The CRISPR/Cas revolution continues: From efficient gene editing for crop breeding to plant synthetic biology[FA]

Jochen Kumlehn[1], Janine Pietralla[2], Goetz Hensel[1], Michael Pacher[2] and Holger Puchta[2]*

1. Plant Reproductive Biology, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), 06466 Seeland OT Gatersleben, Germany
2. Botanical Institute, Molecular Biology and Biochemistry, Karlsruhe Institute of Technology, 76131 Karlsruhe, Germany
doi: 10.1111/jipb.12734

Holger Puchta
*Correspondence:
holger.puchta@kit.edu

Abstract Since the discovery that nucleases of the bacterial CRISPR (clustered regularly interspaced palindromic repeat)-associated (Cas) system can be used as easily programmable tools for genome engineering, their application massively transformed different areas of plant biology. In this review, we assess the current state of their use for crop breeding to incorporate attractive new agronomical traits into specific cultivars of various crop plants. This can be achieved by the use of Cas9/12 nucleases for double-strand break induction, resulting in mutations by non-homologous recombination. Strategies for performing such experiments — from the design of guide RNA to the use of different transformation technologies — are evaluated. Furthermore, we sum up recent developments regarding the use of nuclease-deficient Cas9/12 proteins, as DNA-binding moieties for targeting different kinds of enzyme activities to specific sites within the genome. Progress in base deamination, transcriptional induction and transcriptional repression, as well as in imaging in plants, is also discussed. As different Cas9/12 enzymes are at hand, the simultaneous application of various enzyme activities, to multiple genomic sites, is now in reach to redirect plant metabolism in a multifunctional manner and pave the way for a new level of plant synthetic biology.

INTRODUCTION

A major challenge for crop improvement is the limited genomic variability within elite breeding material. Since the 1950s, the application of genotoxic agents, such as chemicals or irradiation, were used to induce new alleles by random mutagenesis. This technology was time-consuming and ineffective; multiple mutations were introduced in the same genome, simultaneously, and many resulted in adverse effects (Pacher and Puchta 2017). This situation changed only when it became possible to introduce precisely located breaks into DNA, *in vivo*, by the use of site-specific nucleases. Targeted rearrangements require transient opening of the genetic information, with a precise cut, followed by changes catalyzed by the cellular DNA repair machinery. Base editing, directed transcriptional regulation and epigenetic modifications are further developments arising from the ability to target specific genomic sites.

This portfolio of genome modifications became available mainly within the last decade with the discovery of molecular effectors that: (i) can be targeted easily to specific sequences in a genome or transcriptome; (ii) possess intrinsic nuclease activity; or

(iii) are modified to derivatives that deliver defined biochemical reactions or properties to genomic loci or transcripts. This molecular toolbox can be applied both in plant biotechnology, to explore and expand the genetic potential of crops as a major source of nutrition and renewable energy, as well as facilitating and accelerating breeding processes to increase the economic value of products thereof.

In this review, we provide a state-of-the-art overview of the available molecular tools, including their derivatives, their properties and mode of action, their handling in wet-lab activities and advances in plant biotechnological applications, based on these revolutionary techniques. We conclude with an outlook of possible future applications of these tools and considerations as to how the existing toolset might evolve, providing unprecedented possibilities to the growing field of plant synthetic biology.

SEQUENCE-SPECIFIC INDUCTION OF DNA DOUBLE-STRAND BREAKS

The ability to introduce unique genomic site-specific DNA double-strand breaks (DSBs), *in vivo*, dates back to the discovery and characterization of the homing endonuclease I-*Sce*I in *Saccharomyces cerevisiae* (Jacquier and Dujon 1985; Perrin et al. 1993). Expression of this molecular scissor, in plants, harboring its 18mer target sequence, led not only to fundamental progress in the understanding of DSB repair processes, in somatic plant cells (Puchta 2005), but also to the demonstration that genes can be knocked out (Salomon and Puchta 1998), or genomic loci be changed in a controlled manner (Puchta et al. 1996).The vast majority of DSBs are repaired via non-homologous end joining (NHEJ), a highly mutagenic mechanism of partial end-resection and re-ligation of the free ends. Nevertheless, the intrinsic DSB repair machinery has the potential to remove breaks also in an error-free manner, namely homologous recombination (HR), which utilizes allelic or ectopic sequence homologies as a repair template. The equilibrium between HR and NHEJ is strongly biased to the latter in somatic plant cells, as a result of evolutionary requirements to ensure genomic integrity in highly complex and partially repetitive genomes (Cavalier-Smith 2005; Puchta 2005). Thus, the knowledge of repair pathway utilization, key factors involved therein and the superimposed regulatory mechanisms is crucial to any

rational design aiming for efficient generation of modified plant genomes (Puchta and Fauser 2015). So far, both DSB repair pathways have been experimentally addressed to obtain various genetic alterations.

The prevalent DSB repair pathway NHEJ has been largely exploited for obtaining targeted mutations, but furthermore, its potential to specifically invert or delete genome fragments, break genetic linkage groups, or reshuffle chromosome orientations has been recognized (Pacher et al. 2007; Lee et al. 2012; Le Cong et al. 2013; Li et al. 2013; Mali et al. 2013; Wang et al. 2013; Weinthal et al. 2013; Filler Hayut et al. 2017). In addition, HR-mediated DSB repair has been applied for targeted sequence alterations (Steinert et al. 2016). This has been achieved by making use of recombination substrates, such as extrachromosomal templates provided, for example, via an incoming T-DNA from *Agrobacterium tumefaciens*, transfected plasmids, biolistic delivery of DNA sequences, or delivery by viral replicons (Baltes et al. 2014; Čermák et al. 2015; Butler et al. 2016; Čermák et al. 2017; Gil-Humanes et al. 2017). Both the controlled introduction of very minor sequence modifications and the insertion of new genes in transgenic and natural loci have also been achieved with the *in planta* Gene Targeting (GT) method (Fauser et al. 2012; Fauser et al. 2014; Wolter et al. 2018), in combination with the use of viral replicons (Dahan-Meir et al. 2018), or in combination with developmentally-controlled promoters (Miki et al. 2018; Wolter et al. 2018).

While initial efforts for targeted genome rearrangements, applying rare cutters like I-*Sce*I, were limited by the need of artificially inserting the target site as part of a transgene, the development and availability of engineered zinc-finger nucleases (ZFN) (Kim et al. 1996; Smith et al. 2000), transcription activator-like effector nucleases (TALENs) (Boch et al. 2009; Moscou and Bogdanove 2009; Cermak et al. 2011) and, most recently, the CRISPR (clustered regularly interspaced palindromic repeat)-associated (Cas) system, enabled the generation of new genome engineering tools for addressing nearly all genomic loci with unprecedented accuracy and efficiency (Voytas 2013; Mahfouz et al. 2014; Belhaj et al. 2015; Schaeffer and Nakata 2015; Lee et al. 2016; Weeks et al. 2016; Pacher and Puchta 2017; Puchta 2017; Schindele et al. 2018).

The availability of such new tools not only allowed the development of a better understanding of DNA repair pathways (e.g. Schiml et al. 2016), but also

provided means for generation of more than one defined break, simultaneously, within a genome to induce more complex changes, such as deletions, inversions and translocations. The following section provides a state-of-the-art overview on the tool set, its intrinsic properties and artificially designed derivatives thereof, for enhancing the possibilities of plant synthetic biology.

CLASSES OF CAS-ENDONUCLEASES

Clustered regularly interspaced short palindromic repeats and the Cas proteins form a prokaryotic defense mechanism against bacteriophages and mobile genetic elements (Wiedenheft et al. 2012). This adaptive immune system is able to integrate fragments of the invading genomes, as so-called spacers, in the CRISPR array and provides a sequence-based memory of previous infections (Hille and Charpentier 2016; Barrangou and Gersbach 2017; Murugan et al. 2017). Two proteins, Cas1 and Cas2, are essential for most CRISPR/Cas systems to deliver the foreign DNA fragments to the CRISPR array (Koonin et al. 2017). During the second infection, the CRISPR locus, encoding the effector Cas proteins and spacers, is transcribed into the so-called precursor crRNA (pre-crRNA). Various mechanisms exist to process the pre-crRNA, in the different CRISPR/Cas systems, although they are not yet completely understood.

Independent of the manner of processing, the crRNA contains a spacer, which is complementary to viral DNA and provides the target specificity of the Cas system. The crRNA interacts with a Cas effector and guides the effector protein accurately to the DNA or RNA target motif, where the complex performs its cleavage function (Jinek et al. 2012). As a consequence of reciprocal selective pressure, a wide variety of defense strategies against viral invaders has evolved and therefore, there exists a high diversity of structural varieties between the individual CRISPR/Cas systems (Wiedenheft et al. 2012; Makarova et al. 2015).

CRISPR/Cas systems essentially comprise two classes: the most abundant CRISPR/Cas system in bacteria and archaea is referred to as class 1, which contains multi-subunit effector complexes and includes type I, type III and type IV systems. Class 2 consists of three types of single-protein effector modules: type II,

type V and type VI. Each type can also be subdivided into several subtypes (Makarova et al. 2015; Koonin et al. 2017). The most extensively studied Cas protein is the single effector endonuclease, Cas9, of the type II-A system (Jinek et al. 2012; Fonfara et al. 2014). A second RNA-guided effector nuclease was identified with bioinformatics studies (Schunder et al. 2013) and was classified as Cpf1 (now Cas12a), a class 2 type V CRISPR/Cas system (Makarova et al. 2015). Recently, three novel class 2 effectors, C2c1, C2c2 and C2c3, were identified by a computational pipeline (Shmakov et al. 2015). Two of these proteins, C2c1 and C2c3, showed similarity to the already known type V effector, Cas12a, and were recently renamed as Cas12b and Cas12c (Murugan et al. 2017). The third effector, C2c2 (now Cas13a), shows a higher divergence and was assigned to the new class 2 type VI CRISPR/Cas system (Shmakov et al. 2015).

The RNA-guided endonuclease Cas9 of the class 2 type II CRISPR/Cas system from *Streptococcus pyogenes* has become the best characterized single effector protein and an important biotechnological tool for molecular biology (Jinek et al. 2012). Like most class 2 effectors, Cas9 consists of two lobes. A recognition domain (REC) is connected over an arginine-rich bridge helix with the nuclear domain (NUC) (Jinek et al. 2014). The NUC lobe contains two catalytically active subdomains, RuvC and HNH, which are required for the induction of the DSB. The HNH domain is responsible for the cleavage of the DNA strand that is complementary to the spacer region of the guide RNA (gRNA), whereas the RuvC-like domain cleaves the non-complementary DNA strand (Jinek et al. 2012).

In order to find foreign DNA, the Cas9 nuclease requires this gRNA, which is formed by the crRNA and a tracrRNA and a short G-rich sequence motif, termed protospacer adjacent motif (PAM) positioned next to the gRNA-specific part of the target sequence (Figure 1A). Upon formation of the Cas9/gRNA complex, it scans the DNA for the PAM sequence. Due to the nature of the CRISPR/Cas system, the effector protein is able to target simultaneously multiple loci,. Upon PAM identification, the complex separates the DNA double strand and checks whether the spacer on the crRNA is complementary to the sequence next to the PAM site. After formation of a hetero-duplex, the nuclease domains of Cas9 are activated and induce a blunt-end DSB three base pairs (bp) upstream of the PAM (Garneau et al. 2010; Jinek et al. 2012; Chaudhary et al. 2018).
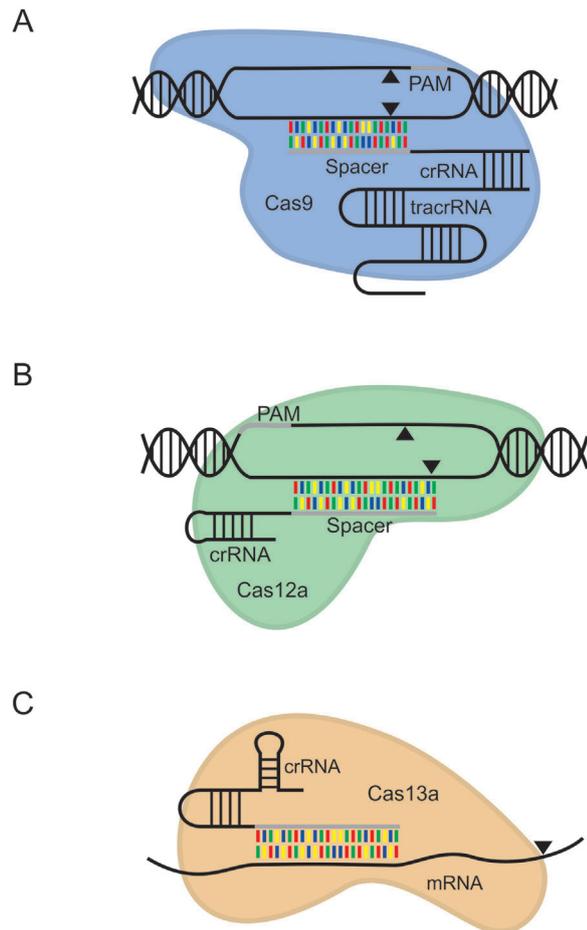
Figure 1. Schematic representation of the three CRISPR Class 2 single-protein effectors, Cas9, Cas12a and Cas13a

(A) After hybridization of the small RNAs, tracrRNA and crRNA, a complex with Cas9 is formed. The crRNA contains a 20 nucleotide guide sequence (Spacer) and mediates sequence-specific DNA binding at the recognition site upstream of the protospacer adjacent motif (PAM). Subsequent to Cas9 recruitment to the target DNA, a sequence-specific double-strand break (DSB) is induced. **(B)** In contrast to Cas9, Cas12a requires only a single crRNA for DNA targeting and the 23–25 nucleotide recognition site is located downstream of the PAM sequence. The Cas12a nuclease-induced DSB possesses 5 nt single-strand DNA overhangs, 18 to 23 nucleotides distal and downstream to the PAM in the spacer region. **(C)** The Cas13a-mediated RNA-specific cleavage activity requires one single crRNA. Binding of the Cas13a/crRNA complex to its target RNA is mediated by the guide sequence of the crRNA. RNA cleavage occurs at the catalytically active sites of Cas13a located on the outer protein surface. **(A–C)** All three single-protein effectors can be specifically directed to almost any target DNA/RNA by exchanging the specific recognition sequences of the crRNA.

