FEBS Letters

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REVIEW ARTICLE

Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13

Patrick Schindele, Felix Wolter and Holger Puchta

Botanical Institute, Karlsruhe Institute of Technology, Germany

Correspondence
H. Puchta, Botanical Institute, Karlsruhe Institute of Technology, POB 6980, 76049, Karlsruhe, Germany
Fax: +49 721 608-44874
Tel: +49 721 608-43833
E-mail: holger.puchta@kit.edu

(Received 15 March 2018, revised 9 April 2018, accepted 23 April 2018, available online 10 May 2018)

doi:10.1002/1873-3468.13073

Edited by Wilhelm Just

Currently, biology is revolutionized by ever growing applications of the CRISPR/Cas system. As discussed in this Review, new avenues have opened up for plant research and breeding by the use of the sequence-specific DNases Cas9 and Cas12 (formerly named Cpf1) and, more recently, the RNase Cas13 (formerly named C2c2). Although double strand break-induced gene editing based on error-prone nonhomologous end joining has been applied to obtain new traits, such as powdery mildew resistance in wheat or improved pathogen resistance and increased yield in tomato, improved technologies based on CRISPR/Cas for programmed change in plant genomes via homologous recombination have recently been developed. Cas9- and Cas12- mediated DNA binding is used to develop tools for many useful applications, such as transcriptional regulation or fluorescence-based imaging of specific chromosomal loci in plant genomes. Cas13 has recently been applied to degrade mRNAs and combat viral RNA replication. By the possibility to address multiple sequences with different guide RNAs and by the simultaneous use of different Cas proteins in a single cell, we should soon be able to achieve complex changes of plant metabolism in a controlled way.

Keywords: crops; gene editing; genome engineering

For a long time, plant research and breeding were limited by the exclusive access to imprecise and barely steerable techniques. The understanding of gene function was based on the unspecific and random generation of mutants via classical mutagenesis techniques or T-DNA insertion and the establishment of new traits was based on time-consuming selection or crossbreeding processes. The emergence of sequence-specific nucleases (SSNs) that enable the targeted induction of double-strand breaks (DSBs) into the genome initiated a revolution in plant biology. By harnessing the endogenous repair mechanisms that are activated after DSB induction, modifications can be introduced into the plant genome at sites of interest. Generally, there are two major pathways of DSB repair in plants: homologous recombination (HR) and nonhomologous end joining (NHEJ), the latter being the major pathway for the repair of DSBs in somatic plant cells [1,2]. The first genome-editing achievements were already accomplished more than two decades ago with the use of site-specific endonucleases [3,4]. The potential of the site-specific induction of DSBs for plant breeding applications was demonstrated by showing that even reciprocal exchanges of chromosome arms can be

Abbreviations
ADAR2dd, adenosine deaminase acting on RNA 2 deaminase domain; CRE, cis-regulatory element; crRNA, CRISPR RNA; dCas13, deadCas13; dCas9, deadCas9; DETECTR, DNA endonuclease targeted CRISPR Trans reporter; DSB, double-strand break; GT, gene targeting; HEPN, higher eukaryotes and prokaryotes nucleotide-binding; HR, homologous recombination; NHEJ, nonhomologous end joining; PAM, protospacer-adjacent motif; RBP, RNA binding protein; sgRNA, single-guide RNA; SSB, single-strand break; SSN, sequence-specific nuclease; TALEN, transcription-activator like effector nuclease; TuMV, turnip mosaic virus.

induced within the plant genome [5]. Since the number of potential target sites was deeply limited by these enzymes, synthetic nucleases were developed, namely zinc finger nucleases and transcription-activator like effector nucleases (TALENs), which enabled for the first time the programmable targeting to the majority of genomic sites [6,7]. The characterization of the CRISPR/Cas9 system, however, provided a biotechnological tool that enables the targeted induction of DSBs at almost any desired site within the genome in the simplest manner [8]. This system utilizes the single effector Cas9 and two RNAs to fulfill its function and can easily be adapted to different sites (Fig. 1A). A small CRISPR RNA (crRNA) that comprises a 20-nucleotide target-dependent sequence constitutes the specificity of this system. A partial complementary trans-activating crRNA (tracrRNA) interacts with the crRNA and mediates the association with the Cas9 enzyme. The assembled complex binds to its recognition site upstream of the so-called protospacer-adjacent motif (PAM) and induces a DSB three bp upstream of the PAM. For simple laboratory use the crRNA and tracrRNA have been fused to a single RNA molecule, the single-guide RNA (sgRNA) [8]. To adapt this system to different targets, only the 20-nucleotide variable sequence of the sgRNA has to be adjusted. CRISPR/Cas systems originate from prokaryotes as adaptive immune systems against invasive foreign DNA and show enormous natural diversity. The latest classification includes two classes that are subdivided in six types and several subtypes. Among them are not only systems to address DNA but also RNA that are useful for biotechnological applications [9].

