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CRISPR/Cas brings plant biology and breeding into the fast lane

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CRISPR/Cas is in the process of inducing the biggest transformation of plant breeding since the green revolution. Whereas initial efforts focused mainly on changing single traits by error prone non-homologous end joining, the last two years saw a tremendous technical progress achieving more complex genetic, epigenetic and transcriptional changes. The efficiencies of inducing directed changes by homologous recombination have been improved significantly and strategies to break genetic linkages by inducing chromosomal rearrangements have been developed. Cas13 systems have been applied to degrade viral and mRNA in plants. Most importantly, a historical breakthrough was accomplished: By introducing multiple genomic changes simultaneously, domestication of wild species in a single generation has been demonstrated, speeding up breeding dramatically.

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Introduction

For a long period of time the induction of genomic changes was limited by the properties of the available editing tools. However, the application of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) nucleases revolutionized plant research and breeding. They combine high efficiency and enhanced flexibility with the possibility of multiplexing, that is, the simultaneous addressing of multiple sites. CRISPR/Cas-systems are divided into two classes with six subtypes. The type II single effector nuclease Cas9 of *Streptococcus pyogenes* (SpCas9) was the first to be remodelled for biotechnological applications in 2012 and used to produce an edited plant in 2013 [1,2]. Over time, additional Cas9 orthologues, such as *Staphylococcus aureus* Cas9 (SaCas9) or *Streptococcus thermophilus* Cas9 (StCas9) were used in plants, providing access to multi-dimensional editing by enabling the simultaneous targeting of different kinds of biochemical activities [3,4]. Further CRISPR/Cas-systems were characterized, such as type V CRISPR/Cas12a with different guide requirements and cleavage patterns, as well as type VI CRISPR/Cas13, which targets RNA instead of DNA [5,6] (Figure 1).

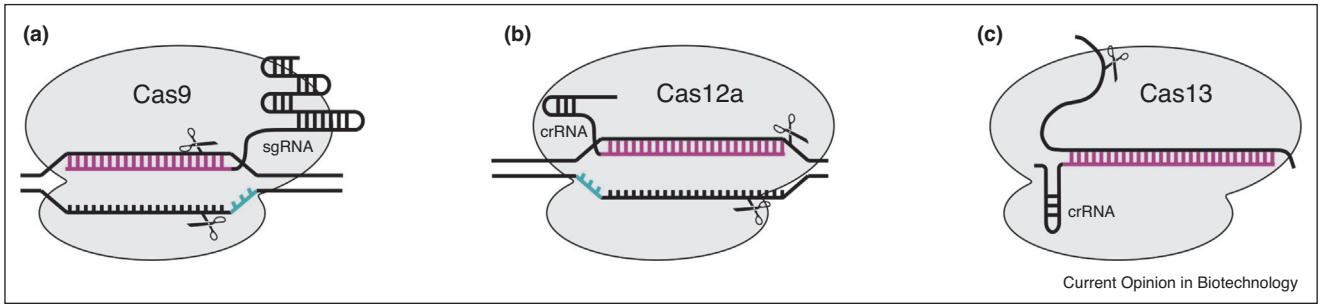
So far, the CRISPR/Cas-system was predominantly exploited for the generation of single knockouts, enabling the introduction of valuable novel traits in crops and phenotypic analysis of mutants in plant biology [7,8]. Here, we will not cover agricultural applications in various crops but concentrate on the technological developments introduced in plants in the last few years, since the same topic was reviewed last in this series [9].

Site-specific manipulation of plant genomes and epigenomes using Cas9 nickases and dCas9

The versatility of the CRISPR/Cas9-system allows not only the induction of targeted DSBs but also the customization as nickase, if one of its two catalytic domains is mutated, or as a programmable DNA-binding protein, if both domains are modified. In both cases, the Cas9 protein can act as scaffold for targeting various enzyme activities (Figure 2). Single base editing independent of DSB induction could be accomplished by fusing a cytidine deaminase or an adenosine deaminase to a Cas9 nickase, converting C/G to T/A and vice versa in rice, wheat and maize [10,11]. More recently, simultaneous base editing of cytosine and adenine was achieved by introducing cytosine and adenine base editors into the rice genome [12]. Nonetheless, recent studies in rice revealed that cytosine base editors induce genome-wide off-target mutations. These mutations were mostly C to T conversions and occur even in the absence of a sgRNA, indicating that this system needs further optimization to be a reliable tool for precise genome editing as the adenine base editors [13•].

