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The CRISPR/Cas9 system for plant genome editing and beyond

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ABSTRACT

Targeted genome editing using artificial nucleases has the potential to accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner. Here we describe the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system, a recently developed tool for the introduction of site-specific double-stranded DNA breaks. We highlight the strengths and weaknesses of this technology compared with two well-established genome editing platforms: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). We summarize recent results obtained in plants using CRISPR/Cas9 technology, discuss possible applications in plant breeding and consider potential future developments.

Contents

Introduction

Genome editing with site-specific nucleases allows reverse genetics, genome engineering and targeted transgene integration experiments to be carried out in an efficient and precise manner. It involves the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, stimulating cellular DNA repair mechanisms. Different genome modifications can be achieved depending on the repair pathway and the availability of a repair template (Fig. 1). Two different DSB repair pathways have been defined: non-homologous end joining (NHEJ) and homologous recombination (HR). In most cases, NHEJ causes random insertions or deletions (indels), which can result in frameshift mutations if they occur in the coding region of a gene, effectively creating a gene knockout. Alternatively, when the DSB generates overhangs, NHEJ can mediate the targeted introduction of a double-stranded DNA template with compatible overhangs (Cristea et al., 2013; Maresca et al., 2013). When a template with regions of homology to the sequence surrounding the DSB is available, the DNA damage can be repaired by HR, and this mechanism can be exploited to achieve precise gene modifications or

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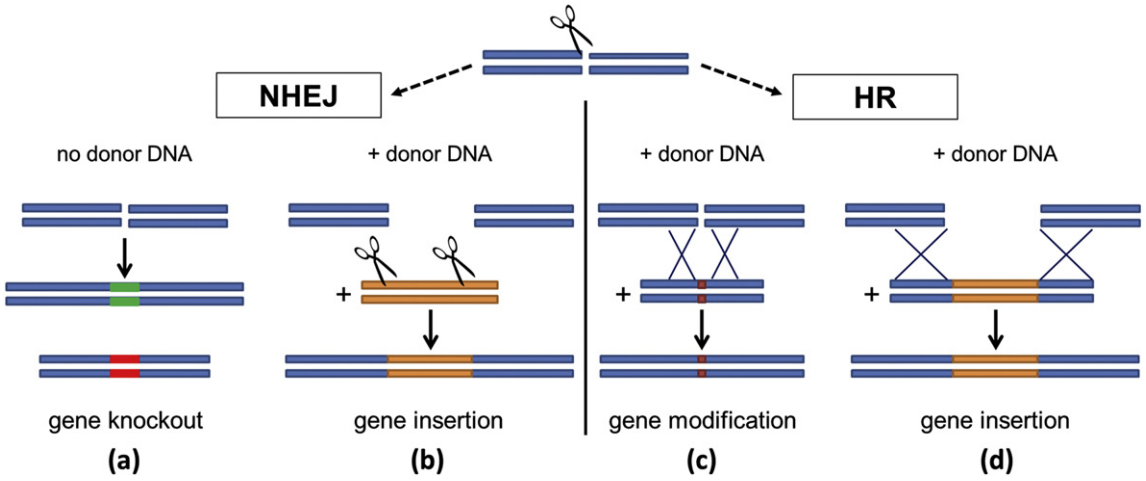

Fig. 1. Genome editing with site-specific nucleases. Double-strand breaks induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). (a) Repair by NHEJ usually results in the insertion (green) or deletion (red) of random base pairs, causing gene knockout by disruption. (b) If a donor DNA is available, which is simultaneously cut by the same nuclease leaving compatible overhangs, gene insertion by NHEJ can also be achieved. (c) HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or (d) to achieve gene insertion.

gene insertions. Even though the generation of breaks in both DNA strands induces recombination at specific genomic loci, NHEJ is by far the most common DSB repair mechanism in most organisms, including higher plants, and the frequency of targeted integration by HR remains much lower than random integration (Puchta, 2005). Strategies such as the overexpression of proteins involved in HR or the use of negative selection markers outside the homology regions of the insertion cassette to prevent the survival of random integration events can achieve moderate improvements in gene targeting efficiency (reviewed in Puchta and Fauser, 2013).