Using Cas9 in plants

Using Cas9 as a nuclease

In its original form, the CRISPR/Cas9 system provides a simple tool for site-specific mutagenesis. By harnessing the different repair mechanisms, genes can be knocked-out or precise genomic alterations can be induced to study gene function or improve agronomical valuable traits in crop plants [10]. The Cas9 orthologue from *Streptococcus pyogenes* (SpCas9) is the predominantly used orthologue among the community but in plants it was shown that the Cas9 orthologue from *Staphylococcus aureus* (SaCas9) provides mutagenesis frequencies that are at least comparable to those for SpCas9, thus providing a second Cas9 available for genome-editing applications [11]. Attempts to increase the rate of mutagenesis mainly focused on the expression of the Cas9 gene, for example, using different types of promoters [12]. In an innovative approach, the sgRNA was flanked by self-processing ribozymes to generate desired termini without any interfering overhangs generated by the commonly used PolIII promoter [13]. By applying this system, sgRNA arrays for multiplexing applications can also be generated since these ribozyme-based arrays undergo self-catalyzed cleavage after transcription which, in the naturally occurring system, requires RNaseIII activity [14,15]. This enables the simultaneous induction of several DSBs without the need for multiple sgRNA expression cassettes. Interestingly, for SpCas9 targeted mutagenesis frequencies could also be strongly enhanced via incubation at 37 °C and this is applicable to different plant species [16]. The targeting range of Cas9 is not limited to protein coding genes, but noncoding RNAs and regulatory elements can also be targeted [17,18]. Gene targeting (GT), the induction of precise genome alterations by HR, is still highly challenging in plants as NHEJ is the preferred mechanism for DSB repair in somatic plant cells and most crops are still lacking efficient transformation and regeneration procedures. Mutation induction could be achieved in plants by applying Cas9 and its sgRNA coded as DNA, as RNA or directly via an RNP complex (for reviews see Altpeter *et al*. [19] and Wolter and Puchta [20]). A GT method independent on high transformation efficiencies is *in planta* GT. Here, the repair template is stably integrated into the plant genome and GT can occur during the life cycle of the plant [21–23]. In another approach, HR-based GT was enhanced by the use of geminivirus-based replicons carrying the homologous donor sequence. After transformation, these constructs start to replicate leading to a strong increase in repair template copy number. This way, GT frequencies could be enhanced by two orders of magnitude [24,25]. Alternative approaches to increase GT efficiency might concentrate on the manipulation of the DSB repair pathways [26]. The downregulation of factors involved in NHEJ could shift DSB repair toward HR and thus increase GT frequencies, as lately demonstrated for *ligase 4* mutant plants [27]. Despite its nonhomologous mechanism, it was shown that NHEJ can also be harnessed for precise genome alterations. In rice, point mutations as well as gene replacements could be obtained by inducing two DSBs in adjacent introns and replacing the excised fragment by a repair template via NHEJ [28].

The potential of the CRISPR/Cas9 system for obtaining new attractive traits was recently demonstrated in several crop plants (Table 1). For instance, it was extensively used to improve quantitative and

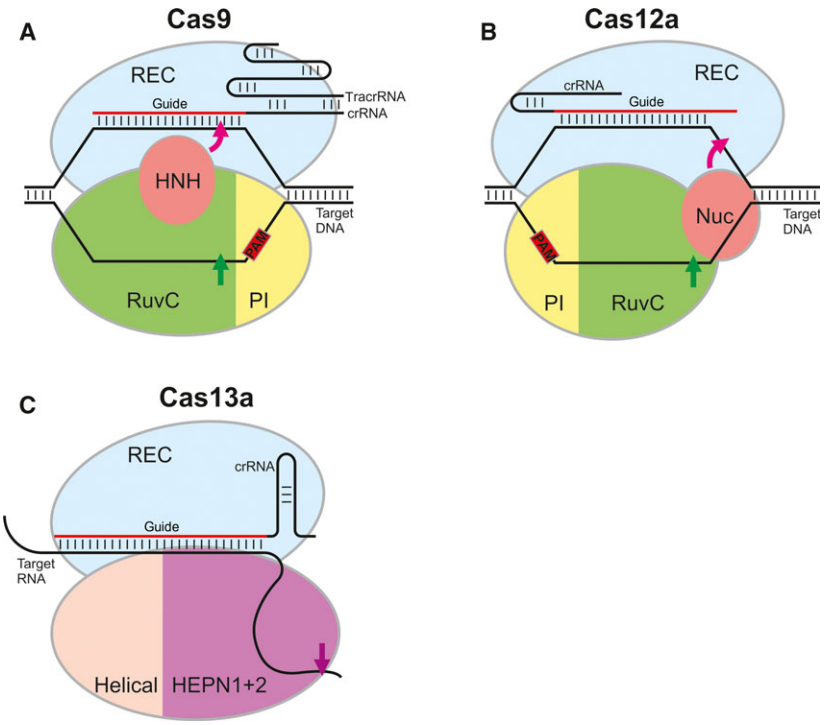

Fig. 1. Schematic comparison of Cas proteins in their native forms. (A) The CRISPR/Cas9 system mediates its function via the single effector Cas9 and two small RNAs, the crRNA and tracrRNA. Upon hybridization, the crRNA::tracrRNA complex associates with the Cas9 nuclease and binds to its recognition site upstream of the PAM sequence. DNA binding is mediated by the 20-nucleotide guide sequence of the crRNA. The Cas9 nuclease induces a blunt-ended DSB 3 bp upstream of the PAM sequence. Recognition of the crRNA::tracrRNA::target complex is mediated by the REC (recognition) lobe, the PI (PAM interacting) domain is responsible for PAM recognition. The DSB is mediated by the HNH and RuvC nuclease domains, with the HNH domain cleaving the target and the RuvC domain cleaving the nontarget strand. (B) The CRISPR/Cas12a system mediates its function via the single effector Cas12a and a single crRNA. Upon association of Cas12a and crRNA, the complex binds to its recognition site downstream of the PAM sequence. DNA binding is mediated by a 23–25-nucleotide guide sequence of the crRNA. The Cas12a nuclease induces a staggered-ended DSB distal from the PAM sequence. Recognition of the crRNA::target complex is mediated by the REC (recognition) lobe, the PI (PAM interacting) domain is responsible for PAM recognition. The DSB is mediated by the Nuc and RuvC domains, with the Nuc domain cleaving the target strand after the 18th bp downstream of the PAM and the RuvC domain cleaving the nontarget strand after the 23rd bp downstream of the PAM (in case of FnCas12a). (C) The CRISPR/Cas13 system (Cas13a shown) mediates its function via the single effector Cas13 and a single crRNA. Upon association of Cas13 and crRNA, the complex binds to its recognition site within the target RNA mediated by the guide sequence of the crRNA. The catalytic site is located at the outside of the protein facing the surrounding solution, leading to cleavage of the target RNA remote from the recognition site. Recognition of the crRNA::target complex is mediated by the REC (recognition) lobe, cleavage of the target RNA by the HEPN domain.