An additional well-characterized single RNA-guided endonuclease is Cas12a (formerly Cpf1), which is assigned to the class 2 type V CRISPR/Cas system. Despite the same function and nearly identical size, there are some important differences compared to the Cas9 protein. In contrast to Cas9, Cas12a requires a shorter crRNA and there is no evidence that a tracrRNA is required (Zetsche et al. 2015). Furthermore, Cas12a is able to process the pre-crRNA itself (Fonfara et al. 2016). As opposed to type II CRISPR-Cas systems, where the crRNA scaffold is located at the 3′ end, the crRNA scaffold of the type 5 CRISPR/Cas systems is located at the 5′ end (Figure 1B) (Zetsche et al. 2015). Additionally, the protein creates a DSB by DNA cleavage distal from the PAM sequence and the seed region, where the crRNA is more tolerant of mismatches (Gao et al. 2016). The cleavage occurs 18 nucleotides downstream of the PAM, at the non-target strand, and 23 nucleotides downstream, at the target strand, which leads to a five nucleotide 5′ overhang (Zetsche et al. 2015). Presumably the overhang is due to the sole cleavage activity of the RuvC-domain (Swarts et al. 2017). Similar to Cas9, Cas12a also appears to have two nuclease domains, RuvC and a Nuc domain (Yamano et al. 2016), but recent structural analyses suggest that the Nuc domain supports DNA binding and lacks catalytic activity (Swarts et al. 2017). However, in contrast to Cas9, Cas12a requires T-rich PAMs, which increases the total number of potential target sites. Recently, a single-stranded DNA (ssDNA) cleavage activity of Cas12a was discovered, whereby Cas12a completely degrades non-specific ssDNA after being activated upon target binding (Chen et al. 2018).

What was previously only possible in the DNA world with Cas9 and Cas12a can now be extended into the RNA world with the RNase Cas13. This type of Cas protein was identified by bioinformatics analyses of bacterial and archaeal genomes (Shmakov et al. 2015). The programmable RNA-guided RNA-targeting CRISPR effector, Cas13a (previously C2c2), cleaves ssRNA molecules in a sequence-specific manner (Figure 1C) (Abudayyeh et al. 2016). Regardless of the similar origin, the protein shows no strong structural correlation to the previously characterized Cas proteins. It also displays a bi-lobed structure, but contains the NUC lobe and two conserved higher eukaryote and prokaryote nucleotide-binding (HEPN) domains.

Cas13a also differs in the location of the catalytic sites; in an activated state, the HEPN domain is located at the external surface, whereas the nuclease domains

of Cas9 and Cas12a are located inside the protein (Liu et al. 2017b; Wolter and Puchta 2018). Like Cas12a, Cas13a can also process the pre-crRNA by itself and possesses no tracrRNA (East-Seletsky et al. 2016). Furthermore, Cas13 shows general non-specific degradation of RNA, after activation, via target binding. This function is not affected by deactivation of the HEPN domains, indicating that there are two different catalytic sites (East-Seletsky et al. 2016). However, the "collateral" cleavage activity seems not to be present in eukaryotic cells (Abudayyeh et al. 2016), making Cas13a a suitable tool for targeting and degrading RNA in a highly specific manner and thus, expanding the toolset to address the transcriptome as well.

Despite the necessity of PAM sequences for Cas9 and Cas12a, such a requirement could not be observed for all Cas13 orthologs, but some Cas13 proteins seem to require a PAM-like motif, called protospacer flanking site (PFS). This has been established so far for the three Cas13 orthologs: Cas13a from *Leptotrichia wadei* (LwaCas13a), Cas13b from *Prevotella* sp. (PspCas13b) and Cas13d from *Eubacterium siraeum* (EsCas13d) (Abudayyeh et al. 2017; Cox et al. 2017; Konermann et al. 2018).

Taken together, the molecular tool set of sequence-specific programmable DNA and RNA nucleases provides unprecedented possibilities for both the targeted generation of DNA breaks, or triggering the degradation of selected RNA molecules, thereby increasing the potential to modify genes, genome structure, or selectively controlling the transcriptome (Schindele et al. 2018). In the following section, we will discuss both the applications of these targeted nucleases, as well as derivatives thereof, which can be generated artificially to change PAM requirements, or enhance the specificity of protospacer recognition to reduce potential off-target cleavage. Additionally, we consider the possibilities to target one Cas effector to more than one locus, simultaneously, or operating different Cas orthologs with variable modifications to induce single-strand breaks (SSBs), or targeting fused protein effectors to loci of interest in multiplex approaches.

SELECTION OF TARGET MOTIFS

Selection criteria
Based upon the interaction of the gRNA and Cas endonuclease and the binding of their complex to target DNA, there are general and sequence-specific requirements, and preferences, to be considered when target motifs are selected. The most decisive limitation for the choice of target motifs derives from the requirement of a PAM, which is the nucleotide triplet specifically bound by the Cas endonuclease. In the case of canonical Cas9, the PAM comprises one flexible nucleotide, followed by two guanosine residues. Utilizing plant species that enable reasonably efficient genetic transformation, simple knockout approaches are fairly straightforward. For site-directed mutagenesis, it may be sufficient to simply select two to three target motifs within the coding sequence of the target gene, with each target comprising any 21 nucleotides upstream of a double G. However, in more challenging approaches, such as multi-target, double Cas or repair template-directed modifications, it is advisable to select target motifs for which high cleavage activity of the gRNA/Cas complex is predicted.

Secondary structure formation of the gRNA plays a crucial role in Cas cleavage activity, because not only must the gRNA 5′ terminal part be capable of base-pairing with the target DNA, but also the binding between Cas endonuclease and gRNA is essentially dependent on two-dimensional (2D) structures formed within the gRNA 3′ terminal scaffold (Ma et al. 2015b). Online tools, such as RNAfold (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) and MFOLD (Zuker 2003), are available to predict the most likely occurring secondary structure variants of gRNAs *in silico*. Taking advantage of this opportunity, substantial interactions between the target-compatible 5′ terminal part of the gRNA and its 3′ scaffold can largely be avoided.

In Figure 2, examples of predicted 2D structures of gRNAs with high (A) and compromised functionality (B) are shown. The extent of intramolecular pairing of nucleobases within gRNAs is also considered in the validation of target motifs by online platforms specifically devoted to the use of customized endonucleases, such as CRISPR-GE (http://skl.scau.edu.cn/) (Xie et al. 2017). Using rice as the experimental model, Liang et al. (2016) have shown that, in efficient gRNAs, no more than 12 nucleotides of the target-specific gRNA 5′ terminal part are involved in base-pairing with the invariable gRNA scaffold. Moreover, a maximum of seven consecutive nucleotides of the variable part are tolerated to pair with complementary bases of the gRNA scaffold.
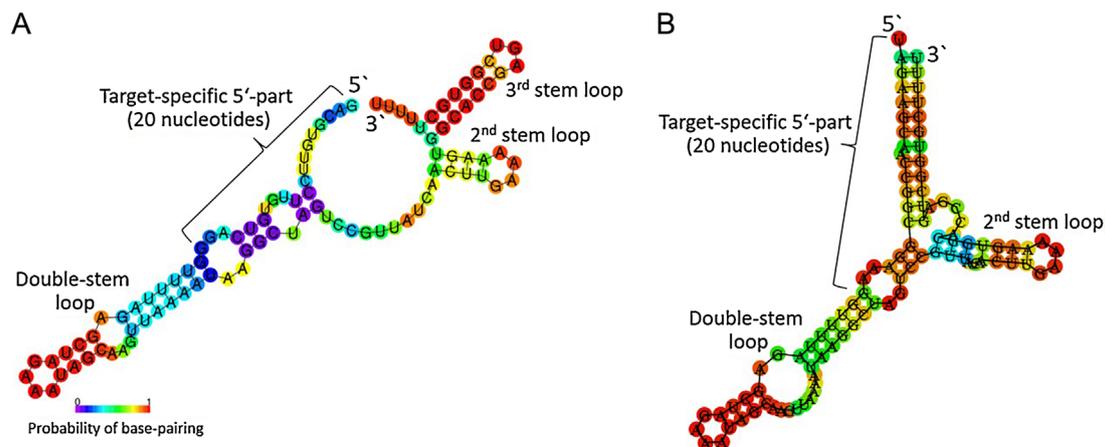
Figure 2. Secondary structure of single-guide RNAs

(**A**) Example of guide RNA with high functionality, due to limited base pairing of the target-specific part and to the presence of all three stem loop structures required to correctly interact with Cas9 endonuclease. Probability of the individual bases to pair is indicated by a color code. (**B**) For comparison, a guide RNA with severely compromised functionality, owing to a high number of paired nucleobases of the target-specific part and to the absence of an essential stem loop structure (namely the third one according to A). The structure models were generated using the RNAfold online platform (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

Importantly, three stem-loops formed within the invariable gRNA scaffold have been proven to be essential for proper interaction of gRNA and Cas endonuclease. By contrast, any further secondary structures, more or less frequently occurring within the gRNA scaffold, were not associated with high cleavage activity of the gRNA/Cas9 complex and, hence, can be neglected when it comes to the selection of target motifs (Liang et al. 2016). Wong et al. (2015) showed that a CTT triplet, residing at the position −1 to −3 immediately upstream of the PAM, severely disturbs the functionality of gRNAs as it is complementary and, thus, readily base-pairs with an AAG triplet present on the gRNA scaffold where one of its functional stem-loops needs to be formed.

Preferences of the various nucleotide positions along the target motifs for particular nucleobases have been elucidated by functionally testing large gRNA libraries, followed by the differentiation of fractions of functional versus non-functional gRNAs, or subpopulations of most efficient gRNAs (Doench et al. 2014; Moreno-Mateos et al. 2015). Conspicuous and independently reoccurring patterns have mostly been detected in the immediate sequence context of the PAM that, itself, constitutes the most striking feature of useful gRNA/Cas target motifs. In the case of the best studied

Cas9, guanine at the position −1 upstream and cytosine at the position +1 downstream of the PAM were shown to be associated with high cleavage activity. On the other hand, the following nucleobases were determined to occur particularly rarely in the given positions of efficiently cleaved target motifs: cytosine and thymine at −1 upstream of the PAM, thymine at the N-position of the PAM and guanine at +1 downstream of the PAM (Xu et al. 2015).

Inconsistent results have been published regarding a number of features of useful target motifs, which is likely to be due to various basic criteria for gRNA selection, as well as to different experimental models, with the latter having specific preferences in terms of DNA repair mechanisms (Xu et al. 2015). For example, an attempt to confirm nucleotide preferences of target motifs established in various vertebrate cell systems failed in rice, where no statistically significant associations of nucleotide composition of the target specific 5′ part of the gRNA with cleavage efficiency were observed, albeit different average occurrences of the four available nucleobases were seen in some positions within the target sequences of efficient versus inefficient gRNAs (Liang et al. 2016).

Likewise, there are inconsistent results as to the role of GC content of the target-specific 5′ part of gRNAs.

Whereas Wang et al. (2014), using human cells, showed that the fractions of gRNAs featuring lower than 45 and higher than 75% GC content of their target-specific 5′ part were associated with reduced cleavage efficacy, Gagnon et al. (2014) reported that the fraction of significantly more efficient gRNAs in zebrafish have a GC content higher than 50%. Using *Drosophila* as an experimental system, Ren et al. (2014) showed that a GC content of over 50% is beneficial only within the six nucleobases positioned immediately upstream of the PAM. Then again, Labuhn et al. (2018) reported a significant effect of the GC content in the domain comprising positions -8 through to −17 upstream of the PAM in human cells.

Whereas the vast majority of approaches to establish selection rules for target motifs have been generated using association principles, rather than causal relationships, some of these rules have been validated at least using hypothesis-driven approaches, for example on the topological interaction of gRNA and target DNA (Xu et al. 2017).

The repair machinery preferentially uses micro-homologies between the free ends produced by DNA cleavage. Therefore, it is possible to take advantage of this phenomenon to predict sequence modifications, or even to use such patterns strategically, be it to enhance cleavage efficiency, to preferentially obtain in- or out-of-frame mutations, or to precisely create particular sequence modifications (Bae et al. 2014).

Online platforms for the selection of efficient and specific target motifs

The selection of target motifs for Cas endonucleases is greatly facilitated by a variety of online platforms. While WU-CRISPR (Wong et al. 2015), SSC (Xu et al. 2015), CRISPR MultiTargeter (Prykhozhij et al. 2015), sgRNA Scorer (Chari et al. 2015) and Desktop Genetics (Doench et al. 2016) are particularly reliable in recommending gRNAs and predicting their cleavage efficacy, other platforms have been tailored for the specific requirements of certain organisms. For example, CRISPR-P 2.0 (Liu et al. 2017a) and CRISPR-GE (Xie et al. 2017) were established for plants. Notwithstanding their utility, the predictive power of these online tools is still limited, since their algorithms have been developed based on the association of target features with cleavage activity, rather than on experimentally established causal relationships. Yet another limitation is that results and

findings which are highly dependent on experimental background conditions and cellular particularities, cannot readily be extrapolated from one experimental system to another.

The establishment of selection criteria for particularly efficiently cleaved target motifs and the development of computational algorithms balancing such criteria, has resulted in the above-mentioned tools for the prediction of useful target sites. A further improved predictability of cleavage activity may be achieved by the elucidation of causative relations between structure and function. To this end, systematic comparisons of two or more targets that differ in just one feature would be required. However, owing to the limited presence of all theoretically possible target motifs in a given organism, and the influence of chromatin structures and their position effects, this will be technically very challenging. Nonetheless, it will be worthwhile to develop experimental concepts in this particular field, since an increased prediction accuracy of target efficacy is key to improved and more sophisticated methods of site-directed genome modification.

Simultaneous modification of multiple genomic targets

Various solutions exist to express multiple gRNAs within the same cell. This principle is of particular value in allopolyploid species where the genetic similarity, across all partial genomes, is often not high enough to allow for the identification of identical and useful target motifs present in all homoalleles of a given target gene. Moreover, it is sometimes required to simultaneously cut more than one target motif in the same gene (e.g. for double nickase setups), or in two or more target genes, to achieve multiple genetic modifications. Approaches to the modification of entire gene families or to pathway engineering are the most challenging in this context.

The first approaches for the simultaneous use of multiple gRNAs were to deliver each gRNA driven by its own promoter (Schiml et al. 2014; Xing et al. 2014; Ma et al. 2015b). The limitation herein is that, with a polymerase (Pol) III promoter for each gRNA, the required constructs become comparatively large. Three different concepts to avoid this challenge are based on the principle of a polycistronic gene. The expression of multiple functional gRNAs as a polycistronic gene, under the control of Pol II promoters in human cells,

was tested by utilizing the RNA-binding and RNA-endonuclease capabilities of Csy4, whereby the gRNAs could be released from their transcript (Nissim et al. 2014). In this context, it is worthy to consider the requirement of a second endonuclease to separate the gRNAs, which also significantly contributes to the length of DNA constructs. Alternatively, a polycistronic construct was developed utilizing the endogenous tRNA processing system in rice. The tRNA sequences are located in between the gRNA sequences and are necessary for the cleavage of two host RNases (Xie et al. 2015).