The dead version of Cas9 can be employed to achieve florescence-imaging [14] or modulate gene expression in a direct or indirect fashion. The direct fusion of dCas9 with four tandem repeats of the transcriptional activator VP16 (VP64) promoted transcriptional activation of endogenous genes in *Arabidopsis* and tobacco. Additionally, a robust repression of gene expression could be achieved by fusing the repressor domain SRDX to dCas9 [15]. Robust

Figure 1



Schematic representation of Cas proteins.
(a) The Cas9 protein forms a complex with the sgRNA (an artificial fusion of crRNA and tracrRNA), to bind the DNA upstream of the G/C-rich PAM recognition site. Binding is enabled by a 20-nucleotide guide sequence of the sgRNA. A blunt-ended DSB is induced 3 bp upstream the PAM sequence. **(b)** The Cas12a and its single crRNA, form a complex to bind the DNA downstream of the A/T-rich PAM recognition site. In contrast to the Cas9, the single effector Cas12a requires a 23-25-nucleotide guide sequence to induce a staggered DSB 18 and 23 nucleotides distal of the PAM, respectively. **(c)** Cas13 targets RNA molecules instead of DNA. The specific binding is mediated by a crRNA, which forms a complex with the single effector nuclease Cas13. The cleavage of the RNA occurs remote from the recognition site and on the outside of the protein surface. For cleavage, no PAM recognition site is required. However, some Cas13 proteins seem to require a protospacer flanking site (PFS), which is a PAM-like motif.

transcriptional repression can also be achieved in plants with CRISPR-Cas12a-based repressors [16]. Indirect fusions are mediated through modified sgRNAs, where small RNA aptamers act as a scaffold for RNA binding proteins [17]. Recent studies displayed an enhanced system for transcriptional activation by combining both strategies, resulting in an improved yet still robust transcriptional activation in *Arabidopsis* and rice [18].

The SunTag represents another promising approach for multiple protein recruitment [19]. The SunTag-system provides a dCas9-fused polypeptide chain that can recruit multiple copies of an antibody-fusion protein. Recently, this system was successfully employed for transcriptional activation and methylation in *Arabidopsis thaliana* [20•]. By the use of the transcriptional activator VP64, robust and specific expression of several genes could be achieved. Moreover, combination of the SunTag-system with the *Nicotiana tabacum* DRM methyltransferase was able to modulate DNA methylation at specific DNA loci very efficiently, opening a new avenue for the epigenetic reprogramming of plant genomes.