qualitative traits or to confer and increase pathogen resistance, respectively [29–39]. Up to now, CRISPR/Cas9-based plant breeding has concentrated on protein coding sequences, and mostly been used for knock-out of genes conferring undesirable traits. In contrast, regulatory sequences have received much less attention to date. Rodriguez-Leal *et al.* [18] now showed that Cas9-induced mutagenesis can also be used for fine-tuning of quantitative traits by targeting cis-regulatory-elements (CREs). Employing multiple sgRNAs simultaneously targeting various regions of promoters, a variety of different alleles could be generated in tomato, each producing plants with slightly different phenotypes. Addressing of CREs in the promoters of *CLAVATA3*, *COMPOUND INFLORESCENCE* and *SELF PRUNING* produced dozens of varieties which differ in the highly yield relevant traits including fruit size, inflorescence branching and shoot architecture, respectively. These results demonstrate that Cas9-mediated engineering has moved far beyond simple knock-out mutations, allowing delicate genomic manipulations like fine-tuning of quantitative traits. Nevertheless, earlier SSN technologies such as TALENs can also be used successfully to establish desirable traits. Albeit bread wheat represents a hexaploid organism, all three alleles of the *TaMLO1* gene

could be mutated, generating powdery mildew resistant plants [40].

Using Cas9 as a nickase

The Cas9 enzyme comprises two nuclease domains for DSB induction, namely the HNH and RuvC domain, each cleaving one strand of the target DNA. By introducing a point mutation into the catalytic site of the HNH or RuvC domain, the Cas9 enzyme can be converted into a nickase [8]. Experiments to determine the efficiency of a Cas9 nickase mediated mutagenesis in plants showed that in contrast to the nuclease, the nickase cannot be used efficiently for NHEJ-based mutagenesis on its own [41]. Different attempts were taken to increase Cas9 specificity and reduce off-target activity, among them an engineered high-fidelity SpCas9 variant which demonstrated strongly reduced off-target activity, while on-target activity remained comparable to wild-type [42]. Alternatively, paired nickases can be used to reduce off-target activity. In this approach, the Cas9 nickase is guided by two sgRNAs, each binding one strand of the DNA in close proximity in such a way that a DSB with 5′-overhangs is generated. As individual single-strand breaks (SSBs) at potential off-targets do not activate error-prone repair via NHEJ, off-target mutagenesis is decreased dramatically [22,43]. Experiments in plants have shown that repair of adjacent SSBs is also a driving force in the evolution of plant genomes, leading to tandem duplications [44].

So far, CRISPR/Cas9-based genome editing predominantly focused on the induction of DSBs to induce mutations into the genome. The latest genome editing approach, however, enables the programmed conversion of single bases without the induction of DSBs by targeting deaminases to the site of interest. By either fusing a cytidine deaminase or an adenosine deaminase to the Cas9 nickase a conversion of C/G to T/A or A/T to G/C could be induced. The efficiency of base-editing is enhanced using a nickase instead of catalytically inactive Cas9 that nicks the unmodified strand, thus stimulating mismatch repair using the unmodified strand as template [45–48]. Just recently, in addition to cytidine deaminases, adenosine deaminases could also be successfully employed for base-editing in plants [49].

Table 1. Traits successfully improved using CRISPR.

Crops	Target gene	Target trait	Outcome	Reference
Orange	*CsLOB1*	Citrus canker resistance	Enhanced citrus canker resistance compared to wild-type	[29]
Cucumber	*eIF4E*	Broad virus resistance	Generation of Cucumber vein yellowing virus immune and Zucchini yellow mosaic virus/Papaya ring spot mosaic virus-W. resistant cucumbers	[30]
Maize	*ARGOS8*	Drought resistance	Increased yields under drought stress during flowering	[31]
Rice	*Gn1a, DEP1, GS3*	Grain number, panicle architecture, grain size	Increased grain number, dense erect panicles, larger grains	[32]
Rice	*OsERF922*	Increased rice blast resistance	Decrease in lesion areas and lesion lengths compared to wild-type	[33]
Rice	*OsHAK1*	Cs^+ uptake	Reduced Cs^+ uptake in high Cs^+ environment compared to wild-type	[34]
Tobacco	CP and Rep sequences of Tomato yellow leaf curl virus (TYLCV)	TYLCV resistance	Transgenic plants with reduced symptoms of infection	[35]
Tomato	*RIN*	Fruit ripening	Generation of incomplete-ripening tomato fruits	[36]
Tomato	*SP5G*	Flowering time	Faster flowering compared to wild-type	[37]
Tomato	*SlIAA9*	Parthenocarpy	Generation of seedless tomato fruits	[38]
Tomato	*SlMlo1*	Powdery mildew resistance	Generation of a transgene-free powdery mildew resistant tomato	[39]
Tomato	*SlS, SlSP*	Inflorescence branching, Shoot architecture	Generation of varieties with a different range of inflorescence branching and shoot architecture	[18]
Wheat	*TaMLO-A1, TaMLO-B1, TaMLO-D1*	Powdery mildew resistance	Generation of powdery-mildew resistant wheat	[40]