With the tandem-like array of self-cleaving ribozymes and gRNAs, the separation of the gRNAs after transcription is also enabled. The advantage here is that no further components are required (Tang et al. 2016). Flanking gRNAs with ribozymes can also be of interest for individual gRNAs, as the gRNA does not necessarily have to be expressed by a Pol III promoter. It will then be possible to use Pol II promoters, which are better studied and can also permit tissue-specific or inducible expression (Gao and Zhao 2014).

Using a polycistronic tRNA-gRNA gene expression strategy in rice, Xie et al. (2015) demonstrated the simultaneous modification of as many as eight target sites. Later, this group took advantage of the same principle to study the roles of four stress-related mitogen-activated protein kinases of rice, via Cas9-mediated knockout (Minkenberg et al. 2017). Lowder et al. (2018) took a multiplex gene activation approach in *Arabidopsis* and rice using a chimeric dCas9-VP64-EDLL, aiming to activate gene-specific expression. However, the authors came to the conclusion that the level of transcriptional activation achieved is remarkably dependent on the individual genes manipulated.

Čermák et al. (2017) compared Cas9 and Cas12 with regard to the simultaneous genome modification at multiple targets in plants. It was shown that Cas12a exhibits endoribonuclease activity and is able to process its pre-crRNA by itself (Fonfara et al. 2016; Swarts et al. 2017). Due to this property, Cas12a is naturally qualified for multiplex gene editing, compared to Cas9. Using an artificial CRISPR array, with alternating direct repeats and crRNA, this functionality was proven in mammalian cells (Zetsche et al. 2017) and plants (Wang et al. 2017b). A further advantage is the smaller size of the Cas12a-crRNA, which means either a smaller size of the

delivered vector or an enhancement of the amount of crRNAs. In general, Cas12a can compete with Cas9 concerning DNA cleavage activity, mutation frequencies and DNA binding (Zetsche et al. 2017). Recently, multiplex gene editing was shown in rice with FnCas12a, LbCas12a and SpyCas9. Nucleases and gRNAs were co-expressed by a Pol II promoter with no additional processing components (Wang et al. 2018).

Delivery systems for Cas endonucleases

For site-directed genome modification of plants, genomic integration of gRNA- and Cas endonuclease-encoding transgene expression units is the most widely used approach. Therefore, conventional plant genetic transformation methods, based upon Agrobacterium, can be applied. In experimental model systems, such as isolated protoplasts, these transgenes can also be introduced via poration of the plasma membrane using voltage pulses or high concentrations of polyethylene glycol. In protoplast systems, transgene expression is only required for a limited period. Therefore, it is sufficient that much of the transgene expression derives from non-integrated DNA.

Alternatively, non-integrating virus replicons carrying units of gRNA, Cas9 and synthetic template (for homology-directed DNA break repair) have been used (Baltes et al. 2014; Ali et al. 2015). The major advantage of this method is that the self-replicating vectors facilitate high transgene expression, which increases the efficiency of site-directed genome modification. However, the challenge remains to find useful viruses that are infectious in particular plant species of choice and to eventually generate modified plants that are entirely virus-free.

Particularly high transgene expression levels can also be achieved during a limited episode upon biolistic gene transfer. Taking this approach, Zhang et al. (2016) produced wheat plants carrying targeted mutations in the yield-related *DEP1* and *GASR7* genes. Of note, no selective conditions were required for mutant regeneration, at reasonable efficiency, and quite a proportion of the primary mutant plants proved to be non-transgenic for the gRNA and Cas9 genes.

In conventional methods, Cas endonucleases and gRNAs act as products of their coding DNA sequences previously transferred into host cells. However, a viable alternative is to *in vitro* transcribe gRNA and *Cas* endonuclease genes, or to pre-produce Cas protein

using microbial expression platforms and, in contrast to transgenesis, to use these non-heritable molecules for transfection. It should be noted here that such reagents collectively constitute nothing but target-specific mutagens. In this context, gRNA and Cas endonuclease can also be assembled prior to transfection to form ribonucleoprotein (RNP) complexes.

The use of RNPs has some advantages over the principle of gRNA and *Cas* transgene expression. First, there is no need to create species- or cell-specifically tailored expression vectors for gRNA and *Cas* endonu-clease genes. Second, the cellular dosage of gRNA and Cas endonuclease is independent of the specificity and strength of available promoters. Third, as the cellular implementation of transgenes into accumulating gene products is rendered unnecessary, gRNA and Cas endonuclease exert their full capacity to generate DSBs immediately after transfection. Fourth, the efficacy of RNP complexes can be strong and is yet confined to a comparatively short temporal window after administration of the reagents, because these are non-replicating molecules that are prone to both dilution, as a result of cell proliferation, and cellular degradation pathways. Consequently, the occurrence of superfluous on-target mutations beyond the germ-line, as well as unwanted off-target mutations in germline cells is much reduced during later stages of plant development. Fifth, once mutants are generated, there is no need to screen the progeny for segregants that have lost the coding sequences for gRNA and *Cas*, that is, all mutant individuals obtained are transgene-free and hence available for any further analyses, investigations and applications.

A first proof of this concept was demonstrated by Woo et al. (2015), who introduced gRNA/Cas9 RNPs to protoplasts from a variety of plant species and, thereby, showed indeed that targeted mutations had been induced. In an attempt to transfect *in vitro* transcribed RNAs for both gRNA and Cas9, 1% of the biolistically transformed cells of bread wheat proved to carry mutations in the *GW2* target gene (Zhang et al. 2016). As a further outcome of this study, about one-third of the obtained plants carried mutations in all six *GW2* alleles that are present in hexaploid wheat, which is particu-larly remarkable, albeit this approach was approxi-mately 60% less efficient than the compared one that involved transient and stable expression of gRNA and *Cas9* transgenes.

In maize, Svitashev et al. (2015) achieved another milestone, in that they mutated four different genes, simultaneously, via biolistic transfer of RNPs into regenerable cells of immature embryos, which was conducted in comparison with gRNA and *Cas9* transgenesis. In this study, both principles (RNPs *vs.* transgenes) were on a par in terms of mutagenesis efficiency (Svitashev et al. 2016). A particularly valuable application of using gRNA/Cas9 RNP complexes was demonstrated by Andersson et al. (2018), who generated an amylopectin potato via knock-out of *GBSS*. In addition, the utilization of Cas12a has been exemplified along with gRNA in the format of RNPs that were transfected into protoplasts to induce targeted mutations in the two *FAD2-1* gene variants of the palaeopolyploid soybean (Kim et al. 2017).

The cleavage efficiency of the enzyme variant derived from *Lachnospiraceae bacterium* (LbCas12a) proved to be about 10 times more efficient as compared to the one of *Acidaminococcus* sp. BV3L6 (AcCas12a). In the same study, tobacco protoplasts were used as well to mutate the AOC gene. However, in this case LbCas12a and AcCas12a caused a comparable mutation rate of about 1%. Cas/gRNA RNPs were also shown to be effective in approaches to homology-driven genome editing using artificial DNA templates that feature the modified sequences to be introduced to the host genome. In this context, protein and DNA have to be co-precipitated to particles used for biolistics or co-transfected via polyethylene glycol-mediated poration of the plasma membrane, which renders this procedure quite challenging. Moreover, owing to the co-transfer of template DNA, a particular advantage of using RNPs is actually lost, namely that otherwise no transgenes are introduced to the host genome.

The use of RNPs to implement the DNA sequence of a repair template was demonstrated in maize, where herbicide tolerance was established by modifying the *ALS2* gene. In this study, Svitashev et al. (2016) used a single-stranded repair template that was 127 DNA polynucleotides in length. In another novel approach, pre-assembled RNP complexes were also used to identify individuals carrying targeted modifications. To this end, target-specific polymerase chain reaction amplicons were *in vitro* digested by corresponding RNPs, which allowed for gel-electrophoretic distinction between wild-type and mutated plants with unprece-dented reliability. This method was demonstrated via

site-directed mutagenesis of the *GW2* and *Cer9* genes of wheat and the *PDS1* gene of rice (Liang et al. 2018b).

This approach may be of particular advantage in species carrying large and complex genomes. In addition, it provides much increased freedom as to the choice of target sequences, in comparison to the use of targets that carry recognition sites of conventional restriction enzymes. A further incentive for the use of RNPs for approaches to targeted genome modification is that the commercial provision of customized gRNA and a variety of Cas proteins is nowadays well established. On the other hand, a broader application of RNPs in the field of genome engineering is still hampered, because suitable transfection methods for regenerable cells have been established only in a limited range of plant species.

APPLICATIONS OF TARGETED GENOME MODIFICATION BY NHEJ

Target sequence-specific genome modification employing RNA-guided Cas endonucleases for DSB induction has been established as routine in many laboratories in which plant research is performed. A comprehensive survey of applications of the technology would thus go beyond the scope of this review, in which only representative, or particularly impressive examples are given.

Fundamental research
Ever since the RNA-guided Cas9 endonuclease emerged as a viable tool for research and development in plants, this technology has been used for the functional validation of gene functions. Recent examples of studies devoted to plant development, using *Arabidopsis* as an experimental model, were given for example by Saito et al. (2018), who elucidated the role of *BZR1* in vascular differentiation, via Cas9-mediated knockout, by Osakabe et al. (2016), who manipulated stomata closure by knockout of *OST2*, by Liang et al. (2018a), who knocked out *GGAT1* so as to alter the photorespiration pathway, and by Yu et al. (2018), who created triple mutants with a single gRNA, which could not be created by conventional crossbreeding.

Comprehensive genomic data, resources and tools are now available in the majority of important cultivated plants, and so the dominance of *Arabidopsis* research has been somewhat declining in recent years. Instead, as one of the major crop species, and representative of agriculturally most important monocots, rice has been playing an increasing role in fundamental research. For example, Zeng et al. (2018) established the function of *OsBBS1*, in the context of leaf senescence, and the response to salt stress, by knocking out this gene. Taking a base-editing approach using nCas9-PBE, Zong et al. (2017) elucidated the role of *OsCDC43* in senescence and programmed cell death.

Further work was devoted to the functional validation via site-directed mutagenesis of the nuclear genes *OsSLA4* and *OsFLN1* involved in chloroplast development (He et al. 2018; Wang et al. 2018c). Site-directed mutagenesis triggered by Cas9 endonucleases was also used in further species to validate gene function. In cotton, for example, the function of *CLA1*, another gene involved in chloroplast formation, was studied via Cas9-mediated knockout (Wang et al. 2018a). In barley, knockout lines of *PM19* were used to study the role of this gene in dormancy. In tomato, a novel mechanism of interspecific incompatibility was recovered by knockout of the pollen-expressed *FPS2* gene (Qin et al. 2018). Other examples are soybean and *Medicago truncatula*, in which the processing of small RNAs in the context of post-transcriptional gene silencing was studied using Cas9-mediated knockout of *GmDrb2a*, *GmDrb2b* and *MtHen1* (Curtin et al. 2018). Zhou et al. (2017) produced rice plants mutated in a variety of micro-RNA genes, thereby providing new insights into micro-RNA function and complex gene regulation pathways.

Herbicide resistance
The establishment of herbicide-resistant crops was among the first applied approaches taking advantage of site-directed genome modification by means of RNA-guided Cas9. On the other hand, the readily selectable herbicide resistance also proved to be particularly useful in establishing Cas endonuclease technology. Whereas resistance to the herbicide Bentazon was simply achieved by knockout of *OsBEL* (Xu et al. 2014), in the case of the herbicide targets acetolactate synthase and 5-enolpyruvylshikimate-3-phosphate synthase, precise modifications were required to prevent their inactivation by the respective herbicides while retaining their essential function for the plant. And since the development of herbicide resistance is one of the most simple genetic modifications that can be associated

with a selective advantage, *in vitro*, this principle was the first to be used to establish methods of homology-directed genome editing, for example in maize (Svitashev et al. 2015), rice (Li et al. 2016a; Sun et al. 2016), and flax (Sauer et al. 2016). Basically the same experimental approach was then also used to establish homology-directed genome modification, via delivery of gRNA and Cas9 in the format of preassembled RNP (Svitashev et al. 2016).

Yield-related traits

Customized RNA-guided Cas9 has also been broadly used to modify traits that are determinants of crop yield. Modern cultivars in numerous crops represent semi-dwarf variants that are less prone to lodging, have an increased capability of implementing nitrogen fertilizers and have an improved harvest index, that is an increase in the portion of harvested seeds or grains from the total above-ground biomass. The generation of more genetic diversity in plant height is still of high interest to further fine-tune this trait for different environmental and regional demands. Dwarf and semi-dwarf plants have been generated via knockout of *BolC.GA4a* in rapeseed (Lawrenson et al. 2015), *ER1*, *ER2* and *SEC3a* in rice (Ma et al. 2018; Zhang et al. 2018) and *Dep1* in wheat (Zhang et al. 2016). Semi-dwarf rapeseed was also created by selecting mutants carrying deletions in the *BaA6.RGA* gene, whereby the translational reading frame is retained (Yang et al. 2017). This principle provides the opportunity to achieve functional modifications rather than an entire loss-of-function of the target gene.

The temporal regulation of the developmental switch from the vegetative to the reproductive phase of both flowering time and fruit maturation, are further important determinants of yield, which have to be considered when tailoring cultivars for different agricultural systems and regional conditions. Accelerated maturity in rice and tomato was achieved via knockout of *OsHd2*, *Hd4*, *Hd5* (Li et al. 2017b) and of *SlSP5G* (Soyk et al. 2017), respectively. By contrast, knockout of *GmFT2* entailed delayed flowering time in soybean (Cai et al. 2018). Li et al. (2018a) demonstrated that mutagenesis of the long non-coding RNA1459 results in delayed tomato fruit ripening.

Grain or seed number and weight represent even more direct determinants of yield. Aiming to improve both of these traits in rice, Li et al. (2016b) produced

mutants for the genes *OsGn1a*, *DEP1*, *GS3* and *IPA1*. Alternatively, grain weight was enhanced via knockout of *GW2*, *GW5* and *TGW6* in rice (Xu et al. 2016), and of *GASR7* in wheat (Zhang et al. 2016). Increased locule and seed number per silique were obtained in rapeseed via knockout of both homoalleles of the *CLV3* gene (Yang et al. 2018). Taking an even more sophisticated approach in tomato, Rodríguez-Leal et al. (2017) demonstrated that a whole range of variability in locule number and fruit size can be obtained by producing an array of *CLV3* promoter variants with different fragment deletions. Cas9-mediated knockout of *AGL6* caused facultative parthenocarpy in tomato, which results in seedless fruits (Klap et al. 2017). Reduced seed shattering was achieved by knocking out the *ALCATRAZ* gene of rapeseed (Braatz et al. 2017).

