

# Applying CRISPR/Cas for genome engineering in plants: the best is yet to come

Holger Puchta



Less than 5 years ago the CRISPR/Cas nuclease was first introduced into eukaryotes, shortly becoming the most efficient and widely used tool for genome engineering. For plants, efforts were centred on obtaining heritable changes in most transformable crop species by inducing mutations into open reading frames of interest, via non-homologous end joining. Now it is important to take the next steps and further develop the technology to reach its full potential. For breeding, besides using DNA-free editing and avoiding off target effects, it will be desirable to apply the system for the mutation of regulatory elements and for more complex genome rearrangements. Targeting enzymatic activities, like transcriptional regulators or DNA modifying enzymes, will be important for plant biology in the future.

## Address

Botanical Institute, Molecular Biology and Biochemistry, Karlsruhe Institute of Technology, Kaiserstr. 12, 76131 Karlsruhe, Germany

Corresponding author: Puchta, Holger ([holger.puchta@kit.edu](mailto:holger.puchta@kit.edu))

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## Introduction

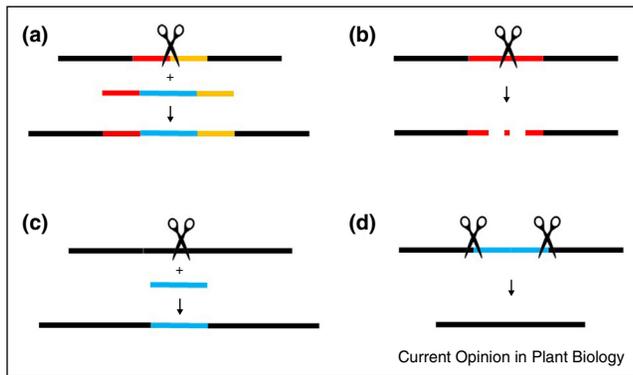
For more than twenty years it has been known for plants that by inducing unique double-strand breaks (DSBs) by a site-specific endonuclease various genomic changes can be achieved [1,2]. There are in principle two different ways how a DSB can be repaired, either by homologous recombination (HR) or by non-homologous end joining (NHEJ). NHEJ by far outperforms HR in somatic plant cells [3] HR can be applied to target predefined changes into specific genomic sites [1]. Via NHEJ it is possible not only to knockout genes [4] but also to excise sequences from [5] or to insert sequences [2] into a genomic site (for details see [Figure 1](#)).

A prerequisite for applying the technology genome-wide is the ability to induce DSBs specifically at the sites of interest. For this purpose, synthetic nucleases, namely

zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which allow the targeting of most genomic sites, have been developed and applied for a long period of time in plants (for reviews see [6–8]). Although with the advent of ZFNs the basic problem of targeting DSBs to specific site in the genome was solved, the system revealed numerous drawbacks. The construction of the enzymes is time consuming and expensive and their specificity is limited, resulting in DSB at genomic site that very similar but not identical to the target site. Thus, unwanted secondary, ‘off-target’ mutations were introduced regularly. After their introduction TALENs outcompeted ZFNs in specificity as well as in cost and time requirements. Still, a reasonable effort was required and only a fraction of the plant laboratory applied the technology for their purposes. However, only with the characterization of the Cas9 protein as an easily reprogrammable nuclease from the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system of the bacterium *Streptococcus pyogenes*, has the field got a tool in hand that can be used quickly and with ease by any molecular biology laboratory [9\*\*]. Thus, genome engineering has become routine. The tool relies on a simple and efficient mechanism of sequence-specific DSB induction. A short CRISPR RNA (crRNA) directly binds to a 20-nt recognition site (RS) on the DNA, the so-called protospacer. The sequence motif ‘NGG’ downstream of the protospacer (termed the protospacer-adjacent motif, PAM) is crucial for binding. A second RNA, termed trans-activating crRNA (tracrRNA), binds the crRNA, and the protein Cas9 is recruited to the complex. Cas9 is an endonuclease that contains two nuclease domains, the RuvC-like domain and the HNH motif, each cleaving one of the two DNA strands that are three base pairs upstream of the PAM. The crRNA and the tracrRNA have been fused to a unique single guiding (sg) RNA, that makes application even more easy ([Figure 2](#)).