Using Cas9 as a DNA binding protein

The CRISPR/Cas9 system enables the simple and efficient targeting of DNA and thus provides a tool to address almost any desired site within the genome. Through the mutation of both, the HNH and RuvC catalytic domains of the Cas9 enzyme it can be converted into a DNA binding protein, called 'deadCas9' (dCas9), which can be deployed as a platform to recruit various effector proteins to sites of interest (Fig. 2). Early approaches concentrated on the modulation of gene expression by fusing regulatory domains to the dCas9 enzyme which is guided to regulatory elements of the gene of interest. The fusion of the repressive KRAB domain to dCas9 was the first to show that both reporter constructs and endogenous genes can be regulated [50]. By fusing the transcriptional activator domain VP64 to dCas9, it was confirmed that this system can also be applied to promote transcriptional activation. In this study, it was further observed that a synergistic effect can be attained when multiple sgRNAs are targeted to the site of interest [51]. The dCas9-VP64 fusion, among other different variants of regulatory domains, could also be successfully applied in plants [52,53]. Piatek *et al*. [53] used the EDLL and TAL activation domain to activate and the SRDX repressor domain to repress reporter constructs or endogenous genes, respectively. Lowder *et al*. [52] fused the VP64 transcriptional activator domain to dCas9 and showed that silenced genes can also be activated, with an increase of mRNA expression for the tested gene up to 400-fold. In this study, it was also confirmed that dCas9-SRDX mediates robust repression. The regulation of gene expression through epigenetic regulators was further shown by the fusion of dCas9 either to the histone acetyltransferase p300 that activates gene expression through the acetylation of histone 3 lysine 27 or to the histone demethylase LSD1 that represses gene expression through the demethylation of H3K4/K9 [54,55]. More recent approaches using the dCas9 system were based on the recruitment of effectors via the sgRNA. Small RNA aptamers that are able to bind specific RNA binding proteins (RBPs) were integrated into the sgRNA. The fusion of regulators to the RBPs enable an indirect recruitment to the site of interest [56]. There are two major advantages of this system: one RNA aptamer binds a dimer of RBPs, thus more copies of the fused regulators are transported to the target and by using various RNA aptamers and corresponding RPBs, multiple targets can be addressed simultaneously without the need of different Cas9 orthologues. The integration of one or more aptamers into the loops of the sgRNA helped to drastically enhance VP64-based transcriptional activation [57,58]. Recently published data show that in plants a combination of the direct and indirect fusion system currently represents the most robust system for transcriptional activation [59].

Fusions of fluorescent proteins to dCas9 were shown to enable the visualization of genomic loci, recently also in living plant cells [60,61]. In a special system called ′SunTag′ a polypeptide chain was fused to dCas9 to serve as a secondary platform for the recruitment of multiple antibody-fused fluorescent proteins [62]. These dCas9-based imaging experiments were lately restricted to repetitive targets due to detection limits. However, with the use of aptamers, here with the addition of multiple RNA aptamers into the loops and the 3′-end of the sgRNA, imaging of low-repeat-containing regions with the use of a single sgRNA could be accomplished [63].

Using Cas12a (formerly named Cpf1) in plants

Using Cas12a as a nuclease

About 2 years ago, a second CRISPR/Cas system was characterized that enables the programmable induction of DSBs with efficiencies comparable to those of SpCas9. This CRISPR/Cas12a system, formerly known as CRISPR/Cpf1, also belongs to the class 2 CRISPR systems and features similar characteristics to the CRISPR/Cas9 system but also has major differences that make it an alternative tool for genome-editing applications (Fig. 1B) [64,65]. In contrast to Cas9, Cas12a solely requires a small crRNA to mediate its activity, target specificity is determined by a longer spacer, requiring at least 22 nt for maximum efficiency [64,66]. Instead of G-rich PAMs required by Cas9, Cas12a recognizes T-rich PAMs and thus further increases the number of potential target sites. Moreover, the PAM is located upstream of the guide sequence. In contrast to Cas9 which generates blunt-ended DSBs proximal to the PAM, Cas12a generates staggered DSBs distal from the PAM. Cleavage of the nontarget strand occurs after the 18th nucleotide downstream of the PAM and cleavage of the target strand after the 23rd nucleotide producing 5-nucleotide 5′-overhangs. Similar to Cas9, only a subset of Cas12a orthologues shows robust activity in eukaryotic cells. Initial screening in human cells identified the orthologues from *Acidaminococcus* spec. *BV3L6* (AsCas12a) and *Lachnospiraceae bacterium ND2006* as active in human cells [64]. For the orthologue from *Francisella novicida U112* (FnCas12a) no efficient