Tolerance to abiotic stress

Using Cas9-induced knockout lines, Huang et al. (2018) have validated the role of rice *NCED3* for salt tolerance. In tomato, Wang et al. (2017a) revealed the implication of *MAPK3* in drought tolerance also by generating and analyzing respective knockout lines. In a particularly sophisticated approach, enhanced drought tolerance was achieved by homology-directed repair of Cas9-induced DNA breaks leading to enhanced expression of the *ARGOS8* gene of maize (Shi et al. 2017).

Resistance to pathogens

Resistance of *Nicotiana benthamiana* to pathogenic geminiviruses was achieved by Baltes et al. (2015) and Ji et al. (2015) via mutagenesis of target motifs residing within the viral genomes. Upon mutational modification of the previously known susceptibility factor *eIF4F*, Chandrasekaran et al. (2016) generated cucumber with resistance to various ipomo- and potyviruses.

Site-directed mutagenesis using RNA-guided Cas endonucleases was also used to establish resistance to bacterial plant diseases. In rice, for example, resistance to bacterial blight and rice blast, respectively, was achieved by knockout of the sugar transporter *SWEET13* (Zhou et al. 2015) and of *ERF922* (Wang et al. 2016). Jia et al. (2017) established resistance to citrus canker by knocking out the *LOB1* gene. And more recently, Wang et al. (2018b) rendered grapevine resistant to *Botrytis cinerea* through the knockout of the transcription factor *WRKY52*. The establishment of powdery mildew resistance in wheat has been among the agriculturally most

relevant achievements using RNA-guided Cas endonucleases (Zhang et al. 2017b). In this study, all three *EDR1* homeoalleles of hexaploid wheat were disrupted. Also in tomato, powdery mildew resistance was achieved by knockout of the susceptibility gene *MLO1* (Nekrasov et al. 2017).

Improvement of nutritional value

Site-directed genome modification has also been shown to be instrumental for the improvement of the nutritional quality of feed and food. For example, Cas9-mediated knockout of the maize *IPK* gene resulted in a reduction of phytic acid biosynthesis, which is a phosphorous storage compound indigestible by mammals (Liang et al. 2014). Targeted knockout of the *SBEI* and *SBEIIb* genes of rice resulted in a substantial reduction of the amylopectin content in favor of the amylose fraction in grains, which may contribute to reduced occurrence of diabetes II, since the resultant, so-called "resistant" starch is preferentially digested in the colon rather than in the small intestine (Sun et al. 2017).

A comparatively challenging approach was taken by Sánchez-León et al. (2018), who simultaneously knocked out as many as 35 of the *α-gliadin* genes of bread wheat, leading to low gluten content of grains with much reduced immunoreactivity. The fatty acid composition has a significant impact on human health. In the emerging oil seed plant *Camelina sativa*, the *FAD2* gene was knocked out thereby increasing the fraction of monounsaturated oleic acid to over 50% at the cost of less desirable polyunsaturated fatty acids (Jiang et al. 2017; Morineau et al. 2017). This approach is another example where the power of Cas9 technology was essential, because three homeoalleles of the hexaploid plant species had to be mutated. In yet another study in *Camelina*, the content of very long fatty acids was reduced by knocking out the *FAE1* gene (Ozseyhan et al. 2018). An enrichment of the nutritionally highly valuable compound lycopene was achieved in tomato fruits via multiplex site-directed mutagenesis of several genes involved in the carotenoid biosynthesis pathway (Li et al. 2018b).

Industrial plant products

The formation of amylopectin-enriched starch was demonstrated by knockout of the potato *GBSS* gene (Andersson et al. 2017, 2018). Due to the resultant reduction in the amylose fraction, a particular starch quality was obtained that is highly useful for the production of paper and other materials. The same principle was also used to produce a new maize variety, in which the *GBSS* gene was knocked out in a current elite genetic background to make waxy corn with higher agricultural performance, as compared to conventional breeds with the same trait. This crop is likely to be among the very first ones produced by Cas endonuclease technology to enter the market (Waltz 2016). An example of generating plants with improved utility for molecular farming approaches was provided by Kapusi et al. (2017), who altered the N-glycosylation of barley-produced proteins by Cas9-mediated knockout of *ENGase*.

Plant breeding technology

Targeted genome modification has also been used to improve plant breeding technology. In the context of hybrid breeding, the opportunity of switching between male fertility and sterility is an essential prerequisite. In numerous studies, different genes of maize (*MS26*, *MS45*) and rice (*CSA*, *TMS5*, *PKS2*) involved in pollen functionality have been knocked out to form male sterility (Svitashev et al. 2015; Li et al. 2016c; Svitashev et al. 2016; Zhou et al. 2016; Zou et al. 2018). In this context, a novel gene dosage-dependent mechanism for indica-japonica rice hybrid male sterility was revealed by knockout of one or two of the three tandem-repeated copies of the *Sc-i* gene of indica rice, which may facilitate the utilization of heterosis in hybrid rice (Shen et al. 2017). Haploid technology also belongs to the most powerful means to support plant breeding programs. A recent study showed that haploidy inducer lines of rice can be established via knockout of the rice ortholog of the maize *MATL* gene. When the *matl* mutant lines were used as pollinators, as much as 6% of the progeny proved to be haploids (Yao et al. 2018).

MODIFICATIONS AND EXTENSIONS OF CAS ENDONUCLEASES

Due to the presence of two individual DNA cleavage domains, both Cas9 and Cas12a enzymes can be converted into nickases (nCas9/nCas12a) by the induction of point mutations in the catalytic sites of either the HNH or RuvC domain of Cas9, or in the Nuc domain of Cas12a, respectively. Thus, these nickases are

able to induce site-specific DNA SSBs (Jinek et al. 2012; Yamano et al. 2016). While publications for the utilization of nCas12a are not yet available, nCas9 was tested in different approaches. To induce a DSB with single-strand overhangs, the nickase can be combined with two gRNAs, each recognizing one target on one of both DNA double strands with a few base pairs distance from each other. Thus, both nickases generate an adjacent SSB, resulting in a DSB with single-strand overhangs (Figure 3).

Attempts to boost *in planta* GT, by nCas-mediated induction of SSBs instead of DSBs, did not result in comparable targeting efficiencies (Wolter et al. 2018). Consequently, it is tempting to speculate whether the utilization of Cas12a, with its intrinsic property to induce short 5′ overhang DSBs, can improve the efficiency of *in planta* (GT), over the frequencies obtained with Cas9 (Wolter et al. 2018). Due to its cleavage activity distal to its PAM, multiple rounds of DSB induction are possible after InDel formation, via NHEJ repair, as the crRNA is more tolerant to mismatches at this side of the target sequence. Therefore, the crRNA might bind again and a stimulation of the targeted

site for HR-mediated GT is still possible (Schindele et al. 2018).

A further advantage of engineering a DSB with paired nickases is the increase in specificity to the target by duplicating the recognition site requirements from 23 to 46 nt in total (Mali et al. 2013; Ran et al. 2013). Moreover, potentially induced SSBs, at off-target sites, can be accurately repaired by the base excision repair pathway, thus any unintended sequence alterations in non-target regions can be abolished (Fauser et al. 2014). For optimal mutagenic results, the two SSBs should be generated at a distance of 50–100 bp to each other, with most mutagenic outcomes being deletions, compared to mostly small insertions with the Cas9 nuclease. The induction of DSBs by paired SSBs leads to single-strand overhangs that are degraded, leading to longer deletions. Besides deletions, the formation of sequence duplications was discovered in plants directly at the DNA breakage, presumably caused in accord the patch-mediated DSB creation model followed by NHEJ (Vaughn and Bennetzen 2014; Schiml et al. 2016).

Early studies with Cas9 revealed a relatively high off-target DNA cleavage frequency in human cells (Fu et al.
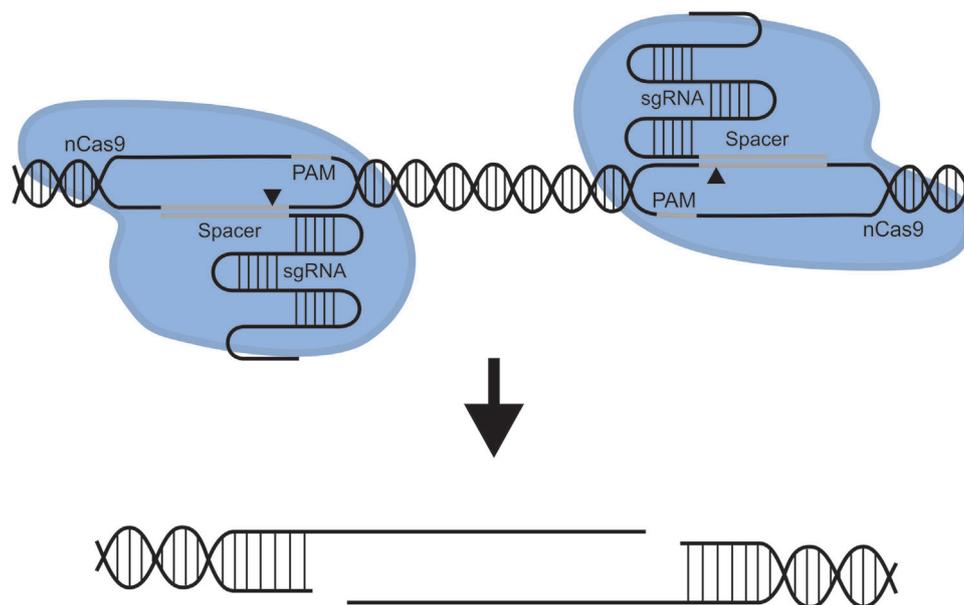

Figure 3. Cas9 paired nickases approach
A single point mutation renders one nuclease domain of Cas9 inactive, resulting in the generation of a single-strand break (SSB)-inducing enzyme (nCas9, nickase). Providing two different gRNAs targeting adjacent positions on opposite DNA strands, two SSBs can be obtained in close proximity, resulting in a double-strand break with single-stranded overhangs.

2013). To reduce such off-target activity, it is advisable to use bioinformatics tools to predict potential off-target sites within the genome. Interestingly, hardly any off-target events were identified so far in plants by sequencing respective sites (Feng et al. 2014). Likewise, large-scale whole-genome sequencing analysis showed barely any off-target events for SpyCas9 and LbCas12a (Tang et al. 2018). Nevertheless, off-target activity remains a concern to be considered, especially for targeted nuclease applications in crops with large and partly polyploid genomes, such as maize, rice and soybean (Scheben et al. 2017), where highly repetitive sequences represent special challenges for site-specific mutagenesis or GT. Therefore, other efforts to improve the on-target/off-target ratio will even further focus on the gRNA-target binding.

In particular, the binding strength between Cas9 and the target DNA appears to influence the specificity of the nuclease. Therefore, several attempts have been undertaken to influence the strength of this bond by truncating the sequence of the gRNA at its 5′ end (Fu et al. 2014), or to destabilize the interaction between the helicase domain and the non-complementary DNA strand by mutations between the HNH and RuvC domains in SpyCas9 (Slaymaker et al. 2016). Truncation of the guide sequences revealed that a length of 17 or 18 nt exhibits efficient on-target recognition and a reduction in off-target cleavage (Fu et al. 2014). Structural analyses of SpyCas9 showed that the nuclease forms contacts, including hydrogen bonds, to the phosphate backbone of the target DNA (Anders et al. 2014; Nishimasu et al. 2014), and it was tested whether the disruption of these contacts is able to influence cleavage of off-target sites (Kleinstiver et al. 2016).

To weaken the binding between protein and target DNA, single amino acids in the SpyCas9-DNA contact sites were exchanged. This disruption might affect the binding energy of the complex, resulting in the induction of DSBs with perfect matches with the target DNA, while the bond is not sufficient to perform a break when mismatches are present. This so-called SpCas9-HF (HF: high fidelity) variant shows comparable on-target DNA cleavage to wild-type SpyCas9, whereas almost no off-target events where observed (Kleinstiver et al. 2016). Previous studies from the same group revealed

that mutations in the PAM interacting domain lead to more specificity in the PAM recognition.

These findings suggest fewer off-target events compared to wild-type SpyCas9. In addition, these mutations cause an alteration in the PAM site to be recognized (Kleinstiver et al. 2015). Further SpyCas9 variants (xCas9) were evolved using phage-assisted continuous evolution (PACE). The enzymes developed in this way recognize a range of different PAM motifs including NG, GAA and GAT, and nevertheless show significantly higher DNA specificity than SpyCas9. Moreover, the off-target activity is considerably reduced compared to the commonly used SpyCas9 (Hu et al. 2018).

In addition to the utilization of orthologous Cas9 proteins, altered PAM sites extend the selection of potential target sites. The commonly used Cas9 nuclease from Streptococcus pyogenes is naturally recognizing "NGG" as a PAM sequence; however, many different Cas9 orthologs with various PAM requirements have also been characterized. The Cas9 orthologs from Streptococcus thermophilus and Staphylococcus aureus seemed most promising for genome editing (Esvelt et al. 2013; Kleinstiver et al. 2015; Ran et al. 2015) due to the variability of their PAM sequences, by which a broader range of accessible targets is provided (Ran et al. 2015). The Cas9 orthologs of Staphylococcus aureus (SauCas9) and Streptococcus thermophilus (Sth1Cas9) require longer PAM sequences than SpyCas9 (SauCas9: NNGGGT; Sth1Cas9: NNRGAA), which should reduce the rate of potential off-target events (Steinert et al. 2015). Another advantage for both of these orthologs is the smaller size with 3.2 kb for SauCas9 and 3.4 kb for Sth1Cas9, compared to the 4.1 kb from SpyCas9, which is especially favorable when the delivery is performed via viral-based systems (Baltes et al. 2014; Ran et al. 2015).

Two different CRISPR open reading frames (ORFs) were identified for SthCas9 orthologous to SpyCas9, called CRISPR1 and CRISPR3. CRISPR1 is smaller and shows a higher activity compared to CRISPR3 (Horvath et al. 2008) and has been successfully used in bacteria, human cells and plants (Jinek et al. 2012; Esvelt et al. 2013; Kleinstiver et al. 2015; Ma et al. 2015a; Ran et al. 2015; Steinert et al. 2015). The application of Sth1- and SauCas9 in plants revealed that both orthologs

achieved targeted mutagenesis, via NHEJ, with similar high efficiency as with SpyCas9, and for SauCas9 an even higher mutagenesis rate was established in *Arabidopsis* (Steinert et al. 2015). SpyCas9, SthCas9 and SauCas9 belong to the class 2 type II CRISPR/Cas system and are also divided into the same subtype A, but are assigned to different clusters (Fonfara et al. 2014; Louwen et al. 2014). The mentioned orthologs differ in the sequences of their associated RNAs (Ran et al. 2015) and form special structures, like hairpins or additional stem loop structures, which are required for species-specific detection (Briner et al. 2014).