After the demonstration of its applicability in plant biology [10–12], the main focus of using the CRISPR/Cas system became the production of NHEJ-mediated heritable mutations in a number of species, by inducing a DSB within ORFs of genes of interest. An impressive example of the power of the strategy for agriculture was the production of powdery mildew resistant wheat plants, by the knockout of all six alleles of the three *MLO* genes present in the hexaploid species [13\*\*]. It is far beyond the scope of this review to give an overview about all crop plants that could successfully be engineered by CRISPR/Cas and the reader is referred to various current reviews

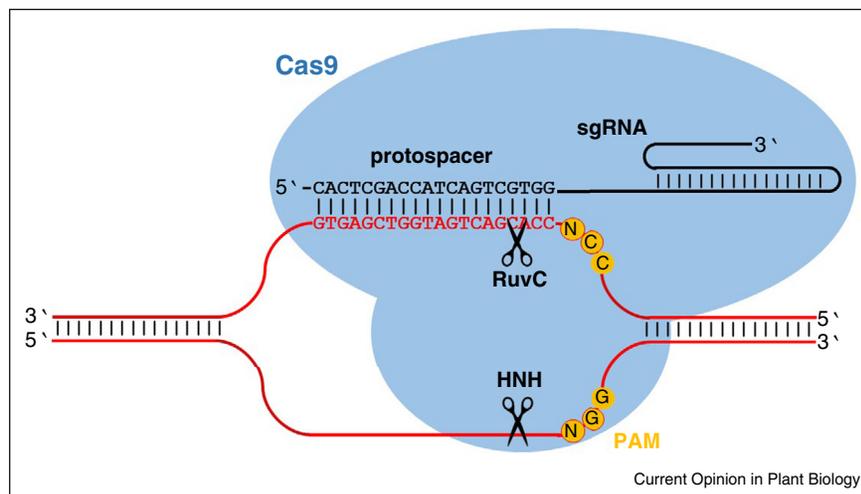
Figure 1



DSB mediate genome engineering in plants: genomic change can be achieved either by inducing homologous recombination (a) or by non-homologous end joining (b)–(d). Gene targeting by homologous recombination (HR) uses regions of homology (in red and orange) that are present in the vector DNA as well as in the genomic sequence for integration. Thus, larger DNA fragments (shown here in blue) can be inserted or single amino acids can be exchanged in a controlled predictable manner. In contrast the simplest way to apply NHEJ is to use its feature that this repair reaction is often imprecise. If a break is induced in an open reading frame due to deletion or insertion the genomic information is often destroyed resulting in a null phenotype. (b) Mutation induction by NHEJ, red: gene to be knocked out; As NHEJ is by far the most prominent way of DSB repair it can also be applied for (c) DNA integration by NHEJ blue: sequence to be integrated into the genome or (d) induction of controlled DNA deletions; blue: genomic sequence to be deleted.

on the topic [14–18]. Interestingly, such plants are not considered as GMOs, at least in some countries. Here, I will not focus on what has been achieved but on some recent developments in applying the technology that will become important for plant biology in the near future.

Figure 2



The CRISPR/Cas system of *S. pyogenes* to induce artificial DSBs: the Cas9 protein is responsible for the cleavage of both strands of the target DNA via its RuvC and HNH domains. The binding specificity is defined by a stretch of 20 bases of the single guiding (sg) RNA which is complementary to the respective target site. Via the protospacer-adjacent motif (PAM) the DNA is interacting with the Cas9 protein. Thus the PAM sequence contributes to the recognition site specificity.

## NHEJ mediated gene knock out: becoming 'DNA-free'

Gene editing by NHEJ has been achieved by transformation of DNA expressing the nuclease into plant cells, which is either transiently expressed or stably integrated. If integrated, the foreign DNA can be eliminated by out-segregation of the progeny of the modified plant [19]. Although, in either case, the product will not contain transgenic DNA, depending on local laws, regulation authorities in a number of countries may still classify the plants as genetically modified organisms due to recombinant nucleic acids being involved in their production [20]. Therefore, over the years, approaches have been carried out to avoid DNA expressing the nuclease in the process of site-specific DSB induction in plant cells. There are two main strategies: either to use RNA viruses as vectors or to directly transfer a functional nuclease into plant cells.

The feasibility of the viral strategy was first demonstrated using a Tobacco rattle virus (TRV)-based expression system for indirect transient delivery of ZFNs into tobacco and petunia [21]. More recently, this technology has also been used for NHEJ-mediated gene editing using the CRISPR/Cas system [22]. Alternatively, one can directly transfer active enzyme molecules or their mRNAs into plant cells, to achieve the desired mutations. In principle different kinds of transfer can be envisaged as there are microinjection, PEG transformation or electroporation as well as particle bombardment. Production of heritable plant mutations was reported using either the purified TALEN protein [23] or its mRNA [24]. In the case of the CRISPR/Cas system, the active Cas9 nuclease has to be formed by combining the protein with its

respective sgRNA(s). Transfecting such a complex into protoplasts of various plant species, demonstrated the applicability and feasibility of DNA-free technology [25\*].