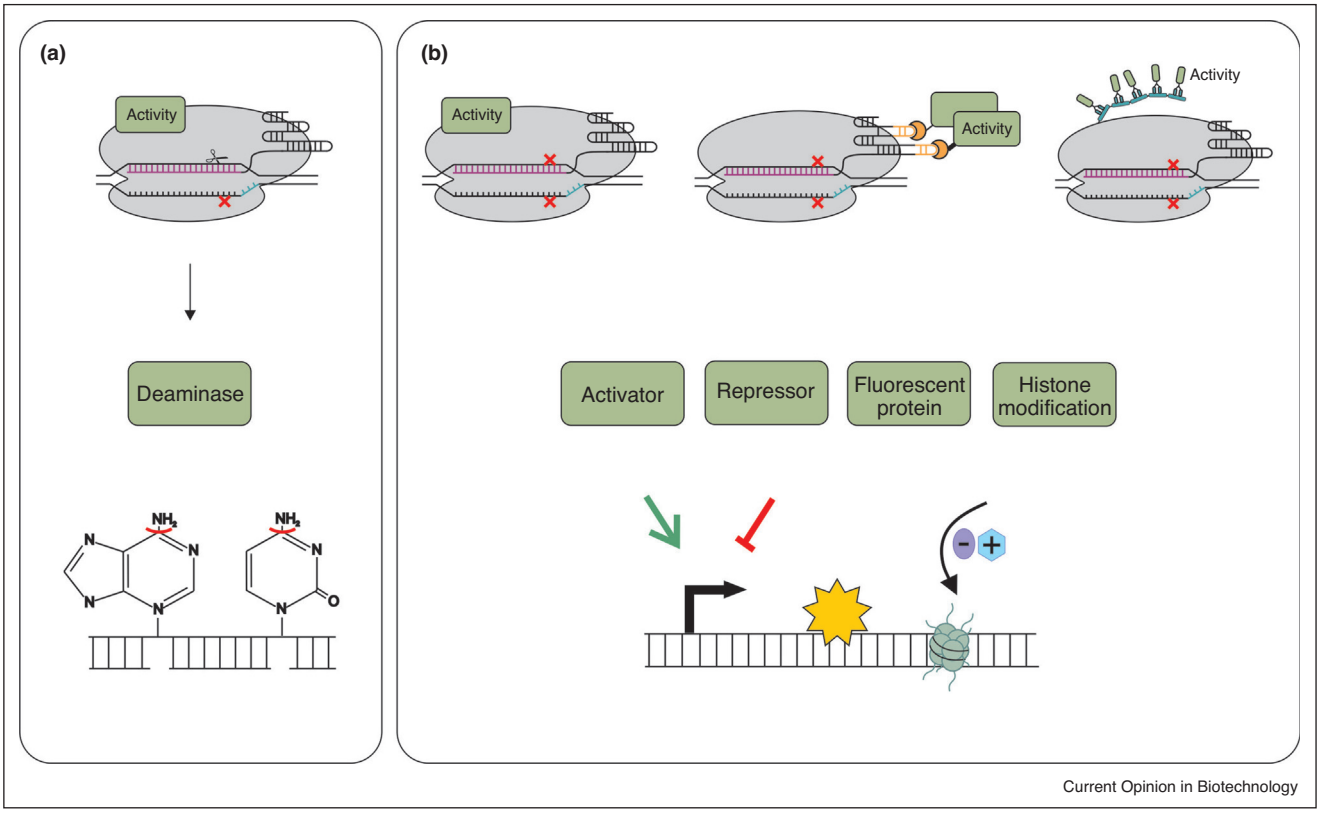
With the use of the CRISPR/Cas13 systems RNA editing also became possible [21]. By now, three different classes, Cas13a, 13b and 13d are applicable for RNA editing. In plant cells, RNA cleavage to degrade mRNA or to block RNA virus infections has been achieved [21,22•]. By using catalytically inactive Cas13 (dCas13) as a scaffold to guide distinct effectors to target RNA, further manipulations like base editing or fluorescent imaging can be envisaged. This technology has a lot of potential to manipulate cellular RNA metabolism on various levels.

CRISPR-mediated gene targeting

NHEJ is the predominant DSB repair pathway in somatic cells of higher plants and at least two orders of magnitude more efficient than homologous recombination (HR). Therefore, gene targeting (GT) by HR poses a challenge. Genome modifications by GT are either sequence insertions or substitutions using a homologous template harboring the desired modification. Multiple strategies have been developed over the years to increase GT frequencies without too much success [23].

Enhancement of GT frequency could be accomplished by increasing the copy number of the repair template. A modified T-DNA with geminiviral features was employed, harbouring two LIR elements flanking the GT template and one SIR element. Co-delivery of Rep/RepA proteins that nick both LIR elements, results in the formation of a double-stranded DNA that serves as template for rolling circle replication to produce high copy numbers of templates [24]. Although this system was demonstrated to work in some crop plants like tomato, partly with astonishing high efficiencies [25,26•,27], a number of other publications show that this system cannot be applied efficiently in other plants [28–30]. Only future experiments will tell how widely the protocol can be applied to obtain fertile plants with the desired change at high frequency. A novel approach involves RNA transcripts as repair templates, as transcription produces multiple RNA molecules of a single DNA copy. Li *et al.* used ribozyme or crRNA flanked repair templates to keep RNA templates in the nucleus [31]. Thus, they were able to achieve RNA-mediated targeting in plants; however, frequencies were lower than by the use of ssOligos as template.