In addition to their DNA cleavage properties, Cas proteins are very versatile tools. As mentioned previously, nuclease domains can be deactivated by point mutations within the active sites. For Cas9, inducing point mutations in both the RuvC and HNH domains is sufficient to obtain a site-specific DNA binding protein, termed "deadCas9" (dCas9) (Jinek et al. 2012; Qi et al. 2013). This is also applicable for Cas12a, although only a point mutation within the RuvC domain is required (dCas12a) (Zetsche et al. 2015). As a result, the Cas proteins can be targeted to almost any desired DNA locus, by exchanging 20-23 nucleotides of the gRNA, for binding without cleavage.

Functional units can be fused to the dCas proteins, allocating a desired effect at a specific locus of the DNA by targeted binding. Thereby, these enzymes can specifically repress gene expression by sterically blocking the transcription machinery, as was shown in *Escherichia coli* and in human cells (Qi et al. 2013; Zhang et al. 2017a), or be used as a platform to guide any enzymatic activities to the desired DNA loci (Figure 4). This property can be used for catalytic activities, such as the visualization of specific genomic loci of living cells by the fusion of a fluorescent protein to a dCas protein (Chen et al. 2013). The application of multiple Cas9 orthologs fused with fluorescent proteins of different colors enabled the multicolored detection of various genomic loci within mammalian cells (Anton et al. 2014; Ma et al. 2015a; Chen et al. 2016), and also recently in plant cells (Dreissig et al. 2017).

With the intention of obtaining a detectable fluorescent signal, a novel repeating peptide array, called SunTag, was developed to serve as a platform for multiple fluorescent protein-antibody fusions (Tanenbaum et al. 2014). The linkage of a catalytic effector to Cas9 can either be achieved by direct fusion

or indirect fusion, which includes a modified gRNA with sequence-specific aptamer motifs enabling the binding of the catalytic effectors to the gRNA. Protein domains such as MS2, originating from an RNA phage coat protein, recognize and specifically bind this RNA sequence motif (Peabody 1993). By fusing the respective aptamer-binding domain to a catalytic effector, the complex is also directed to the DNA in a sequence-specific manner by gRNA-Cas9 interaction.

Aptamer-binding domains bind as dimers on the respective aptamer sequence, whereby twice as many effectors can bind, compared to a direct Cas fusion. Another advantage is that various aptamers and their corresponding binding domains can be used, enabling simultaneous utilization of several effector proteins (Konermann et al. 2015). Thus, an approach similar to the SunTag system, to increase the fluorescent signal by the integration of up to 16 MS2 binding motifs to the gRNA, is especially suitable for imaging non-repetitive targets (Qin et al. 2017).

In order to improve the gene repression properties of dCas9 in human cells, a repressive KRAB domain was fused to Spy-dCas9 and showed reduced expression of the targeted genes. To test the so-called CRISPRi platform, as a versatile tool for specific transcriptional regulation, the same group fused the transcription activator domain VP64 to dCas9 and was able to activate reporter gene expression (Gilbert et al. 2013). Targeting multiple loci of one gene of interest by applying various gRNAs can enhance the desired effect (Perez-Pinera et al. 2013). Cas9-based specific transcriptional regulators fused with the activator domains VP64, EDLL and TAL effectors or the SRDX repressor domain were also successfully applied in plants, to either activate or suppress endogenous genes in *Nicotiana benthamiana* leaves and *Arabidopsis* plants (Lowder et al. 2015; Piatek et al. 2015).

It was shown that the highest repression was obtained by using three different gRNAs, targeting the promotor region and first exon of the endogenous gene in *N. benthamiana* leaves (Piatek et al. 2015). The VP64 transcriptional activator domain fused to dCas9 leads to a strong activated expression of a reporter gene in tobacco leaves, as well as in protein-coding and non-protein-coding genes in *Arabidopsis* plants (Lowder et al. 2015). Through recruitment of multiple transcriptional activators, the dCas-based transcriptional activator system can be improved further (Konermann et al.
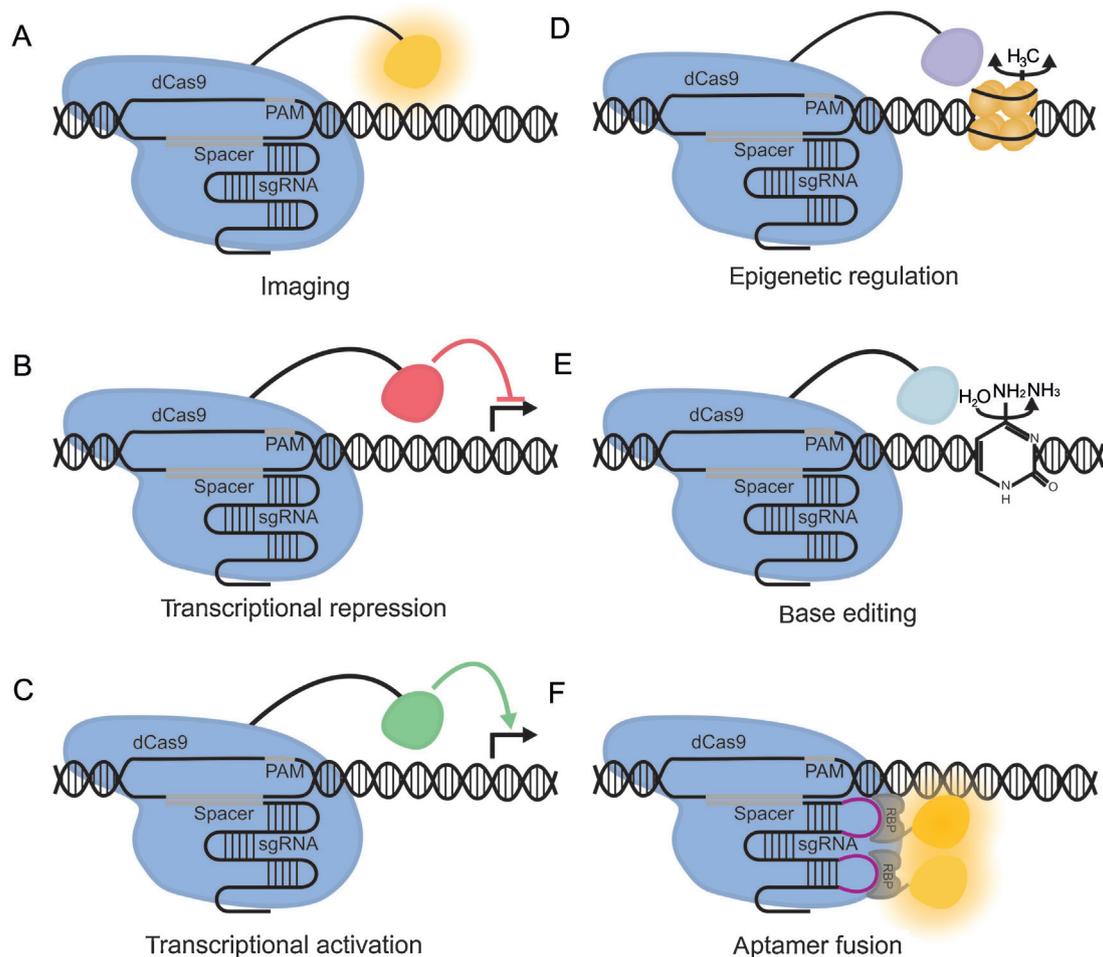
Figure 4. Applications for catalytically inactive Cas9 DNA-binding proteins

The nuclease domains of Cas9 can be deactivated by point mutations to convert the protein into a sequence-specific DNA-binding protein. The resultant inactive Cas9 (dCas9) can serve as a platform for targeting other effectors to the DNA by adapting the single-guide RNA (sgRNA). These effectors can be fused to dCas9, via a short peptide linker, and perform various functions on the DNA. By fusing fluorescent proteins to dCas9 **(A)**, specific DNA sequences can be labeled. The fusion of transcriptional repressors **(B)**, or activators **(C)**, allows for targeted modulation of gene expression. Gene activity can also be regulated, epigenetically, by fusing histone modifiers to dCas9 **(D)**. Single bases can be edited by the targeted activity of deaminases **(E)**. Alternatively, factors can be indirectly bound to extended crRNAs (aptamers), via compatible peptide motifs (RBP) **(F)**. Instead of direct protein-linked mediated fusion of effectors to dCas9, factors can be indirectly bound to extended crRNAs (aptamers), via compatible peptide motifs.

2015; Zalatan et al. 2015), as shown using the SunTag system (Tanenbaum et al. 2014). Furthermore, indirect fusions of transcriptional activators, like VP64 to modified gRNAs, have allowed robust transcriptional multiplex gene activation, *in planta* (Lowder et al. 2018).

In parallel, Cas12a is also suitable as a DNA-binding protein and for delivering catalytic effectors to targeted genomic locations. Lb-dCas12a was fused to either the strong synthetic VPR activator derived from the VP16 activator, Rta- or p65 activator domains, for robust transcriptional activation in human cells. In the same study, it was demonstrated that transcriptional activation is inducible by drug treatment, using the split Lb-dCas12a-DmrA complex and a DmrC-VPR or -p65 complex. Taking advantage that Cas12a can process its own crRNA in a multiplex single transcript, the same group confirmed the synergistic activation when targeting either the same or different promoters

(Tak et al. 2017). In *Arabidopsis*, dCas12a was already successfully applied as a transcriptional repressor fused with the SRDX repressor domain. The results indicate that Lb-dCas12a, and particularly As-dCas12a, can efficiently bind to the DNA and regulate gene expression (Tang et al. 2017).

Regulation of gene expression can also be achieved by the fusion of epigenetic regulators to Cas effectors causing chromatin manipulation. The fusion of histone acetyltransferase p300 to the C-terminus of dCas9 enabled transcriptional activation of targeted genes, in human cells, by catalyzing acetylation of histone H3 lysine 27 (H3K27). Binding of the dCas effector protein, at the selected promoter or enhancer region, activates the targeted gene by specific chromatin modification (Hilton et al. 2015). Fusion of the p300 core domain to Lb-dCas12a showed robust and specific gene activation in human cells and was able to activate three genes, simultaneously, using three different gRNAs (Zhang et al. 2017a). *Vice versa*, targeted epigenetic repression in mammalian cells can be achieved by demethylation of H3K4/K9 through the histone demethylase LSD1 (Kearns et al. 2015). The potential of developing new epialleles for generating traits of interest has been successfully demonstrated in *Arabidopsis* by targeted demethylation of a specific cytosine residue in the promoter of the *FWA* gene, resulting in a heritable late-flowering phenotype (Gallego-Bartolomé et al. 2018).

In plants, genome editing, based on DSB-induced HR, to incorporate a template with a desired sequence modification can be a tedious and inefficient process; thus, the newly developed Cas-dependent "base editing" could be an adjuvant solution for some traits to be developed. This method is based on the properties of cytidine deaminases or adenosine deaminases and allows for the directed conversion of one targeted DNA base. The fusion of a cytidine deaminase enzyme to a dCas9 effector, or a Cas9 nickase, mediates the targeted conversion of cytidine to uridine, resulting in a C-to-T substitution. Therefore, converting base pairs C/G to T/A does not require any DSBs or HR-mediated repair processes. Cytidine deaminases require RNA or ssDNA as substrate, therefore the Cas effector is not only the key for targeted DNA binding, but also for unravelling of the DNA during R-loop formation.

Experimentally, the cytidine deaminase rAPOBEC1 was fused to the N-terminus of Spy-dCas9 (BE1) and Spy-nCas9. Fusion of the effector to a nickase causes the incorporation of the new base in the non-edited strand, while the SSB-induced mismatch repair mechanism (MMR) synthesizes along the edited strand. To counteract the intrinsic cellular repair mechanism and optimize the editing result, a uracil DNA glycosylase inhibitor (UGI) was fused to the C-terminus of the targeting tool to suppress base-excision repair (BE2/BE3) (BER) (Komor et al. 2016) by preventing a possible removal of uracil, as the most deaminated bases are restored by BER (Liu (Man) 2009).

Currently, the commonly used tool for base editing is APOBEC-XTEN-nCas9-UGI (BE3) (Komor et al. 2016). Whereas the BE3 approach catalyzes cytidine deamination within a window of 5 bp around the −15 position upstream of the PAM sequence (Komor et al. 2016), similar results were obtained with a C-terminal fusion of an activation-induced cytidine deaminase (AID), which catalyzes deamination 3–5 bp around −18 position upstream of the PAM sequence (Nishida et al. 2016). Based on the BE3 approach, with APOBEC or the one with AID, many different base editing attempts have been undertaken, for example by targeted induction of STOP codons for gene disruption in human cells, silkworm or *E. coli* (Billon et al. 2017; Kuscu et al. 2017; Li et al. 2018d; Zheng et al. 2018). Furthermore, base editing attempts were also achieved with the BE3 system in such crop plants as rice, wheat and maize (Li et al. 2017a; Lu and Zhu 2017; Zong et al. 2017; Ren et al. 2018; Yan et al. 2018). Thus, herbicide-resistant rice as well as marker-free edited tomato plants were obtained through the BE-AID approach (Shimatani et al. 2017).

To expand base editing applications, further variants (BE4) have been developed using a Cas9 ortholog of *Staphylococcus aureus* (SaBE4), which increases the efficiency of the conversion from C/G to T/A. The unintended formation of InDels during editing could be successfully reduced by fusing the DSB binding bacteriophage Mu protein Gam, to BE3 and BE4 systems (Komor et al. 2017). The challenge of off-target editing events was addressed by combining the increased target sequence specificity requirements of Cas9-HF with the BE3 system in a study delivering the editor as RNPs (Rees et al. 2017). Although many different cytidine deaminases exist, no naturally adenine deaminases are known (Gaudelli et al. 2017). The development of a tRNA adenosine deaminase to mediate the conversion of A/T to G/C in genomic DNA expands the base editing tool portfolio by combining it with the BE3

and BE4 base editing designs, respectively (Gaudelli et al. 2017). The application of LbCas12a and AsCas12a as "dead-" and nickase-variants in the aforementioned BE-variants also provides efficient options to alter the DNA sequence specifically without the induction of DSBs (Li et al. 2018c).

Next to the targeted editing of individual bases to modify genes, the Cas effector tools developed so far also allow for sequence-specific regulation of transcription, utilizing the recently discovered Cas13 effector, which operates exclusively on RNA and mediates its cleavage. Three Cas13 orthologs studied so far showed efficient targeted RNA knockdown comparable to RNAi, whereas these proteins exhibit a higher specificity. LwaCas13a was applied for targeted RNA knockdown in rice protoplasts with up to 50% knockdown efficiency, 48 h post-transformation.