### **NHEJ-mediated gene knock out: becoming more precise**

Off-target effects of synthetic nucleases, including Cas9, have been of general concern over the years. For plants, several reports exist stating that such effects can be found with Cas9 [26], although the opposite has also been reported [27]. There are different level to detect such off target effects, obviously the determination of the complete genomic sequence of the mutated plant is the most reliable one [26] whereas other groups tested only the sequence of sites with close homology to the target [28]. Secondary sites can also be detected by digesting purified genome DNA with the respective purified nuclease and consecutive whole genome sequencing [29]. Besides selecting for non-redundant sites for sgRNA construction, there are two recent technical developments that drastically reduce off-target effects. On the one hand, engineered Cas9s have been constructed, although not yet applied in plants, that are more specific in their sequence recognition than the natural enzyme. The most prominent example is SpCas9-HF1, harbouring alterations designed to reduce non-specific DNA contacts while retaining on-target activities comparable to wild-type SpCas9 [30]. On the other hand, Cas9 nuclease can be transformed into a nickase by a point mutation in the active centre of either the RuvC or the HNH domain (Figure 2). By the use of two sgRNAs, paired single strand breaks can be induced at a specific distance in both strands, enhancing the specific recognition from 20 to 40 bps [31]. This approach has been applied in plants [32], where it could be demonstrated that off-target effects were minimized [28]. A recent study showed that the paired nickase approach can efficiently be used for inducing mutations in both intragenic and intergenic regions, as well as in heterochromatin, in Arabidopsis [33\*].

### **New and more complex targets for genome engineering**

Besides modifying ORFs and genes using NHEJ-induced mutations, Cas9 is also a valuable tool for modifying genomic sequences involved in regulation (Figure 3) [34]. Although such applications in plants are only just beginning to be used [35], in mammals, the setup of a multiplexed editing regulatory assay enabled for the screening of thousands of mutations in terms of their influence on gene activity. Therefore, new regulatory elements can be identified that do not have typical features [36,37]. For breeding applications, the introduction of new SNPs at defined genomic positions by NHEJ-mediated DSB repair to modulate expression of factors

influencing yield quality and quantity, might also be attractive.

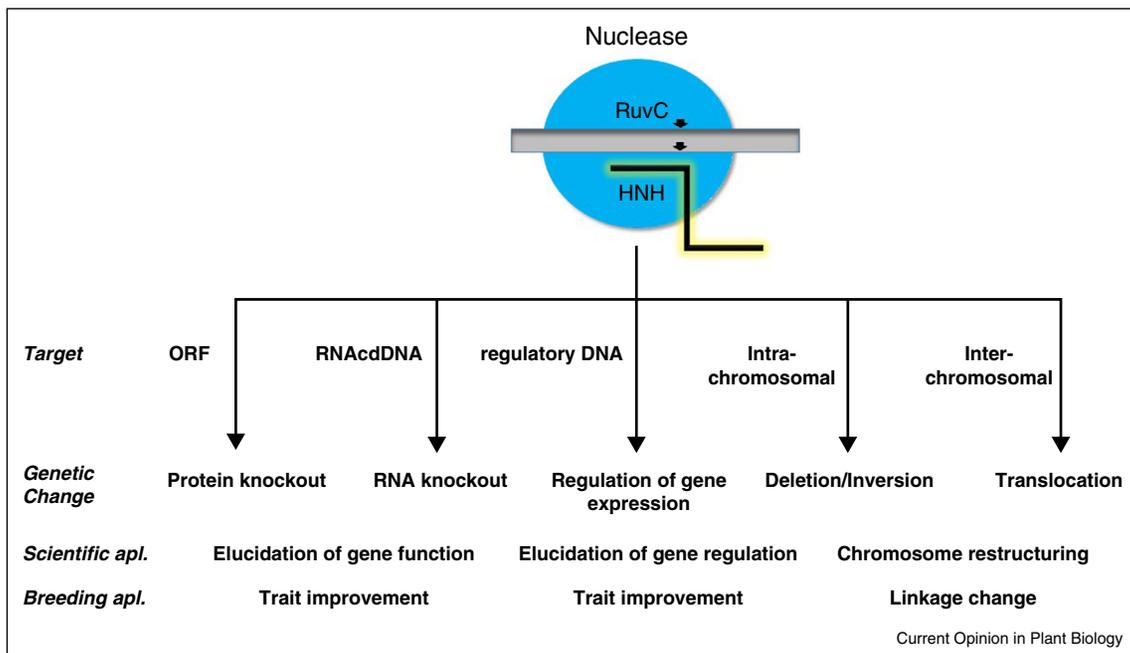
By inducing two genomic DSBs in close proximity, it has become routine to induce smaller deletions that can be transferred to the next generation in plants [38,39]. However, although possible, there have not yet been any reports on the production of larger heritable inversions [40,41] nor reciprocal chromosomal translocations [42], by the use of synthetic nucleases. For breeding, reversion of inversions on homologues might help with the transfer of traits from wild relatives to elite cultivars. Inducing translocations could potentially enable new linkages between beneficial traits to be made or to break undesired linkages.