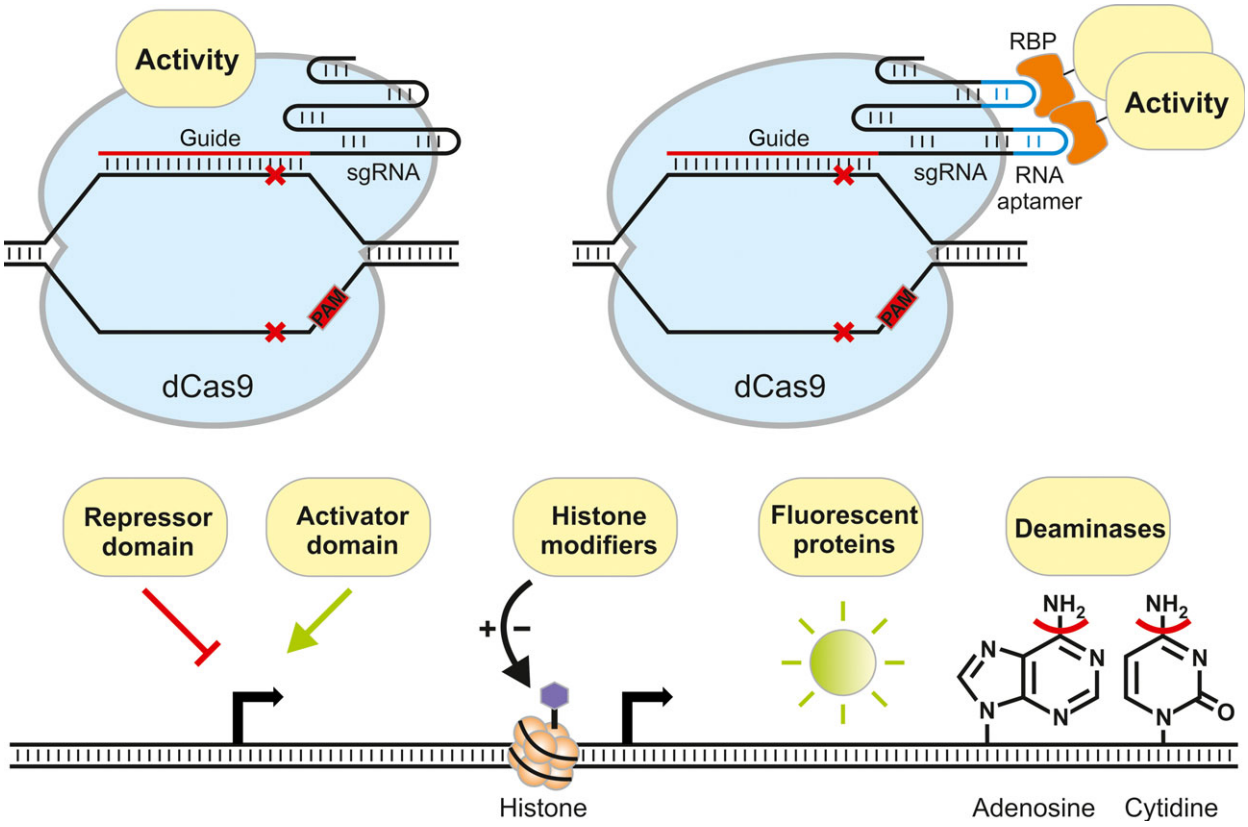

Fig. 2. Applications for DNA binding proteins dCas9 and dCas12a. By inactivating the catalytic domains of Cas9 or Cas12a, the enzymes are converted into DNA binding proteins that can serve as platforms for the recruitment of distinct activities to sites of interest. The respective enzymes can either be fused directly to the catalytic inactive protein (left) or mediated through a RBP, which can bind to specific aptamers integrated within the guide RNA (right). Depending on the fused factors respective activities can be applied to the target: By fusing transcriptional repressor or activator domains gene expression can be regulated. The fusion of histone modifiers can regulate gene expression on the epigenetic level. By fusing fluorescent proteins imaging of genomic loci can be achieved. The use of deaminases enables the irreversible editing of single bases.

mutagenesis could be detected at the tested target locus. However, more recent experiments revealed that FnCas12a possesses robust DNA cleavage activity in human cells as well with frequencies comparable to those of the other orthologues [67]. The Cas12a orthologues recognize T-rich PAMs, which are 5′-TTV(A/C/G)-3′ for FnCas12a and 5′-TTTV-3′ for AsCas12a and LbCas12a, although 5′-TTN-3′ and 5′-TTTN-3′ can also be recognized at lower efficiency [68]. Recent publications in yeast and rice have revealed that a PAM of 5′-TTTV-3′ is also strongly preferred by FnCas12a [69,70]. Recently, the targeting range for Cas12a has been expanded greatly by engineered versions that recognize 5′-TYCV-3′ and 5′-TATV-3′ PAMs without sacrificing efficiency or specificity [71]. The first experiments on Cas12a-mediated mutagenesis in plants were performed in rice and tobacco. In these studies it could be shown that via the CRISPR/Cas12a system targeted mutagenesis can be accomplished and that the appropriate mutations are heritable [72,73]. Both, LbCas12a and FnCas12a were successfully tested, however, for LbCas12a, it has been reported that the combination with pre-crRNAs instead of mature crRNAs leads to increased efficiencies. For FnCas12a, similar frequencies in combination with mature crRNAs could be obtained. To increase mutagenesis frequencies in plants, Tang *et al.* [74] used a ribozyme-based system for both AsCas12a and LbCas12a. The study revealed that LbCas12a promotes higher mutation frequencies than AsCas12a and, moreover, it was reported that nearly all of the mutations generated were biallelic, proving the potential of Cas12a for mutagenesis in plants [74]. Interestingly, analogous to SpCas9, the activity of AsCas12a and LbCas12a can be enhanced through incubation at 37 °C. This effect was stronger for AsCas12, explaining its reduced efficiency in plants