Post-transcriptional gene knockdown, instead of transcriptional regulation, can be interesting when only certain splicing variants are to be regulated, as all isoforms are repressed during transcriptional regulation (Mahas et al. 2018). Additionally, gene activity suppression by post-transcriptional regulation provides a fast, effective and highly specific means of regulation: existing cytoplasmic mRNAs get cleaved, whereas transcriptional regulation suppresses the production of further mRNA generations, while already produced mRNAs remain active (Schindele et al. 2018; Wolter and Puchta 2018). An applied example of such post-transcriptional repression was recently shown to successfully combat an RNA virus in plants, using the Cas13a ribonuclease from *Leptotrichia shahii* (LshCas13a). *Nicotiana benthamiana* leaves were infiltrated with a *Turnip mosaic virus* (TuMV), LshCas13a and crRNAs targeting the virus. Seven days post-inoculation, the viral GFP reporter signal was reduced by 50%, whereas plant vitality was not affected. However, there was a strong variance in the effectiveness of tested crRNAs, indicating a significant influence of the secondary structure of the mRNA on the cleavage activity (Aman et al. 2018). Compared to LwaCas13a and PspCas13b, the recently discovered RNA-binding effector EsCas13d has so far achieved the strongest gene knockdown in human cells (Konermann et al. 2018). Whether Cas13d is as efficient in plants has yet to be tested.

Similar to Cas9 and Cas12, the catalytic residues in the HEPN domains of Cas13 can be deactivated by

induction of point mutations resulting in a dCas13, a site-specific RNA binding protein. These RNA binding proteins can also be fused to functional units, for example fluorescent proteins to allow for specific imaging of RNAs in live cells (Abudayyeh et al. 2017). Furthermore, dCas13 enables precise RNA base editing by the fusion of an adenosine deaminase, as previously shown with Cas9 (Cox et al. 2017). In this way, sequence alterations can be incorporated, at the transcript level, without a permanent modification of the encoding genome sequence.

PERSPECTIVE

Multiple Cas applications in parallel (3D principle)

The intrinsic specificity of Cas orthologs for their corresponding gRNA, combined with the availability of modified proteins (nickase, dCas) and the feasibility of directly and indirectly fusing effector proteins, or functional domains, allows for not only simultaneous targeting of various loci in a genome, but also delivering locally different enzymatic activities to elevate, or suppress transcription of genes, edit individual bases and, thus, modify the ORF of a given gene to improve the encoded protein functions, or enable epigenetic modifications. Together with the site-specific induction of SSBs or DSBs and the possibility to transiently modify the required DNA repair machinery, we now have efficient and specific multifunctional tools available that can be applied to modulate complete pathways and thus, change the metabolome to synthetically improve crop traits. To improve the efficiency of such highly sophisticated genome reshuffling efforts, 3D portfolio properties of Cas orthologs could be exploited (Puchta 2016). Such an approach could enable boosting specific DSB repair pathways, by transient upregulation of desired repair factors, or downregulating competing pathways, which might shift the general DSB repair equilibrium in favor of the desired outcome.

Approaches of this nature for orthogonal regulation of gene expression became even more flexible with the availability of fusion gRNAs (fgRNAs) that can recruit different Cas orthologs, simultaneously, and thus, facilitate multiplexing with no detectable increase in off-target activity (Kweon et al. 2017). Additionally, the successful application of an orthogonal tri-functional

CRISPR system in *S. cerevisiae* for metabolic engineering, unambiguously demonstrates the versatility of combining different targeted activities, simultaneously, in one cell (Lian et al. 2017). Another intriguing possibility has been described as the proxy-CRISPR approach, which combines dCas9 orthologs to change chromatin structure, in a genomic region of interest, to restore therein the nuclease activity of another targeted effector.

This targeted activation bears strong potential to selectively edit a single gene in a genome harboring multiple identical copies, by adapting the adjacent chromatin microenvironment (Chen et al. 2017). Furthermore, modulation of gene expression can be achieved by targeting engineered bivalent dCas9 complexes to distal cis-regulatory elements in order to support the formation of large DNA loops and thereby, improving enhancer-promoter contact, as demonstrated in *E. coli* reporter strains (Hao et al. 2017). Additionally, specific recombination factors might be targeted to the loci needed, to locally enhance the availability of required repair processes, as recently demonstrated in mammalian cells for the key HR factor CtIP fused to Cas9 (Charpentier et al. 2018). Furthermore, we can envisage combining the aforementioned factors and even physically enhancing the probability of desired recombination events by directly or indirectly fusing Cas effectors to tether chromosomal fragments of choice.

Applications for highly sophisticated genome restructuring and breeding acceleration

The ability to induce multiple simultaneous DSBs in one genome, by targeting Cas9 nucleases to more than one locus, allows for the precise induction of segmental chromosome inversions, or reciprocal translocations. Inversions of genomic sequences would not change the overall gene pool of a given species; however, it might pose a means to render certain regions silent for meiotic recombination, due to the rearranged sequence context. Such artificially created recombination blocks might be suitable to stabilize advantageous alleles in breeding processes and thus, might reduce developmental costs. Next to that, targeted induction of reciprocal translocations between non-homologous chromosomes can be applied to create new tight linkage groups between advantageous traits to stabilize these as one unit for breeding purposes. *Vice versa*, undesired traits occurring in natural close linkages can

be broken to allow simple separation by segregation. The technology can be applied in a way that the obtained recombinants do not contain any transgenic sequences. Thus, the molecular tools described above should allow for reshaping genomes in order to better exploit the natural gene pool and additionally, to accelerate crop breeding processes.

REFERENCES

Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox D, Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, Zhang F (2017) RNA targeting with CRISPR-Cas13. **Nature** 550: 280–284

Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox D, Shmakov S, Makarova KS, Semenova E, Minakhin L, Severinov K, Regev A, Lander ES, Koonin EV, Zhang F (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. **Science** 353: aaf5573

Ali Z, Abul-faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, Aouida M, Piatek A, Baltes NJ, Voytas DF, Dinesh-Kumar S, Mahfouz MM (2015) Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. **Mol Plant** 8: 1288–1291

Aman R, Ali Z, Butt H, Mahas A, Aljedaani F, Khan MZ, Ding S, Mahfouz M (2018) RNA virus interference via CRISPR/Cas13a system in plants. **Genome Biol** 19: 1

Anders C, Niewoehner O, Duerst A, Jinek M (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. **Nature** 513: 569–573

Andersson M, Turesson H, Nicolia A, Fält A, Samuelsson M, Hofvander P (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. **Plant Cell Rep** 36: 117–128

Andersson M, Turesson H, Olsson N, Fält A, Ohlsson P, Gonzalez MN, Samuelsson M, Hofvander P (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. **Physiol Plant** https://doi.org/10.1111/ppl.12731

Anton T, Bultmann S, Leonhardt H, Markaki Y (2014) Visualization of specific DNA sequences in living mouse embryonic stem cells with a programmable fluorescent CRISPR/Cas system. **Nucleus** 5: 163–172

Bae S, Kweon J, Kim HS, Kim J (2014) Microhomology-based choice of Cas9 nuclease target sites. **Nat Methods** 11: 705–706

Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF (2014) DNA replicons for plant genome engineering. **Plant Cell** 26: 151–163

Baltes NJ, Hummel AW, Konecna E, Cegan R, Bruns AN, Bisaro DM, Voytas DF (2015) Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. **Nat Plants** 1: 15145 EP -

Barrangou R, Gersbach CA (2017) Expanding the CRISPR Toolbox: Targeting RNA with Cas13b. **Mol Cell** 65: 582–584

Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V (2015) Editing plant genomes with CRISPR/Cas9. **Curr Opin Biotech** 32: 76–84

Billon P, Bryant EE, Joseph SA, Nambiar TS, Hayward SB, Rothstein R, Ciccia A (2017) CRISPR-mediated base editing enables efficient disruption of eukaryotic genes through induction of STOP codons. **Mol Cell** 67: 1068–1079.e4

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. **Science** 326: 1509–1512

Braatz J, Harloff H, Mascher M, Stein N, Himmelbach A, Jung C (2017) CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). **Plant Physiol** 174: 935–942

Briner AE, Donohoue PD, Gomaa AA, Selle K, Slorach EM, Nye CH, Haurwitz RE, Beisel CL, May AP, Barrangou R (2014) Guide RNA functional modules direct Cas9 activity and orthogonality. **Mol Cell** 56: 333–339

Butler NM, Baltes NJ, Voytas DF, Douches DS (2016) Geminivirus-mediated genome editing in potato (*Solanum tuberosum* L.) Using sequence-specific nucleases. **Front Plant Sci** 7: 1045

Cai Y, Chen L, Liu X, Guo C, Sun S, Wu C, Jiang B, Han T, Hou W (2018) CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. **Plant Biotechnol J** 16: 176–185

Cavalier-Smith T (2005) Economy, speed and size matter: Evolutionary forces driving nuclear genome miniaturization and expansion. **Ann Bot-London** 95: 147–175

Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. **Nucleic Acids Res** 39: e82

Čermák T, Baltes NJ, Čegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. **Genome Biol** 16: 232

Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono TJY, Konečná E, Belanto JJ, Starker CG, Mathre JW, Greenstein RL, Voytas DF (2017) A multipurpose toolkit to enable advanced genome engineering in plants. **Plant Cell** 29: 1196–1217

Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. **Mol Plant Pathol** 17: 1140–1153

Chari R, Mali P, Moosburner M, Church GM (2015) Unraveling CRISPR-Cas9 genome engineering parameters via a library-on-library approach. **Nat Methods** 12: 823–826

Charpentier M, Khedher AHY, Menoret S, Brion A, Lamribet K, Dardillac E, Boix C, Perrouault L, Tesson L, Geny S, de Cian A, Itier JM, Anegon I, Lopez B, Giovannangeli C, Concordet JP (2018) CtIP fusion to Cas9 enhances transgene integration by homology-dependent repair. **Nat Commun** 9: 1133

Chaudhary K, Chattopadhyay A, Pratap D (2018) The evolution of CRISPR/Cas9 and their cousins: Hope or hype? **Biotechnol Lett** 40: 465–477

Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li G, Park J, Blackburn EH, Weissman JS, Qi LS, Huang B (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. **Cell** 155: 1479–1491

Chen B, Hu J, Almeida R, Liu H, Balakrishnan S, Covill-Cooke C, Lim WA, Huang B (2016) Expanding the CRISPR imaging toolset with *Staphylococcus aureus* Cas9 for simultaneous imaging of multiple genomic loci. **Nucleic Acids Res** 44: e75

Chen F, Ding X, Feng Y, Seebeck T, Jiang Y, Davis GD (2017) Targeted activation of diverse CRISPR-Cas systems for mammalian genome editing via proximal CRISPR targeting. **Nat Commun** 8: 14958

Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM, Doudna JA (2018) CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. **Science** 360: 436–439

Cox D, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F (2017) RNA editing with CRISPR-Cas13. **Science** 358: 1019–1027

Curtin SJ, Xiong Y, Michno J, Campbell BW, Stec AO, Čermák T, Starker C, Voytas DF, Eamens AL, Stupar RM (2018) CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of *Glycine max* and *Medicago truncatula*. **Plant Biotechnol J** 16: 1125–1137

Dahan-Meir T, Filler-Hayut S, Melamed-Bessudo C, Bocobza S, Czosnek H, Aharoni A, Levy AA (2018) Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. **Plant J** 95: 5–16

Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, Virgin HW, Listgarten J, Root DE (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. **Nat Biotechnol** 34: 184–191

Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE (2014) Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. **Nat Biotechnol** 32: 1262–1267

Dreissig S, Schiml S, Schindele P, Weiss O, Rutten T, Schubert V, Gladilin E, Mette MF, Puchta H, Houben A (2017) Live-cell CRISPR imaging in plants reveals dynamic telomere movements. **Plant J** 91: 565–573

East-Seletsky A, O'Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, Doudna JA (2016) Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. **Nature** 538: 270–273

Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM (2013) Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. **Nat Methods** 10: 1116–1121

Fauser F, Roth N, Pacher M, Ilg G, Sánchez-Fernández R, Biesgen C, Puchta H (2012) In planta gene targeting. **Proc Natl Acad Sci USA** 109: 7535–7540

Fauser F, Schiml S, Puchta H (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. **Plant J** 79: 348–359

Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang D, Wang Z, Zhang Z, Zheng R, Yang L, Zeng L, Liu X, Zhu J (2014) Multi-generation analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. **Proc Natl Acad Sci USA** 111: 4632–4637

Filler Hayut S, Melamed Bessudo C, Levy AA (2017) Targeted recombination between homologous chromosomes for precise breeding in tomato. **Nat Commun** 8: 15605

Fonfara I, Le Rhun A, Chylinski K, Makarova KS, Lécrivain A, Bzdrenga J, Koonin EV, Charpentier E (2014) Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. **Nucleic Acids Res** 42: 2577–2590

Fonfara I, Richter H, Bratovič M, Le Rhun A, Charpentier E (2016) The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. **Nature** 532: 517–521

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. **Nat Biotechnol** 31: 822–826

Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. **Nat Biotechnol** 32: 279–284

Gagnon JA, Valen E, Thyme SB, Huang P, Akhmetova L, Ahkmetova L, Pauli A, Montague TG, Zimmerman S, Richter C, Schier AF (2014) Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. **PLoS ONE** 9: e98186

Gallego-Bartolomé J, Gardiner J, Liu W, Papikian A, Ghoshal B, Kuo HY, Zhao JM, Segal DJ, Jacobsen SE (2018) Targeted DNA demethylation of the *Arabidopsis* genome using the human TET1 catalytic domain. **Proc Natl Acad Sci USA** 115: E2125–E2134

Gao Y, Zhao Y (2014) Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. **J Integr Plant Biol** 56: 343–349

Gao P, Yang H, Rajashankar KR, Huang Z, Patel DJ (2016) Type V CRISPR-Cas Cpf1 endonuclease employs a unique mechanism for crRNA-mediated target DNA recognition. **Cell Res** 26: 901–913

Garneau JE, Dupuis M, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. **Nature** 468: 67–71

Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR (2017) Programmable base editing of A·T to G·C in genomic DNA without DNA cleavage. **Nature** 551: 464–471

Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. **Cell** 154: 442–451

Gil-Humanes J, Wang Y, Liang Z, Shan Q, Ozuna CV, Sánchez-León S, Baltes NJ, Starker C, Barro F, Gao C, Voytas DF (2017) High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. **Plant J** 89: 1251–1262