In meiosis natural induction of DSBs by the Spo11 protein results in at least one crossover (CO) per parental homologue pair. Thus, linkages between traits that are present only in one or the other parental chromosome can be broken. The plant breeder uses this phenomenon to single out adverse traits or combine attractive traits in specific cultivates. However, CO are not distributed evenly over chromosomes and many traits cannot be unlinked [43]. Therefore it would be highly desirable to induce CO by artificial DSB induction between homologues at predefined sites in plants. In yeast, this has been achieved in different ways: In a pioneering study already more than 10 years ago, a specific DNA binding domain was fused to the Spo11 protein, to directly enhance meiotic recombination [44\*]. Furthermore, mitotic crossovers could also recently be obtained by Cas9-induced HR [45].

### **HR-mediated gene targeting: still room for improvements**

Induction of DSB increases integration of a DNA carrying homologies to the target locus by orders of magnitude in plants [1]. A number of recent papers have been published that demonstrate that via Cas9-mediated induction of DSBs HR-mediated gene targeting frequencies can be enhanced drastically (for a recent review see [46]). Nevertheless, whereas mutation induction by NHEJ reaches efficiencies of sometimes close to 100% of plants in the progeny, most reports indicate a HR efficiency merely in the low percentage range. Thus, the amount of work that has to be invested to achieve HR-mediated gene targeting is at least one to two orders of magnitude higher than with NHEJ, which is especially disappointing for crop plants with low transformation efficiencies. The reason for this is that in somatic plant cells, NHEJ is much more efficient than HR in repairing DSBs [47]. Various approaches have been carried out to try to improve this situation, for instance by excising an integrated vector out of the genome [19,48\*,49]. In another promising approach the vector DNA transformed into plant cells that carries outside the homologous region a geminiviral replicon and

Figure 3



Multiple types of genomic targets for Cas9 nuclease-mediated NHEJ and their applications (apl.). The classical nuclease is depicted with a single guiding RNA. The HNH and RuvC domains that are responsible for inducing single strand breaks in one or the other DNA strand — resulting in a DSB — are also shown. Applications useful for plant biology, as well as plant breeding, are listed. Besides the knockout of proteins or RNA coding genes, regulatory elements can be manipulated by the technology. On the other side, multiple breaks in one or several chromosomes can be used to induce genomic rearrangements like deletions, inversions or translocations, that are also attractive for changing genetic linkages in breeding (blue: Cas9 protein, black: sgRNA; grey bar: DNA; RNAcdDNA: structural RNA coding DNA).

the replication factor Rep. Thus replication of the vector DNA is initiated. The resulting increase in copy number of the target vector as well as the induction of a replication-prone status of the cell by the geminiviral Rep protein both contribute to enhanced DSB-induced targeting frequencies [50,51]. Additionally, inhibition of NHEJ, for example, by knocking out Ligase 4 [52] can help to improve GT frequencies. The transfection of DNA oligonucleotides [53,54] has also been applied successfully for DSB-induced gene correction in plants. Despite all these efforts, it is feasible but still laborious to modify genomes of plants via DSB-induced HR, especially if transformation efficiencies of the respective species is low. It will be interesting to see whether the situation can be further improved by the development of more sophisticated technologies in the near future.