Figure 2



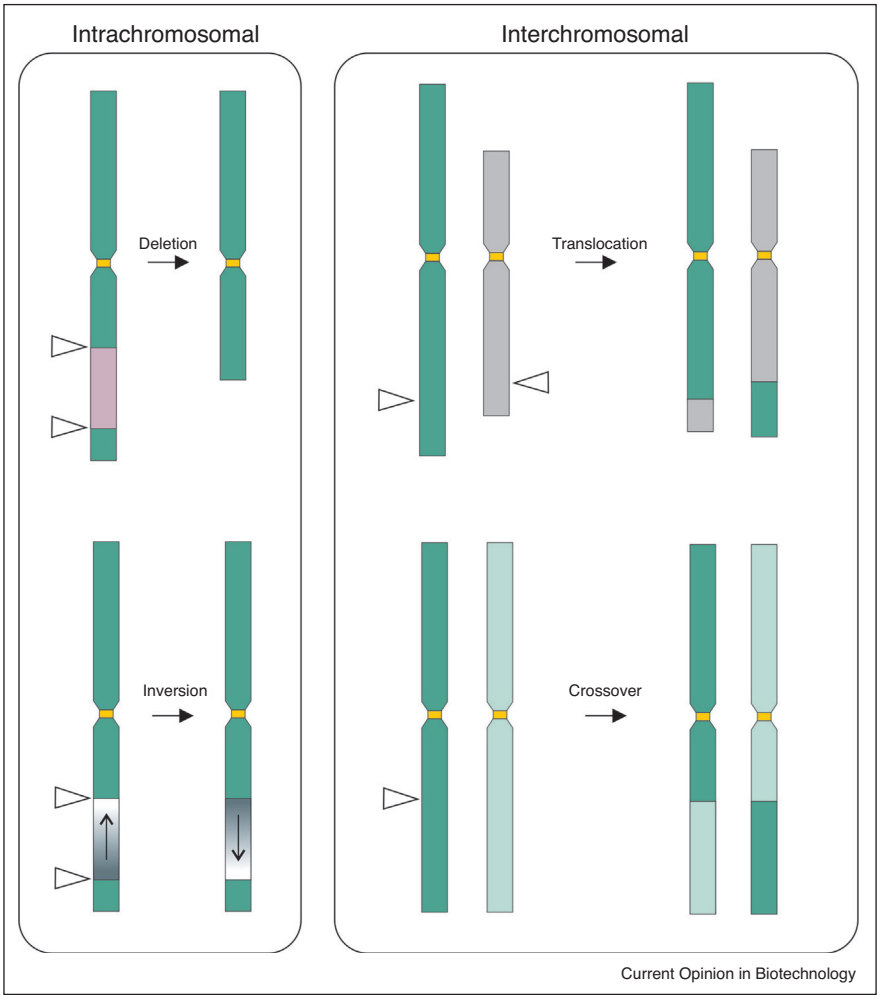
Applications of nCas9 and dCas9-proteins.
(a) Cas9 nickase, which is mutated in one of the two SSB cleavage domains, is used to mediate base editing by fusing cytidine deaminases or adenosine deaminases directly to the Cas9 protein. **(b)** Dead Cas9 proteins carry mutations in both SSB cleavage domains, converting the protein into a DNA binding protein and thus a scaffold for different activities to modulate the site of interest. The respective activity can be fused either directly to the Cas9 protein or to RNA-binding-proteins that bind to aptamer sequences incorporated in the respective sgRNAs. The SunTag provides a polypeptide chain as a platform for the recruitment of multiple copies of antibody-fusion proteins. Biochemical activities used in these fusions were transcriptional activators such as VP16, or repressors like SRDX. Additionally, by fusing fluorescent proteins to the Cas9 scaffold imaging of genomic loci is possible. Moreover, epigenetic states can be influenced by the fusion of epigenetic modulators, such as histone acetyltransferases or cytosine demethylases.

Another kind of template activation is used by the '*in planta* gene targeting' strategy [32]. Here, a transgenic template is excised out of the genome simultaneously to DSB induction in the target. Recently, GT frequencies in *Arabidopsis* could be improved by the use of the Cas9 orthologue SaCas9 instead of SpCas9 [33•]. This surprising effect is supported by biochemical data that SaCas9, in contrast to SpCas9, has a multiple turnover activity, allowing frequent cleavage of the target substrate [34]. However, the most important improvement for GT in *Arabidopsis* was the use of the egg-cell-specific EC1 transcriptional cassette for nuclease expression that has been shown to be efficient in NHEJ-based editing before its use for GT in plants [35]. Here, in the best cases, lines with 5–10% positive events could be obtained [33•,36•,37]. Because of early activity of the promoter in plant development and/or reproductive tissue, frequently heritable events and homozygous GT events could already be detected in T2 seedlings. Of special importance is the application of an efficient bulk screening protocol to identify the T2 lines with the highest GT efficiency to obtain positive individuals with minimal efforts [36•].