Hao N, Shearwin KE, Dodd IB (2017) Programmable DNA looping using engineered bivalent dCas9 complexes. **Nat Commun** 8: 1628

He L, Zhang S, Qiu Z, Zhao J, Nie W, Lin H, Zhu Z, Zeng D, Qian Q, Zhu L (2018) FRUCTOKINASE-LIKE PROTEIN 1 interacts with TRXz to regulate chloroplast development in rice. **J Integr Plant Biol** 60: 94–111

Hille F, Charpentier E (2016) CRISPR-Cas: Biology, mechanisms and relevance. **Philos Trans R Soc B Biol Sci** 371: https://doi.org/10.1098/rstb.2015.0496

Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. **Nat Biotechnol** 33: 510–517

Horvath P, Romero DA, Coûté-Monvoisin A, Richards M, Deveau H, Moineau S, Boyaval P, Fremaux C, Barrangou R (2008) Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. **J Bacteriol** 190: 1401–1412

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, Liu DR (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. **Nature** 556: 57–63

Huang Y, Guo Y, Liu Y, Zhang F, Wang Z, Wang H, Wang F, Li D, Mao D, Luan S, Liang M, Chen L (2018) 9-cis-Epoxycarotenoid dioxygenase 3 regulates plant growth and enhances multi-abiotic stress tolerance in rice. **Front Plant Sci** 9: 162

Jacquier A, Dujon B (1985) An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. **Cell** 41: 383–394

Ji X, Zhang H, Zhang Y, Wang Y, Gao C (2015) Establishing a CRISPR–Cas-like immune system conferring DNA virus resistance in plants. **Nat Plants** 1: 15144 EP -

Jia H, Zhang Y, Orbović V, Xu J, White FF, Jones JB, Wang N (2017) Genome editing of the disease susceptibility gene *CsLOB1* in citrus confers resistance to citrus canker. **Plant Biotechnol J** 15: 817–823

Jiang WZ, Henry IM, Lynagh PG, Comai L, Cahoon EB, Weeks DP (2017) Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. **Plant Biotechnol J** 15: 648–657

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. **Science** 337: 816–821

Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E, Doudna JA (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. **Science** 343: 1247997

Kapusi E, Corcuera-Gómez M, Melnik S, Stoger E (2017) Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. **Front Plant Sci** 8: 540

Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, Garber M, Maehr R (2015) Functional annotation of native enhancers with a Cas9-histone demethylase fusion. **Nat Methods** 12: 401–403

Kim H, Kim S, Ryu J, Kang B, Kim J, Kim S (2017) CRISPR/Cpf1-mediated DNA-free plant genome editing. **Nat Commun** 8: 14406

Kim YG, Cha J, Chandrasegaran S (1996) Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. **Proc Natl Acad Sci USA** 93: 1156–1160

Klap C, Yeshayahou E, Bolger AM, Arazi T, Gupta SK, Shabtai S, Usadel B, Salts Y, Barg R (2017) Tomato facultative parthenocarpy results from SlAGAMOUS-LIKE 6 loss of function. **Plant Biotechnol J** 15: 634–647

Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. **Nature** 529: 490–495

Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JJ, Aryee MJ, Joung JK (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. **Nature** 523: 481–485

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. **Nature** 533: 420–424

Komor AC, Zhao KT, Packer MS, Gaudelli NM, Waterbury AL, Koblan LW, Kim YB, Badran AH, Liu DR (2017) Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. **Sci Adv** 3: eaao4774

Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. **Nature** 517: 583–588

Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD (2018) Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. **Cell** 173: 665–676.e14

Koonin EV, Makarova KS, Zhang F (2017) Diversity, classification and evolution of CRISPR-Cas systems. **Curr Opin Microbiol** 37: 67–78

Kuscu C, Parlak M, Tufan T, Yang J, Szlachta K, Wei X, Mammadov R, Adli M (2017) CRISPR-STOP: Gene silencing through base-editing-induced nonsense mutations. **Nat Methods** 14: 710–712

Kweon J, Jang A, Kim D, Yang JW, Yoon M, Rim Shin H, Kim J, Kim Y (2017) Fusion guide RNAs for orthogonal gene manipulation with Cas9 and Cpf1. **Nat Commun** 8: 1723

Labuhn M, Adams FF, Ng M, Knoess S, Schambach A, Charpentier EM, Schwarzer A, Mateo JL, Klusmann J, Heckl D (2018) Refined sgRNA efficacy prediction improves large- and small-scale CRISPR-Cas9 applications. **Nucleic Acids Res** 46: 1375–1385

Lawrenson T, Shorinola O, Stacey N, Li C, Østergaard L, Patron N, Uauy C, Harwood W (2015) Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. **Genome Biol** 16: 258

Le Cong, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. **Science** 339: 819–823

Lee HJ, Kweon J, Kim E, Kim S, Kim J (2012) Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. **Genome Res** 22: 539–548

Lee J, Chung J, Kim HM, Kim D, Kim H (2016) Designed nucleases for targeted genome editing. **Plant Biotechnol J** 14: 448–462

Li J, Meng X, Zong Y, Chen K, Zhang H, Liu J, Li J, Gao C (2016a) Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. **Nat Plants** 2: 16139

Li J, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. **Nat Biotechnol** 31: 688–691

Li J, Sun Y, Du J, Zhao Y, Xia L (2017a) Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. **Mol Plant** 10: 526–529

Li M, Li X, Zhou Z, Wu P, Fang M, Pan X, Lin Q, Luo W, Wu G, Li H (2016b) Reassessment of the four yield-related genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in rice using a CRISPR/Cas9 system. **Front Plant Sci** 7: 377

Li Q, Zhang D, Chen M, Liang W, Wei J, Qi Y, Yuan Z (2016c) Development of japonica photo-sensitive genic male sterile rice lines by editing carbon starved anther using CRISPR/Cas9. **J Genet Genomics** 43: 415–419

Li R, Fu D, Zhu B, Luo Y, Zhu H (2018a) CRISPR/Cas9-mediated mutagenesis of lncRNA1459 alters tomato fruit ripening. **Plant J** 94: 513–524

Li X, Wang Y, Chen S, Tian H, Fu D, Zhu B, Luo Y, Zhu H (2018b) Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. **Front Plant Sci** 9: 559

Li X, Wang Y, Liu Y, Yang B, Wang X, Wei J, Lu Z, Zhang Y, Wu J, Huang X, Yang L, Chen J (2018c) Base editing with a Cpf1-cytidine deaminase fusion. **Nat Biotechnol** 36: 324–327

Li X, Zhou W, Ren Y, Tian X, Lv T, Wang Z, Fang J, Chu C, Yang J, Bu Q (2017b) High-efficiency breeding of early-maturing

rice cultivars via CRISPR/Cas9-mediated genome editing. **J Genet Genomics** 44: 175–178

Li Y, Ma S, Le Sun, Zhang T, Chang J, Lu W, Chen X, Liu Y, Wang X, Shi R, Zhao P, Xia Q (2018d) Programmable single and multiplex base-editing in *Bombyx mori* using RNA-guided cytidine deaminases. **G3-Genes Genom Genet** 8: 1701–1709

Lian J, Hamedi Rad M, Hu S, Zhao H (2017) Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system. **Nat Commun** 8: 1688

Liang G, Zhang H, Lou D, Yu D (2016) Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. **Sci Rep** 6: 21451

Liang Y, Zeng X, Peng X, Hou X (2018a) *Arabidopsis glutamate: Glyoxylate aminotransferase 1* (Ler) mutants generated by CRISPR/Cas9 and their characteristics. **Transgenic Res** 27: 61–74

Liang Z, Chen K, Yan Y, Zhang Y, Gao C (2018b) Genotyping genome-edited mutations in plants using CRISPR ribonucleoprotein complexes. **Plant Biotechnol J** https://doi.org/10.1111/pbi.12938

Liang Z, Zhang K, Chen K, Gao C (2014) Targeted mutagenesis in *Zea mays* using TALENS and the CRISPR/Cas system. **J Genet Genomics** 41: 63–68

Liu H, Ding Y, Zhou Y, Jin W, Xie K, Chen L (2017a) CRISPR-P 2.0: An improved CRISPR-Cas9 tool for genome editing in plants. **Mol Plant** 10: 530–532

Liu L, Li X, Wang J, Wang M, Chen P, Yin M, Li J, Sheng G, Wang Y (2017b) Two distant catalytic sites are responsible for C2c2 RNase activities. **Cell** 168: 121–134

Louwen R, Staals RH, Endtz HP, van Baarlen P, van der Oost J (2014) The role of CRISPR-Cas systems in virulence of pathogenic bacteria. **Microbiol Mol Biol R** 78: 74–88

Lowder LG, Zhang D, Baltes NJ, Paul JW, Tang X, Zheng X, Voytas DF, Hsieh T, Zhang Y, Qi Y (2015) A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. **Plant Physiol** 169: 971–985

Lowder LG, Zhou J, Zhang Y, Malzahn A, Zhong Z, Hsieh T, Voytas DF, Zhang Y, Qi Y (2018) Robust transcriptional activation in plants using multiplexed CRISPR-Act2.0 and mTALE-Act systems. **Mol Plant** 11: 245–256

Lu Y, Zhu J (2017) Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 System. **Mol Plant** 10: 523–525

Ma H, Naseri A, Reyes-Gutierrez P, Wolfe SA, Zhang S, Pederson T (2015a) Multicolor CRISPR labeling of chromosomal loci in human cells. **Proc Natl Acad Sci USA** 112: 3002–3007

Ma J, Chen J, Wang M, Ren Y, Wang S, Lei C, Cheng Z, Sodmergen (2018) Disruption of OsSEC3A increases the content of salicylic acid and induces plant defense responses in rice. **J Exp Bot** 69: 1051–1064

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X, Dong Z, Liu Y (2015b) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. **Mol Plant** 8: 1274–1284

Mahas A, Neal Stewart C, Mahfouz MM (2018) Harnessing CRISPR/Cas systems for programmable transcriptional and post-transcriptional regulation. **Biotechnol Adv** 36: 295–310

Mahfouz MM, Piatek A, Stewart CN (2014) Genome engineering via TALENs and CRISPR/Cas9 systems: Challenges and perspectives. **Plant Biotechnol J** 12: 1006–1014

Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJ, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, van der Oost J, Backofen R, Koonin EV (2015) An updated evolutionary classification of CRISPR-Cas systems. **Nat Rev Microbiol** 13: 722–736

Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA-guided human genome engineering via Cas9. **Science** 339: 823–826

Miki D, Zhang W, Zeng W, Feng Z, Zhu J (2018) CRISPR/Cas9-mediated gene targeting in *Arabidopsis* using sequential transformation. **Nat Commun** 9: 1967

Minkenberg B, Xie K, Yang Y (2017) Discovery of rice essential genes by characterizing a CRISPR-edited mutation of closely related rice MAP kinase genes. **Plant J** 89: 636–648

Moreno-Mateos MA, Vejnar CE, Beaudoin J, Fernandez JP, Mis EK, Khokha MK, Giraldez AJ (2015) CRISPRscan: Designing highly efficient sgRNAs for CRISPR-Cas9 targeting *in vivo*. **Nat Methods** 12: 982–988

Morineau C, Bellec Y, Tellier F, Gissot L, Kelemen Z, Nogué F, Faure J (2017) Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid *Camelina sativa*. **Plant Biotechnol J** 15: 729–739

Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. **Science** 326: 1501

Murugan K, Babu K, Sundaresan R, Rajan R, Sashital DG (2017) The revolution continues: Newly discovered systems expand the CRISPR-Cas toolkit. **Mol Cell** 68: 15–25

Nekrasov V, Wang C, Win J, Lanz C, Weigel D, Kamoun S (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. **Sci Rep** 7: 482

Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY, Shimatani Z, Kondo A (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. **Science** 353: 6305

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. **Cell** 156: 935–949

Nissim L, Perli SD, Fridkin A, Perez-Pinera P, Lu TK (2014) Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. **Mol Cell** 54: 698–710

Osakabe Y, Watanabe T, Sugano SS, Ueta R, Ishihara R, Shinozaki K, Osakabe K (2016) Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. **Sci Rep** 6: 26685

Ozseyhan ME, Kang J, Mu X, Lu C (2018) Mutagenesis of the FAE1 genes significantly changes fatty acid composition in seeds of *Camelina sativa*. **Plant Physiol Biochem** 123: 1–7

Pacher M, Puchta H (2017) From classical mutagenesis to nuclease-based breeding - directing natural DNA repair for a natural end-product. **Plant J** 90: 819–833

Pacher M, Schmidt-Puchta W, Puchta H (2007) Two unlinked double-strand breaks can induce reciprocal exchanges in plant genomes via homologous recombination and nonhomologous end joining. **Genetics** 175: 21–29

Peabody DS (1993) The RNA binding site of bacteriophage MS2 coat protein. **EMBO J** 12: 595–600

Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW, Guilak F, Crawford GE, Reddy TE, Gersbach CA (2013) RNA-guided gene activation by CRISPR-Cas9-based transcription factors. **Nat Methods** 10: 973–976

Perrin A, Buckle M, Dujon B (1993) Asymmetrical recognition and activity of the I-SceI endonuclease on its site and on intron-exon junctions. **EMBO J** 12: 2939–2947

Piatek A, Ali Z, Baazim H, Li L, Abulfaraj A, Al-Shareef S, Aouida M, Mahfouz MM (2015) RNA-guided transcriptional regulation in planta via synthetic dCas9-based transcription factors. **Plant Biotechnol J** 13: 578–589

Prykhozhij SV, Rajan V, Gaston D, Berman JN (2015) CRISPR multitargeter: A web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. **PLoS ONE** 10: e0119372

Puchta H (2005) The repair of double-strand breaks in plants: Mechanisms and consequences for genome evolution. **J Exp Bot** 56: 1–14

Puchta H (2016) Using CRISPR/Cas in three dimensions: Towards synthetic plant genomes, transcriptomes and epigenomes. **Plant J** 87: 5–15

Puchta H (2017) Applying CRISPR/Cas for genome engineering in plants: The best is yet to come. **Curr Opin Plant Biol** 36: 1–8

Puchta H, Dujon B, Hohn B (1996) 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** 93: 5055–5060

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. **Cell** 152: 1173–1183

Qin P, Parlak M, Kuscu C, Bandaria J, Mir M, Szlachta K, Singh R, Darzacq X, Yildiz A, Adli M (2017) Live cell imaging of low- and non-repetitive chromosome loci using CRISPR-Cas9. **Nat Commun** 8: 14725

Qin X, Li W, Liu Y, Tan M, Ganal M, Chetelat RT (2018) A farnesyl pyrophosphate synthase gene expressed in pollen functions in S-RNase-independent unilateral incompatibility. **Plant J** 93: 417–430

Ran FA, Hsu PD, Lin C, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. **Cell** 154: 1380–1389