and other organisms that grow at lower temperatures [75]. The CRISPR/Cas12a system might provide an important tool for plants to promote the induction of genomic alterations via HR since complementary overhangs could slightly shift the repair from NHEJ to HR. In addition, as cleavage occurs remote from the PAM and seed region, even after NHEJ-based small InDels are generated, further cleavage might still occur, which could still induce HR. In rice, using FnCas12a and LbCas12a targeted insertions via HR were accomplished and, at least for FnCas12a, with higher rates than SpCas9-based experiments [76]. Furthermore, when delivered as pre-assembled ribonucleoprotein complexes, LbCas12a was highly efficient at homology-directed DNA replacement in Chlamydomonas, which has been recalcitrant to efficient editing before [77]. A recent study in human cells about the correlation between Cas9-induced breaks and DNA repair showed that 5′-overhangs promote HR-mediated repair and insertions, supporting this hypothesis [78]. To enable the expression of multiple guides from a single transcript, ribozyme- or tRNA-based systems were developed for Cas9. In its natural mechanism, the tracrRNA as well as RNaseIII activity are required for the processing of the pre-crRNAs. For Cas12a, it was shown that the enzyme itself processes the pre-crRNA using a catalytic site exhibiting endoribonuclease activity [79,80]. Therefore, it naturally enables multiplex gene editing with a single array. This was proven in mammalian cells by the use of an artificial pre-crRNA array consisting of multiple guides and recently also for plant cells [81,82]. The latest findings about Cas12a surprisingly revealed that after target binding of either dsDNA or ssDNA Cas12a induces the indiscriminate cleavage of ssDNA in the surrounding solution [83]. This newly discovered activity was demonstrated for all commonly used Cas12a orthologues. Similar to a recently developed detection method based on CRISPR/Cas13a, Chen *et al.* [83] developed a DNA detection method, named DNA endonuclease targeted CRISPR Trans reporter (DETECTR). Via this method rare DNA molecules can be detected: fluorescence of a ssDNA-fluorophore-quencher reporter is only released by cleavage when Cas12a is activated by target binding. The combination with isothermal amplification enables the detection of targets with attomolar sensitivity.

Using Cas12a as a DNA binding protein

At first, Cas12a was predicted to bind as dimer to its target site to induce a DSB since sequence analysis only revealed one RuvC nuclease domain in its structure. However, a recent structural study discovered a second nuclease domain, the Nuc domain, which together with the RuvC domain is responsible for target site cleavage [84]. An interesting aspect of target site cleavage by Cas12a is that apparently, cleavage of the nontarget strand is essential for cleavage of the target strand, a contrast to Cas9-mediated cleavage [84,85]. The advantage of this set-up is that only the RuvC domain has to be inactivated to generate a catalytically inactive version of Cas12a (dCas12a). Like dCas9, dCas12a can be used as a platform to recruit different enzymes to sites of interest (Fig. 2). Currently, there are only few studies available showing the use of dCas12a as specific DNA binding protein. In human cells it was reported that dCas12a-based transcription factors can be used for transcriptional activation, through either regulation on the transcriptional or epigenetic level [86,87]. By fusing a synthetic activator complex consisting of the VP16, p65, and Rta activator domains to dLbCas12a robust transcriptional activation could be achieved. Moreover, it was demonstrated that inducible dCas12a-based transcription factors can be generated by fusing domains to dCas12a that interact with drugs. This way, transcriptional activation could be induced upon drug treatment. In the same study, it was also confirmed that a single crRNA array consisting of more than one crRNA is able to induce synergistic activation when targeted to the same promoter or multiplex activation when targeted to different promoters [86]. In another experiment, dLbCas12a and dAsCas12a have been fused to the histone acetyltransferase p300 to activate gene expression, comparable to the dCas9-based approach. By targeting the promoter region or the enhancer, gene expression could be enhanced. However, significant effects could only be obtained for dLbCas12a-p300, whereas dAsCas12a-p300 induced merely marginal activation [87]. Transcriptional repression mediated by dCas12a was demonstrated in plants with the use of dAsCas12a and dLbCas12a [74]. By fusing them to three copies of the SRDX repressor domain, expression of a specific target locus could be downregulated to less than 10% compared to the wild-type. The analysis of multiple independent lines revealed a similar efficiency for both orthologs, albeit dAsCas12a-based repression showed less variations between the lines.

Using Cas13 (formerly named C2c2) in plants

A very recent and highly useful addition to the CRISPR toolbox is Cas13, formerly known as C2c2

and C2c6 in case of Cas13a and Cas13b, respectively. After its bioinformatic prediction, it was assigned to a new type because of its unique features, class II type VI [88]. It is the first class II effector that acts exclusively on RNA which is catalyzed by its two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains (Fig. 1C) [89]. As the HEPN catalytic site of activated Cas13a is located on the outer surface, RNAs other than the target RNA in the solution are cleaved as well in an unspecific manner, suggesting that Cas13 elicits programmed cell death or dormancy in the natural system [90,91]. However, as an important finding with respect to genome engineering, this promiscuous RNA cleavage upon activation could not be detected in eukaryotic cells, at least with the Cas13 orthologues that showed high activity in these cells [92,93]. Furthermore, just like Cas12a, Cas13 proteins exhibit the ability to autonomously process pre-crRNA without the involvement of a tracrRNA, which is catalyzed by a distinct domain, the Helical1 domain. This crRNA maturation activity could be harnessed for simple and efficient addressing of multiple targets simultaneously [92]. The availability of an efficient and specific RNA targeting CRISPR system now enables a wide range of targeted RNA manipulations.