Efficient restructuring of plant genomes using the CRISPR/Cas system

It has been shown long before that site-specific nucleases can be used to induce chromosomal changes like deletions and reciprocal translocations [38,39] (Figure 3). The CRISPR/Cas system has been efficiently applied for the induction of large deletions in plant genomes. Recently, the expression of Cas9 nuclease under a tissue-specific or cell-stage-specific promoter was shown to promote inheritable deletion events in *Arabidopsis*, resulting in high frequencies of deletions up to 13 kb and lower frequencies of deletions of 120 kb in following generations [40–42]. Additionally, a high frequency of

Figure 3



Chromosomal rearrangements after DSB induction.
Two DSBs in the same chromosome result either in a sequence deletion between the break sites or in an inversion of the excised region after reintegration. Next to this, two DSBs on two different chromosomes might lead to a reciprocal translocation, resulting in the exchange of chromosome arms. By induction of a single DSB, crossovers with the homologue might be achieved.

deletions <100 bp was achieved in *Nicotiana benthamiana* in six independent loci [41].

Apart from deletions, contemporaneous cleavage of two sequences on the same chromosome can also lead to inversions. Natural inversions occur across all species and represent the most common large-scale chromosomal rearrangements. These events can lead to major obstacles for breeding, due to the inaccessibility of inverted regions for recombination, resulting in the establishment of trait linkages. A very recent study applying SaCas9 under the expression of the egg-cell specific promoter demonstrated the transmission of inversions up to 18 kb into the next generation of *Arabidopsis*. For some T1 lines, up to 10% of their progeny were found to carry inversions, making this approach attractive for breeding applications in crop plants [43•].

The most direct way to break genetic linkage is the artificial induction of crossovers between homologous chromosomes. Although some time ago a study already demonstrated that HR between homologues can be induced in plant cells at low frequency, via DSB induction [44], it was only recently that a ground-breaking study of the group of Avraham Levy addressed this question using Cas9 [45••]. They developed a selection system, using hybrids of two tomato accessions, distinguished by SNPs and fruit color, which enables them to identify HR between homologous chromosomes. By inducing an allele-specific DSB, they were able to identify surprisingly large numbers of somatic gene conversions where a small segment of the homologue was used for repair. One putative crossover event was identified that unfortunately could not be transferred to the next

generation. This study indicates that allelic HR occurs much more frequently than previously anticipated and that the induction of somatic COs could be achieved in the near future.

CRISPR-mediated mutagenesis enhances genetic diversity in various ways

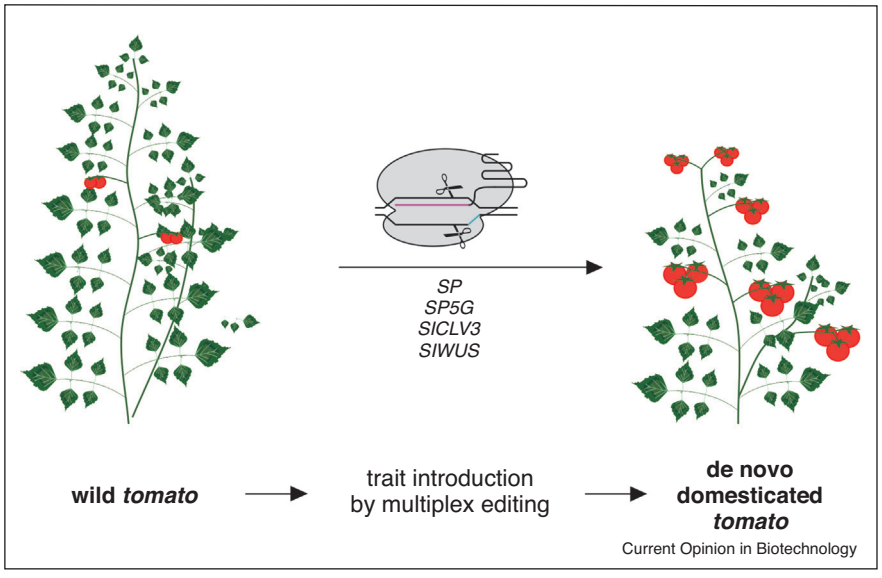
One of the major advantages of the CRISPR/Cas system is that it can be used to induce multiple DSBs and thus multiple mutations within a single plant generation. In the last two years, ground-breaking studies showed how breeding can not only be accelerated massively but that also outcomes can be produced that could not be achieved by classical breeding at all.

In rice for instance, the editing of up to eight agronomically important genes in a parallel fashion could be achieved. Thereby, heterozygous and homozygous mutants of individual genes, as well as homozygous sextuple, septuple, and octuple mutants, were obtained within one generation [46]. In further studies, the highly efficient multiplexing system was employed for clonal reproduction of F1 rice hybrids by simultaneously targeting three meiotic genes and one gene involved in fertilization (*REC8*, *PAIR1*, *OSD1* and *MTL*), leading to the fixation of hybrid heterozygosity and production of clonal seeds without the peril of increasing ploidy [47]. Approaches like this could enable the ultimate exploitation of the heterosis effect without the expensive and elaborate seed production of hybrids.