Ran FA, Le Cong, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F (2015) *In vivo* genome editing using *Staphylococcus aureus* Cas9. **Nature** 520: 186–191

Rees HA, Komor AC, Yeh W, Caetano-Lopes J, Warman M, Edge ASB, Liu DR (2017) Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery. **Nat Commun** 8: 15790

Ren B, Yan F, Kuang Y, Li N, Zhang D, Zhou X, Lin H, Zhou H (2018) Improved base editor for efficiently inducing genetic variations in rice with CRISPR/Cas9-guided hyperactive hAID Mutant. **Mol Plant** 11: 623–626

Ren X, Yang Z, Xu J, Sun J, Mao D, Hu Y, Yang S, Qiao H, Wang X, Hu Q, Deng P, Liu L, Ji J, Li JB, Ni J (2014) Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in Drosophila. **Cell Rep** 9: 1151–1162

Rodríguez-Leal D, Lemmon ZH, Man J, Bartlett ME, Lippman ZB (2017) Engineering quantitative trait variation for crop improvement by genome editing. **Cell** 171: 470–480

Saito M, Kondo Y, Fukuda H (2018) BES1 and BZR1 redundantly promote phloem and xylem differentiation. **Plant Cell Physiol** 59: 590–600

Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. **EMBO J** 17: 6086–6095

Sánchez-León S, Gil-Humanes J, Ozuna CV, Giménez MJ, Sousa C, Voytas DF, Barro F (2018) Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. **Plant Biotechnol J** 16: 902–910

Sauer NJ, Narváez-Vásquez J, Mozoruk J, Miller RB, Warburg ZJ, Woodward MJ, Mihiret YA, Lincoln TA, Segami RE, Sanders SL, Walker KA, Beetham PR, Schöpke CR, Gocal GF (2016) Oligonucleotide-mediated genome editing provides precision and function to engineered nucleases and antibiotics in plants. **Plant Physiol** 170: 1917–1928

Schaeffer SM, Nakata PA (2015) CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field. **Plant Sci** 240: 130–142

Scheben A, Wolter F, Batley J, Puchta H, Edwards D (2017) Towards CRISPR/Cas crops - bringing together genomics and genome editing. **New Phytol** 216: 682–698

Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in *Arabidopsis* resulting in heritable progeny. **Plant J** 80: 1139–1150

Schiml S, Fauser F, Puchta H (2016) Repair of adjacent single-strand breaks is often accompanied by the formation of tandem sequence duplications in plant genomes. **Proc Natl Acad Sci USA** 113: 7266–7271

Schindele P, Wolter F, Puchta H (2018) Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13. **FEBS Lett** 592: 1954–1967

Schunder E, Rydzewski K, Grunow R, Heuner K (2013) First indication for a functional CRISPR/Cas system in *Francisella tularensis*. **Int J Med Microbiol** 303: 51–60

Shen R, Wang L, Liu X, Wu J, Jin W, Zhao X, Xie X, Zhu Q, Tang H, Li Q, Chen L, Liu Y (2017) Genomic structural variation-mediated allelic suppression causes hybrid male sterility in rice. **Nat Commun** 8: 1310

Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. **Plant Biotechnol J** 15: 207–216

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H, Nishida K, Ariizumi T, Kondo A (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. **Nat Biotechnol** 35: 441–443

Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, Zhang F, Koonin EV (2015) Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. **Mol Cell** 60: 385–397

Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F (2016) Rationally engineered Cas9 nucleases with improved specificity. **Science** 351: 84–88

Smith J, Bibikova M, Whitby FG, Reddy AR, Chandrasegaran S, Carroll D (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. **Nucleic Acids Res** 28: 3361–3369

Soyk S, Müller NA, Park SJ, Schmalenbach I, Jiang K, Hayama R, Zhang L, van Eck J, Jiménez-Gómez JM, Lippman ZB (2017) Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. **Nat Genet** 49: 162–168

Steinert J, Schiml S, Fauser F, Puchta H (2015) Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. **Plant J** 84: 1295–1305

Steinert J, Schiml S, Puchta H (2016) Homology-based double-strand break-induced genome engineering in plants. **Plant Cell Rep** 35: 1429–1438

Sun Y, Jiao G, Liu Z, Zhang X, Li J, Guo X, Du W, Du J, Francis F, Zhao Y, Xia L (2017) Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. **Front Plant Sci** 8: 298

Sun Y, Zhang X, Wu C, He Y, Ma Y, Hou H, Guo X, Du W, Zhao Y, Xia L (2016) Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. **Mol Plant** 9: 628–631

Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. **Nat Commun** 7: 13274

Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM (2015) Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. **Plant Physiol** 169: 931–945

Swarts DC, van der Oost J, Jinek M (2017) Structural basis for guide rna processing and seed-dependent DNA targeting by CRISPR-Cas12a. **Mol Cell** 66: 221–233

Tak YE, Kleinstiver BP, Nuñez JK, Hsu JY, Horng JE, Gong J, Weissman JS, Joung JK (2017) Inducible and multiplex gene regulation using CRISPR-Cpf1-based transcription factors. **Nat Methods** 14: 1163–1166

Tanenbaum ME, Gilbert LA, Qi LS, Weissman JS, Vale RD (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. **Cell** 159: 635–646

Tang X, Liu G, Zhou J, Ren Q, You Q, Tian L, Xin X, Zhong Z, Liu B, Zheng X, Zhang D, Malzahn A, Gong Z, Qi Y, Zhang T, Zhang Y (2018) A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. **Genome Biol** 19: 84

Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER, Zhang Y, Qi Y (2017) A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. **Nat Plants** 3: 17018

Tang X, Zheng X, Qi Y, Zhang D, Cheng Y, Tang A, Voytas DF, Zhang Y (2016) A single transcript CRISPR-Cas9 system for efficient genome editing in plants. **Mol Plant** 9: 1088–1091

Vaughn JN, Bennetzen JL (2014) Natural insertions in rice commonly form tandem duplications indicative of patch-mediated double-strand break induction and repair. **Proc Natl Acad Sci USA** 111: 6684–6689

Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. **Annu Rev Plant Biol** 64: 327–350

Waltz E (2016) CRISPR-edited crops free to enter market, skip regulation. **Nat Biotechnol** 34: 582

Wang F, Wang C, Liu P, Lei C, Hao W, Gao Y, Liu Y, Zhao K (2016) Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene *OsERF922*. **PLoS ONE** 11: e0154027

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. **Cell** 153: 910–918

Wang L, Chen L, Li R, Zhao R, Yang M, Sheng J, Shen L (2017a) Reduced drought tolerance by CRISPR/Cas9-mediated SlMAPK3 mutagenesis in tomato plants. **J Agr Food Chem** 65: 8674–8682

Wang M, Mao Y, Lu Y, Tao X, Zhu J (2017b) Multiplex gene editing in rice using the CRISPR-Cpf1 system. **Mol Plant** 10: 1011–1013

Wang M, Mao Y, Lu Y, Wang Z (2018) Multiplex gene editing in rice with simplified CRISPR-Cpf1 and CRISPR-Cas9 systems. **J Integr Plant Biol** 60: 626–631

Wang P, Zhang J, Sun L, Ma Y, Xu J, Liang S, Deng J, Tan J, Zhang Q, Tu L, Daniell H, Jin S, Zhang X (2018a) High efficient multisites genome editing in allotetraploid cotton (*Gossypium hirsutum*) using CRISPR/Cas9 system. **Plant Biotechnol J** 16: 137–150

Wang T, Wei JJ, Sabatini DM, Lander ES (2014) Genetic screens in human cells using the CRISPR-Cas9 system. **Science** 343: 80–84

Wang X, Tu M, Wang D, Liu J, Li Y, Li Z, Wang Y, Wang X (2018b) CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. **Plant Biotechnol J** 16: 844–855

Wang Z, Lv J, Xie S, Zhang Y, Qiu Z, Chen P, Cui Y, Niu Y, Hu S, Jiang H, Ge S, Trinh H, Lei K, Bai W, Zhang Y, Guo L, Ren D (2018c) OsSLA4 encodes a pentatricopeptide repeat protein essential for early chloroplast development and seedling growth in rice. **Plant Growth Regul** 84: 249–260

Weeks DP, Spalding MH, Yang B (2016) Use of designer nucleases for targeted gene and genome editing in plants. **Plant Biotechnol J** 14: 483–495

Weinthal DM, Taylor RA, Tzfira T (2013) Nonhomologous end joining-mediated gene replacement in plant cells. **Plant Physiol** 162: 390–400

Wiedenheft B, Sternberg SH, Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. **Nature** 482: 331–338

Wolter F, Klemm J, Puchta H (2018) Efficient in planta gene targeting in *Arabidopsis* using egg-cell specific expression of the Cas9 nuclease of *Staphylococcus aureus*. **Plant J** 94: 735–746

Wolter F, Puchta H (2018) The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists. **Plant J** 94: 767–775

Wong N, Liu W, Wang X (2015) WU-CRISPR: Characteristics of functional guide RNAs for the CRISPR/Cas9 system. **Genome Biol** 16: 218

Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim S, Kim S, Choe S, Kim J (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. **Nature Biotechnol** 33: 1162–1164

Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. **Proc Natl Acad Sci USA** 112: 3570–3575

Xie X, Ma X, Zhu Q, Zeng D, Li G, Liu Y (2017) CRISPR-GE: A convenient software toolkit for CRISPR-based genome editing. **Mol Plant** 10: 1246–1249

Xing H, Dong L, Wang Z, Zhang H, Han C, Liu B, Wang X, Chen Q (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. **BMC Plant Biol** 14: 327

Xu H, Xiao T, Chen C, Li W, Meyer CA, Wu Q, Di Wu, Le Cong, Zhang F, Liu JS, Brown M, Liu XS (2015) Sequence determinants of improved CRISPR sgRNA design. **Genome Res** 25: 1147–1157

Xu R, Li H, Qin R, Wang L, Li L, Wei P, Yang J (2014) Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice. **Rice** 7: 5

Xu R, Yang Y, Qin R, Li H, Qiu C, Li L, Wei P, Yang J (2016) Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. **J Genet Genomics** 43: 529–532

Xu X, Duan D, Chen S (2017) CRISPR-Cas9 cleavage efficiency correlates strongly with target-sgRNA folding stability: From physical mechanism to off-target assessment. **Sci Rep** 7: 143

Yamano T, Nishimasu H, Zetsche B, Hirano H, Slaymaker IM, Li Y, Fedorova I, Nakane T, Makarova KS, Koonin EV, Ishitani R, Zhang F, Nureki O (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. **Cell** 165: 949–962

Yan F, Kuang Y, Ren B, Wang J, Zhang D, Lin H, Yang B, Zhou X, Zhou H (2018) Highly efficient A·T to G·C base editing by Cas9n-Guided tRNA adenosine deaminase in rice. **Mol Plant** 11: 631–634

Yang H, Wu J, Tang T, Liu K, Dai C (2017) CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in *Brassica napus*. **Sci Rep** 7: 7489

Yang Y, Zhu K, Li H, Han S, Meng Q, Khan SU, Fan C, Xie K, Zhou Y (2018) Precise editing of *CLAVATA* genes in *Brassica napus* L. regulates multilocular silique development. **Plant Biotechnol J** 16: 1322–1335

Yao L, Zhang Y, Liu C, Liu Y, Wang Y, Liang D, Liu J, Sahoo G, Kelliher T (2018) OsMATL mutation induces haploid seed formation in indica rice. **Nat Plants** 4: 530–533

Yu Z, Chen Q, Chen W, Zhang X, Mei F, Zhang P, Zhao M, Wang X, Shi N, Jackson S, Hong Y (2018) Multigene editing via CRISPR/Cas9 guided by a single-sgRNA seed in *Arabidopsis*. **J Integr Plant Biol** 60: 376–381

Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, Lim WA (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. **Cell** 160: 339–350

Zeng D, Yang C, Qin R, Alamin M, Yue E, Jin X, Shi C (2018) A guanine insert in OsBBS1 leads to early leaf senescence and salt stress sensitivity in rice (*Oryza sativa* L.). **Plant Cell Rep** 37: 933–946

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. **Cell** 163: 759–771

Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY, Scott DA, Severinov K, van der Oost J, Zhang F (2017) Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. **Nat Biotechnol** 35: 31–34

Zhang X, Wang J, Cheng Q, Zheng X, Zhao G, Wang J (2017a) Multiplex gene regulation by CRISPR-ddCpf1. **Cell Discov** 3: 17018

Zhang Y, Bai Y, Wu G, Zou S, Chen Y, Gao C, Tang D (2017b) Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. **Plant J** 91: 714–724

Zhang Y, Li S, Xue S, Yang S, Huang J, Wang L (2018) Phylogenetic and CRISPR/Cas9 studies in deciphering the

evolutionary trajectory and phenotypic impacts of rice *ERECTA* genes. **Front Plant Sci** 9: 473

Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu J, Gao C (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. **Nat Commun** 7: 12617

Zheng Y, Shen W, Zhang J, Yang B, Liu Y, Qi H, Yu X, Lu S, Chen Y, Xu Y, Li Y, Gage FH, Mi S, Yao J (2018) CRISPR interference-based specific and efficient gene inactivation in the brain. **Nature Neurosci** 21: 447–454

Zhou H, He M, Li J, Chen L, Huang Z, Zheng S, Zhu L, Ni E, Jiang D, Zhao B, Zhuang C (2016) Development of commercial thermo-sensitive genic male sterile rice accelerates hybrid rice breeding using the CRISPR/Cas9-mediated TMS5 editing system. **Sci Rep** 6: 37395

Zhou J, Deng K, Cheng Y, Zhong Z, Tian L, Tang X, Tang A, Zheng X, Zhang T, Qi Y, Zhang Y (2017) CRISPR-Cas9 based

genome editing reveals new insights into microrna function and regulation in rice. **Front Plant Sci** 8: 1598

Zhou J, Peng Z, Long J, Sosso D, Liu B, Eom J, Huang S, Liu S, Vera Cruz C, Frommer WB, White FF, Yang B (2015) Gene targeting by the TAL effector PthXo2 reveals cryptic resistance gene for bacterial blight of rice. **Plant J** 82: 632–643

Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu J, Wang D, Gao C (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. **Nat Biotechnol** 35: 438–440

Zou T, Liu M, Xiao Q, Wang T, Chen D, Luo T, Yuan G, Li Q, Zhu J, Liang Y, Deng Q, Wang S, Zheng A, Wang L, Li P, Li S (2018) OsPKS2 is required for rice male fertility by participating in pollen wall formation. **Plant Cell Rep** 37: 759–773

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. **Nucleic Acids Res** 31: 3406–3415

Scan using WeChat with your smartphone to view JIPB online

Scan with iPhone or iPad to view JIPB online