Using Cas13 for posttranscriptional repression

In its native form, Cas13 can be employed for targeted RNA cleavage, for example, for knockdown of specific transcripts (Fig. 3A). Two of the most active orthologues identified for this purpose were Cas13a from *Leptotrichia wadei* and Cas13b from *Prevotella* sp. *P5–125* [92,93]. Although some Cas13 orthologues require a protospacer flanking site analogous to the PAM, there was no such restriction for LwaCas13a and PspCas13b. Concerning cellular localization, LwaCas13a was most efficient for targeted RNA knockdown when combined with a nuclear localization signal, whereas PspCas13b was most active when combined with a nuclear export signal [92,93]. Compared to RNAi, the efficiency of RNA knockdown was comparable but in terms of specificity, the Cas13 system was clearly superior. At least for the LwaCas13a orthologue, the functionality of targeted RNA knockdown was also confirmed for plants, with most guides exceeding 50% knockdown in rice protoplasts at only 48 h after transformation, suggesting that a broad range of organisms can be edited using this system [92]. Importantly, Cas13 facilitates targeting of specific splicing isoforms, whereas transcriptional regulation on the DNA level indifferently affects all isoforms. This way, aberrant or pathogenic splicing isoforms could be eliminated without affecting wild-type transcripts [94]. Another advantage of posttranscriptional regulation is the temporal dimension: Cas13 enables a much faster reduction in gene expression by directly eliminating cytoplasmic mRNAs present, whereas transcriptional regulation only impedes further mRNA generation. Besides targeting mRNAs, a promising application of Cas13 for advances in basic research is the knockdown of noncoding RNAs (ncRNAs). In plants, ncRNAs fulfill a wide range of functions, including regulation of gene expression, assembly of protein complexes, guiding of protein translocation, regulation of splicing and more [95]. Despite their relevance, functional studies on ncRNAs are impeded by a lack of mutants [96]. Cas13 now enables to directly cleave these RNAs *in vivo*, providing greater flexibility in experimental manipulations compared to complete knockout on the DNA level using Cas9 or Cas12a. For example using Cas13, it is now feasible to repress ncRNAs in the tissue of interest using a tissue specific promoter, in cases where complete knockout mutants are lethal. Furthermore, different knockdown intensities can be analyzed in addition to complete knockout. Advantageous to RNAi, Cas13 is not restricted to cytoplasmic transcripts, but nuclear transcripts can also be targeted by localizing Cas13 to the nucleus via a NLS tag.

Using Cas13 for combating RNA viruses

Previously, it was demonstrated that Cas9 can be used for interference against plant DNA viruses [35]. Now, the RNA targeting ability of Cas13 can be used to combat RNA viruses which are the most common form of plant viruses, and even for DNA retroviruses which replicates via an RNA intermediate during their life cycle (Fig. 3B) [97]. Employing LshCas13a, Aman *et al.* [97] achieved interference against turnip mosaic virus (TuMV), a Potyvirus, in *Nicotiana benthamiana*. Delivering TuMV, Cas13 and crRNAs via leaf infiltration, they achieved 50% reduction of viral GFP signal 7 days post infiltration. As no adverse effect on plant vitality was observed, collateral RNA degradation seems to be negligible or absent in plant cells for LshCas13a. However, not all crRNAs were effective, suggesting that RNA secondary structure strongly influences cleavage activity. It is thus advisable to test multiple target sequences for virus defense. In the end, only if all infecting viral RNAs are cut by the RNase, efficient viral resistance can be obtained. It can be speculated that using the more recently characterized LwaCas13a and