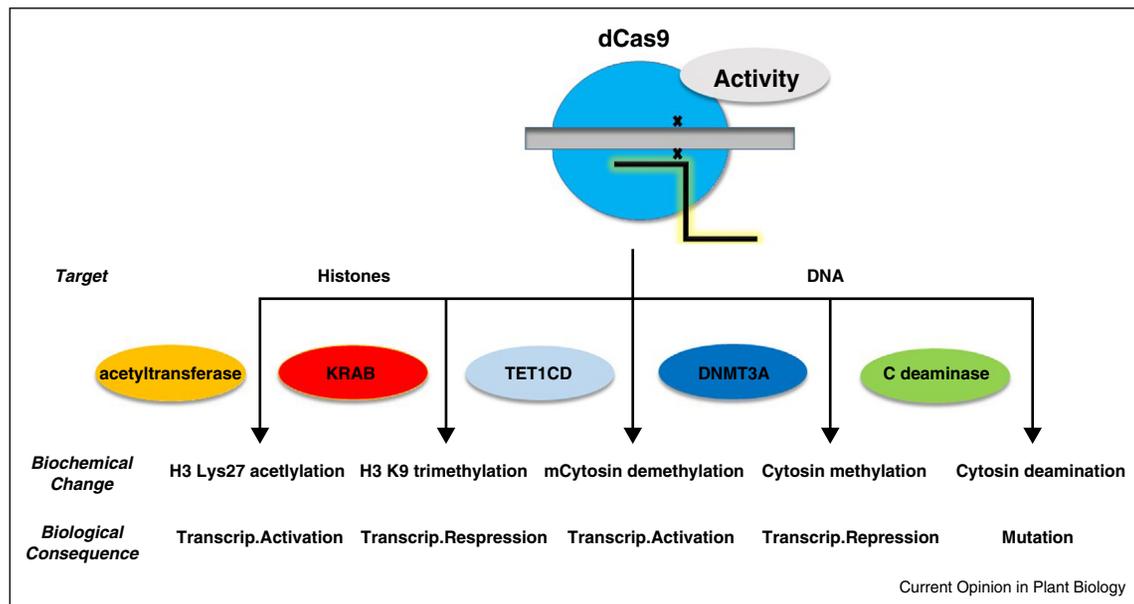
In principle DSB induction can also be used for site-specific integration via NHEJ [2]. Recently such a CRISPR-Cas-mediated transgene knock in was reported [55] with an efficiency, which makes this approach a valuable alternative to gene targeting via HR (see also Figure 1c). The authors targeted an exon containing construct into an intronic region. Thus resulting small changes introduced by NHEJ during the intron-knock in are unlikely to affect subsequent mRNA splicing or expression of the knocked in exon. Using this approach,

a 1.6 kb gene construct was introduced with an efficiency of 2.2%. However, one has to keep in mind that integration of the fragment might also occur elsewhere in the genome. Therefore, the obtained recombinant lines have to be analyzed whether they contain extra copies of the insert at ectopic sites.

### Using dCas9 fusion for transcriptional regulation and *in vivo* labelling

Converting Cas9 to a DNA binding protein [dead or dCas9], eliminating its nuclease activity by two simultaneous point mutation in the active centre of both the RuvC and the HNH domain (Figure 2), and combining it by translational fusion with an enzymatic function, enables the development of a tool that can selectively target any kind of activity to any specific genomic sequence (Figure 4). The respective enzymatic domain can either be fused directly to Cas9 [56] or indirectly to an RNA binding protein, that is able to interact with an aptamer sequence integrated within the respective sgRNA [57]. Thus, the CRISPR/Cas system can be used to switch on and off genes, which can be achieved directly by blocking accessibility or recruiting relevant factors, or indirectly by modification of the histone code or by changes in DNA methylation at specific genomic sites [58]. Histone modification can be achieved by fusing the catalytic core of the human acetyltransferase p300 to

Figure 4



Multiple applications of dCas9 fusions for histone and DNA modifications. The dead dCas9 as DNA-binding domain, the specificity of which is defined by its sgRNA fused to another protein domain with activity to modify the respective target molecule. Thus, activation or repression of transcription via epigenetic change, for example, by covalent modification of histone H3 or by methylation of cytosine or demethylation of m-cytosine, can be achieved. By deamination of cytosine a uracil arises, which – if not eliminated by DNA repair before – will pair in the next round of DNA replication to an adenine instead of guanine, leading to a point mutation in one sister cell (blue: dCas9 protein, black: sgRNA; grey bar: DNA).