By the induction of several breaks, *cis*-regulatory elements (CREs) can be changed in complex ways. CREs comprises promoter, enhancer and silencer sequences regulating gene transcription. Thus, the manipulation of CREs allows a fine-tuning of gene expression. In tomato, a successful alteration of inflorescence architecture with improved flower production and yield was obtained by mediating reduced expression of the respective quantitative trait loci (QTLs) [48]. Moreover, multiplex editing of CREs provides access to a broad variation of alleles for rapid alteration of crop traits. In tomato, the group of Zac Lippman generated a biallelic promoter mutant for a trait regulating gene and transformed it with Cas9, targeting various sites in the native wild type but not in the mutant promoter. After backcrossing with WT plants, targeting of the WT allele resulted in a broad range of allele variations with an immediate impact on plant phenotype [49••]. Thus, the alteration of gene expression based on editing of CREs of developmental key transcription factors promises to be an attractive tool for the generation of trait variants.

However, further improvement of elite crops, such as tomato, has its limitations due to reduced genetic diversity caused by inbreeding over millennia. As a result, beneficial traits, such as disease resistance and stress tolerance, as well as nutritional value and taste were lost [50]. In contrast, wild species or uncultured varieties from elite crop species still harbor enormous genetic diversity, making them attractive for rapid domestication using genome editing. Over time, the number of identified genes which are connected to

Figure 4



De novo domestication of wild tomato.
To generate genomic diversity in crops like tomato, domestication genes, such as *SP*, *SP5G*, *SICLV3* and *SIWUS* were edited via a CRISPR/Cas9 multiplex approach [52••]. The simultaneous targeting of plant architecture genes resulted in determinate growth architecture, earlier and synchronized flowering and increased fruit size, without the loss of beneficial wild traits such as stress tolerance.

domestication events, expended dramatically, making it easier to apply the CRISPR/Cas technology to accelerate the domestication process [51].

The groups of Dan Voytas and Lazaro Peres succeeded in combining agronomically desirable traits with beneficial traits of wild crops by employing CRISPR/Cas9 technology [52••]. In their strategy, they used six independent genes, which are involved in yield and productivity in domesticated tomato, and targeted them in wild *Solanum pimpinellifolium* to enable *de novo* domestication. The engineered lines showed a threefold increase in fruit size and a tenfold increase in fruit number in a single generation. A similar approach was performed by Li *et al.* in four *S. pimpinellifolium* accessions, which are either salt tolerant or highly resistant to bacterial spot diseases [53•] (Figure 4). A recent study showed that domestication is also possible in another member of the *Solanaceae* family, *Physalia pruinosa*, which was an outstanding acquisition due to the lack of a reference genome, gene annotation data and transformation protocol [54•]. As target genes for genome editing, the *P. pruinosa* orthologues of *SP*, *SP5G* and CLV1 were selected. While the null alleles of *Ppr-sp* resulted in a limitation of fruit production, *Ppr-sp5g* mutants depicted moderate shoot termination and an up to 50% higher fruit number along each shoot. Additionally, a *Ppr-clv1* null allele took a huge effect on fruit size, with a 24% increase in fruit mass, accentuating that semi-domesticated and wild crops can be domesticated with CRISPR/Cas technology.

Conclusion

The progress obtained with CRISPR/Cas in the last few years is breath taking. Because of concerted efforts of the scientific community and the ever-growing numbers of users, the technology is accelerating the pace of achievable genome change in an unprecedented manner. The transformation of a cultivated crop out of a wild plant in a single generation is an impressive example of how far the technology can take us in the field of breeding, just seven years after its first application. Mankind might be able to obtain novel kinds of crop plants within the next decade. Recently, the first CRISPR/Cas-based directed evolution platform could also be set up for plants [55]. One can be optimistic that in the future, novel developments of the technology will also result in a harvest far beyond our expectations.

Conflict of interest statement

Nothing declared.

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Our excuses to all those colleagues we were not able to cite with their exciting work. Owing to space limitations, we could only discuss a fraction of the work relevant to the topic. We want to thank all colleagues of our group for sharing the enthusiasm on the CRISPR/Cas systems, Amy Whitbread for critical reading of the manuscript and the European Research Council (ERC advance grant "CRISBREED" to HP) for generously funding the research on genome engineering in plants.

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- •• of outstanding interest

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