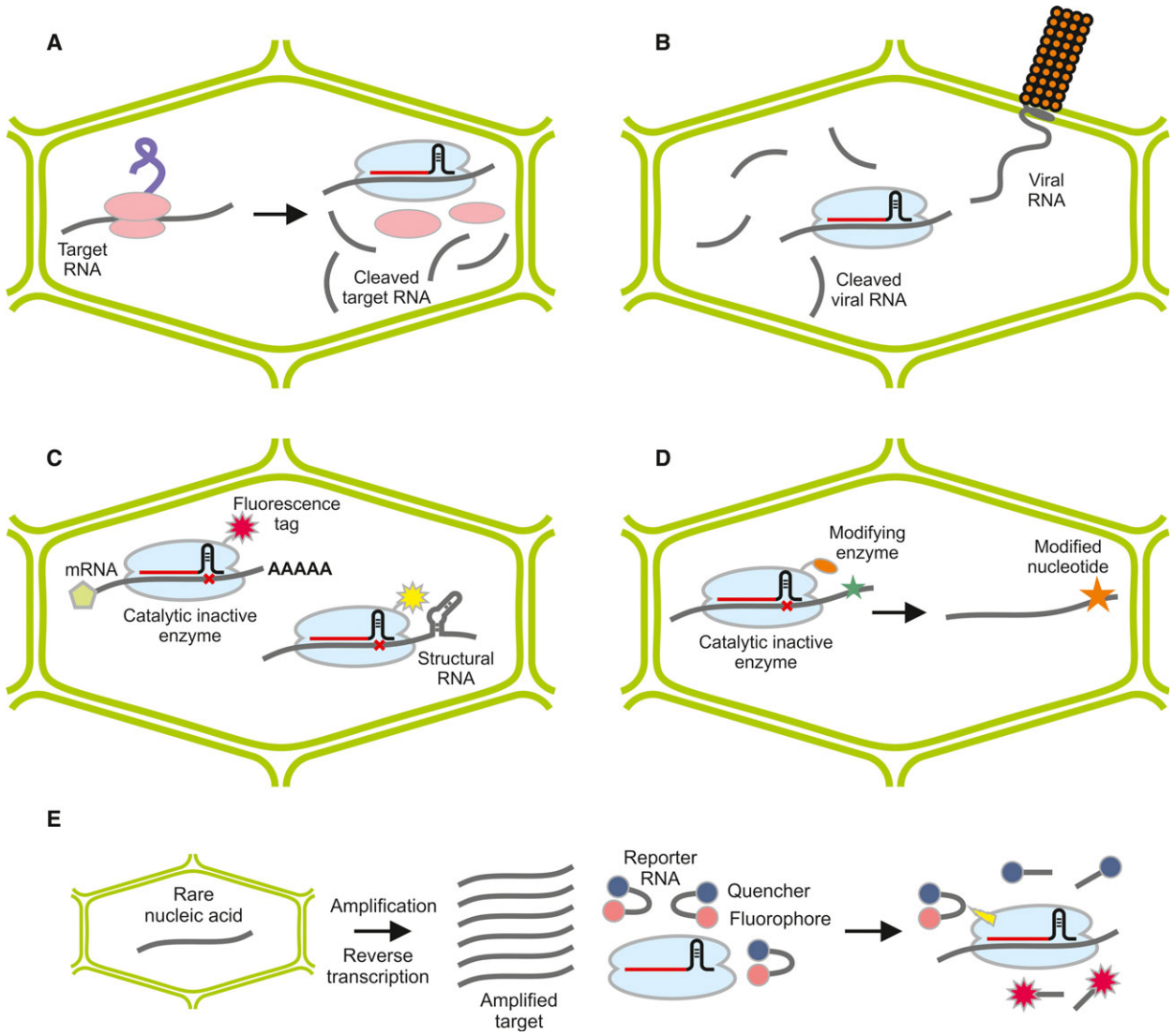

Fig. 3. Applications for Cas13. (A) Cas13 can be used to target protein-coding RNAs to downregulate gene expression (B) or to target invading viral RNA as defense against plant RNA viruses. (C) Catalytically inactive Cas13 (dCas13) can be fused to fluorescent tags to image different types of RNAs in living cells (D) or can be used as platform to guide distinct enzymatic domains to target RNAs to induce reversible modifications at specific sites. (E) *In vitro*, Cas13 exhibits collateral cleavage of RNA, a mechanism that can be harnessed for the detection of rare nucleic acids. After isothermal amplification and reverse transcription, Cas13 binds to the target RNA which then activates its collateral RNA cleavage activity. This leads to the cleavage of a RNA quencher-fluorophore-reporter that releases the active fluorophore whose fluorescence is detected.

PspCas13b variants, the efficiency of virus interference might be further improved.

Using Cas13 as a RNA binding protein

Analogous to Cas9 and Cas12a, Cas13 retains its RNA binding ability when the two catalytic residues in the HEPN domains of Cas13 are deactivated ('dCas13'). This way, a programmable RBP is obtained. Abudayyeh *et al.* [92] achieved specific imaging of transcripts in live cells using dLwaCas13a fused to fluorescent proteins (Fig. 3C). This allowed mRNA translocation into stress granules to be followed in live cells. Another exciting possibility enabled by Cas13 is precise RNA base editing (Fig. 3D). As shown before on the DNA level, precise point mutations can be generated by fusion of a deaminase domain to Cas9 [45,46]. Now, RNA can be edited in an analogous manner, as shown by Cox *et al.* [93] using PspCas13b fused to the adenosine deaminase

acting on RNA 2 deaminase domain (ADAR2dd). This enzyme catalyzes deamination of adenosine to inosine, which is functionally equivalent to guanine in translation. RNA editing rates at or above 50% were consistently achieved in reporter systems and two genes related to human diseases were also successfully edited at 35 and 23% efficiency. However, RNA seq analysis revealed thousands of off-targets across the transcriptome. Specificity could be enhanced dramatically by introducing point mutations into ADAR2dd destabilizing unspecific RNA binding, while on-target editing remained almost unaffected. Importantly, as opposed to DNA editing, RNA editing is not stable but reversible. This could enable a delicate temporal control over the editing process [93]. Furthermore, when editing RNA both edited and non-edited transcripts can be present simultaneously, which could enable fine-tuning of the edited transcript amount, whereas DNA editing affects all transcripts. Many other RNA manipulations are conceivable. Fusing translational enhancers or repressors to dCas13 could be used to specifically regulate translation. dCas13 could also be used to translocate targeted RNAs to specific cellular locations by fusing a trafficking agent. For basic research, dCas13 could help to identify proteins bound by targeted RNAs by fusing it to an affinity tag, leading to coprecipitation of bound proteins [94]. Further concerning basic research, by bringing modifying effectors to specific tRNAs to manipulate their diverse chemical modifications, dCas13 could be used to improve our understanding of the complex influence on translational regulation by post-transcriptional tRNA modifications.

Conclusions and perspectives

The continuous identification and characterization of highly useful new CRISPR proteins reveal the enormous potential behind CRISPR/Cas. While CRISPR/Cas9 initially allowed the guidance of one specific enzymatic activity to one or, by the use of multiple sgRNAs, to various target sites, it is now possible to realize multidimensional approaches that enable the parallel employment of a number of activities [98]. This was recently demonstrated through the simultaneous application of different Cas9 orthologues either for imaging or GT experiments [23,61]. The establishment of aptamer-based sgRNAs that can recruit a variety of potential RBPs allows considerable flexibility for employment of different enzymatic activities simultaneously (e.g. [99]). The constantly increasing set of applicable CRISPR proteins expands the possible number and flexibility of parallel genomic manipulations even further. In particular, the availability of Cas12a opens up new target sites and Cas13 now enables the manipulation of RNA on several levels. New CRISPR based methods enabling often unexpected new applications are continuously being developed, as recently evidenced by SHERLOCK and DETECTR using CRISPR for highly sensitive nucleic acid detection (Fig. 3E) [83,100]. Through the combination of DNA and RNA editing systems, the cellular transcriptome can now be manipulated on the transcriptional and posttranscriptional level simultaneously, allowing delicate and also reversible fine-tuning of gene expression. Previous experiments only indicated the possibilities to edit genomes or influence cell metabolism, and current applications merely give us a hint of the amount of further discoveries that await to change molecular biology.

Acknowledgements

The authors want to thank Amy Whitbread for critical comments on the manuscript and the European Research Council for funding our work on the application of CRISPR/Cas for plant breeding (ERC Advanced Grant 'CRISBREED' to HP).

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