dCas9. The fusion protein catalyzes acetylation of histone H3 lysine 27 at its target sites, leading to robust transcriptional activation of target genes [59]. Fusions of dCas9 to the Krüppel-associated box (KRAB) repressor (dCas9-KRAB) can silence target gene expression by inducing highly specific induction of H3K9 trimethylation (H3K9me3), at the target region [60]. Alternatively, gene activation can also be achieved via cytosine demethylation following fusion of the dCas9 to the catalytic domain of the mammalian Ten-Eleven Translocation (TET) dioxygenase1 (TET1CD) [61]. In contrast to mammals in plants the ROS1 glycosylase-lyase family is involved in demethylation [62] making this enzyme class also a good candidate for fusions. Whereas DNA methylation can be induced to shut down gene expression by fusing dCas9 to the DNA methyltransferase DNMT3A [63]. dCas9-mediated regulation of gene expression has rarely been used for plants at present [64,65], however one does not have to be a prophet to foresee that it will become more widely applied in the future. Similarly, direct or RNA-mediated fusion of dCas9 with fluorescent marker proteins can be used to visualize genomic loci, in living cells, to investigate chromosome dynamics [66,67], a technique that will also be attractive for plant cytology.

### Introducing mutations without breaking DNA

Very recently, a new approach to genome editing that enables the direct, irreversible programmed conversion of one target DNA base into another, called ‘base editing’,

was developed for mammalian cells [68,69]. dCas9 was fused to cytidine deaminase enzyme, mediating the direct conversion of cytidine to uridine (Figure 2) so that following replication of the duplex a C/G bp was changed into a T/A bp, in one of the daughter cells. Thus, without DSB induction, point mutations within a window of 5 nucleotides can be introduced. However, as Uracil is also incorporated into DNA by mistake or can naturally arise by deamination of cytosine, relatively efficient DNA repair mechanisms exist in eukaryotic cells that eliminate uracil, which ultimately have to be blocked in order to obtain reasonable mutation frequencies. It will be interesting to see whether such a relative sophisticated technology is also attractive for application in plants.

### New game players: Cas9 orthologues and Cpf1

Different kinds of CRISPR/Cas systems can be found in different bacteria. Only type II is characterized by an active nuclease entity consisting of a single protein (Cas9) and the crRNA-tracrRNA complex [70]. Multiple orthologues of Cas9 in bacteria are attractive for use in genome engineering, as they differ in their protospacer adjacent motif (PAM) sequences, which limits the flexibility of sequence recognition. Nucleases from *Staphylococcus aureus* (SauCas9) and *Streptococcus thermophilus* (Sth1Cas9) have been used recently, at least as efficiently as the *S. pyogenes* Cas9 (SpyCas9), for genome engineering in plants [71–73]. Importantly, it could be demonstrated

that SauCas9 and SpyCas9, and their specific sgRNA, do not interfere with each other in plant cells [71]. As a result, it is possible to apply these enzymes simultaneously in one plant cell to target differently modified Cas9 orthologues to different sites in the same genome. Thus, at the same time DSBs and SSB can be induced in a genome, or transcription of different sets of gene can be switched on and off simultaneously in the same cell. In the long run, building up synthetic entities by the use of the CRISPR/Cas will become possible for plants (for review see [74]).

Besides the classical Cas9 orthologues, the nuclease Cpf1 was recently characterized. This enzyme produces 4 bp overhangs [75,76], as opposed to the blunt ends the classical Cas9 orthologues generate, making it an exceptionally attractive alternative when wishing to achieve more complex genome rearrangements. With sticking ends the efficiency genomic insertions into a complementary site in the genome as well deletion formation might be enhanced in comparison to blunt ends. Interestingly, recent studies with Cpf1 in the human genome, demonstrate drastically fewer off target effects than for classical Cas9 nucleases [77,78]. Consequently, Cpf1 proves extremely attractive for use in plant biology and its successful application will only be a matter of time.

## Conclusions

The very recent discovery of bacterial enzymes of the C2c2 class [79], opens up opportunities for site-specific RNA manipulations, an area that has hardly addressable till now for eukaryotes. In general, the development of the CRISPR/Cas field is so fast, that it is hard to foresee all the potential applications, even in the near future. However, without doubt, due to CRISPR/Cas, molecular biology has never been more exciting than now.

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My excuses to all those colleagues I was not able to cite with their exciting work. Due to space limitations, I could only discuss a fraction of the work relevant to the topic. I want to thank my group for sharing the enthusiasm on the CRISPR/Cas system, Manfred Focke, Simon Schiml, Amy Whitbread and Felix Wolter for critical reading of the manuscript and the European Research Council (ERC) for generously funding my research on genome engineering.

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