, Xiao Q, Zhao S, Zhang L, Kang G, Wang F, Yi H, *et al.*: **A design optimized prime editor with expanded scope and capability in plants**. *Nat Plants* 2022, **8**:45-52.

52. • Zong Y, Liu Y, Xue C, Li B, Li X, Wang Y, Li J, Liu G, Huang X, Cao X, Gao C: **An engineered prime editor with enhanced editing efficiency in plants**. *Nat Biotechnol* 2022, **40**:1394-1402.
Improvement of prime editing in plants by eliminating the ribonuclease H domain of the RT and adding a viral protein with nucleic acid chaperone activity.

53. Li Z, Ma R, Liu D, Wang M, Zhu T, Deng Y: **A straightforward plant prime editing system enabled highly efficient precise editing of rice Waxy gene**. *Plant Sci* 2022, **323**:111400.

54. Jiang Y-Y, Chai Y-P, Lu M-H, Han X-L, Lin Q, Zhang Y, Zhang Q, Zhou Y, Wang X-C, Gao C, Chen Q-J: **Prime editing efficiently generates W542L and S621I double mutations in two ALS genes in maize**. *Genome Biol* 2020, **21**:257.

55. • Lin Q, Jin S, Zong Y, Yu H, Zhu Z, Liu G, Kou L, Wang Y, Qiu J-L, Li J, Gao C: **High-efficiency prime editing with optimized, paired pegRNAs in plants**. *Nat Biotechnol* 2021, **39**:923-927.
Demonstration that prime editing efficiencies can be improved by using two pegRNAs that encode the same edits.

56. Anzalone AV, Gao XD, Podracky CJ, Nelson AT, Koblan LW, Raguram A, Levy JM, Mercer JAM, Liu DR: **Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing**. *Nat Biotechnol* 2022, **40**:731-740.

57. Schmidt C, Fransz P, Rönspies M, Dreissig S, Fuchs J, Heckmann
•• S, Houben A, Puchta H: **Changing local recombination patterns in Arabidopsis by CRISPR/Cas mediated chromosome engineering**. *Nat Commun* 2020, **11**:4418.
Demonstration that CRISPR/Cas-mediated chromosomal inversions can be utilized to unlock specific regions for recombination.

58. Beying N, Schmidt C, Pacher M, Houben A, Puchta H: **CRISPR-**
•• **Cas9-mediated induction of heritable chromosomal translocations in Arabidopsis**. *Nat Plants* 2020, **6**:638-645.
First achievement of heritable chromosomal translocations.

59. Lu Y, Wang J, Chen B, Mo S, Lian L, Luo Y, Ding D, Ding Y, Cao Q,
• Li Y, *et al*.: **A donor-DNA-free CRISPR/Cas-based approach to gene knock-up in rice**. *Nat Plants* 2021, **7**:1445-1452.
Demonstration that the precise induction of inversions or duplications can be used to swap promoters between genes.

60. Schwartz C, Lenderts B, Feigenbutz L, Barone P, Llaca V, Fengler K, Svitashev S: **CRISPR-Cas9-mediated 75.5-Mb inversion in maize**. *Nat Plants* 2020, **6**:1427-1431.

61. Rönspies M, Schmidt C, Schindele P, Lieberman-Lazarovich M,
• Houben A, Puchta H: **Massive crossover suppression by CRISPR–Cas-mediated plant chromosome engineering**. *Nat Plants* 2022, **8**:1153-1159.
Demonstration that CRISPR/Cas-mediated chromosomal inversions can be utilized to block specific regions for recombination.

62. Rönspies M, Dorn A, Schindele P, Puchta H: **CRISPR-Cas-mediated chromosome engineering for crop improvement and synthetic biology**. *Nat Plants* 2021, **7**:566-573.

63. Schindele A, Gehrke F, Schmidt C, Röhrig S, Dorn A, Puchta H:
•• **Using CRISPR-Kill for organ specific cell elimination by cleavage of tandem repeats**. *Nat Commun* 2022, **13**:1502.
Demonstration that tissue specific induction of multiple DSB in repetitive sequences enables elimination of distinct organs in pants

64. Schindele P, Merker L, Schreiber T, Prange A, Tissier A, Puchta H: **Enhancing gene editing and gene targeting efficiencies in *Arabidopsis thaliana* by using an intron-containing version of ttLbCas12a**. *Plant Biotechnol J* 2022,.