The CRISPR/Cas9 system

Until 2013, the dominant genome editing tools were zinc finger nucleases (ZFNs; Kim et al., 1996) and transcription activator-like effector nucleases (TALENs, Christian et al., 2010). Both are artificial fusion proteins comprising an engineered DNA-binding domain fused to the nonspecific nuclease domain of the restriction enzyme FokI, and they have been used successfully in many organisms including plants (reviewed in Jankele and Svoboda, 2014; Palpant and Dudzinski, 2013). The latest ground-breaking technology for genome editing is based on RNA-guided engineered nucleases, which already hold great promise due to their simplicity, efficiency and versatility. The most widely used system is the type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated) system from *Streptococcus pyogenes* (Jinek et al., 2012). CRISPR/Cas systems are part of the adaptive immune system of bacteria and archaea, protecting them against invading nucleic acids such as viruses by cleaving the foreign DNA in a sequence-dependent manner. The immunity is acquired by the integration of short fragments of the invading DNA known as spacers between two adjacent repeats at the proximal end of a CRISPR locus. The CRISPR arrays, including the spacers, are transcribed during subsequent encounters with invasive DNA and are processed into small interfering CRISPR RNAs (crRNAs) approximately 40 nt in length, which combine with the transactivating CRISPR RNA (tracrRNA) to activate and guide the Cas9 nuclease (Barrangou et al., 2007). This cleaves homologous double-stranded DNA sequences known as protospacers in the invading DNA (Barrangou et al., 2007). A prerequisite for cleavage is the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5′-NGG-3′ (Gasiunas et al., 2012; Jinek et al., 2012) but less frequently NAG (Hsu et al., 2013). Specificity is provided by the so-called 'seed sequence' approximately 12 bases upstream of the PAM, which must match between the RNA and target DNA (Fig. 2).

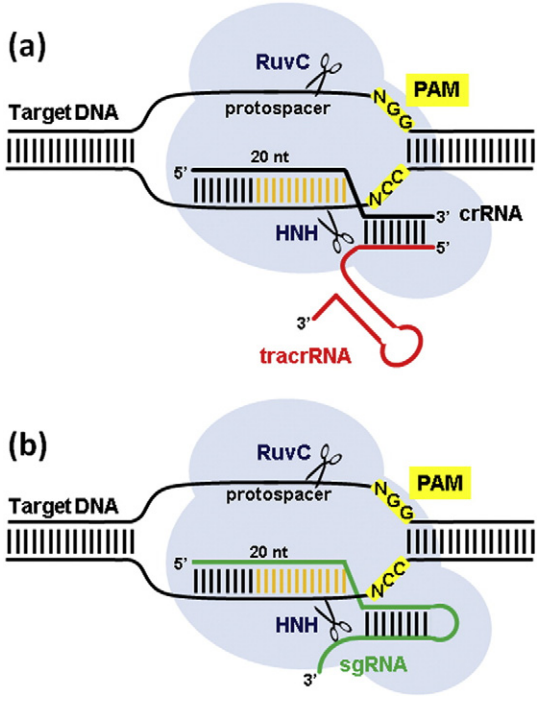

Fig. 2. RNA-guided DNA cleavage by Cas9. (a) In the native system, the Cas9 protein (light blue) is guided by a structure formed by a CRISPR RNA (crRNA, in black), which contains a 20-nt segment determining target specificity, and a trans-activating CRISPR RNA (tracrRNA, in red), which stabilizes the structure and activates Cas9 to cleave the target DNA (protospacer). The presence of a protospacer-adjacent motif (PAM, in yellow), i.e., an NGG (or less frequently NAG) sequence directly downstream from the target DNA, is a prerequisite for DNA cleavage by Cas9. Among the 20 RNA nucleotides determining target specificity, the so-called seed sequence of approximately 12 nt (in orange) upstream of the PAM is thought to be particularly important for the pairing between RNA and target DNA. (b) Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule (gRNA, in green), a chimera generated by fusing the 3′ end of the crRNA to the 5′ end of the tracrRNA.

The rise of a genome editing wonder

Although CRISPR arrays were first identified in the *Escherichia coli* genome in 1987 (Ishino et al., 1987), their biological function was not understood until 2005, when it was shown that the spacers were homologous to viral and plasmid sequences suggesting a role in adaptive immunity (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Two years later, CRISPR arrays were confirmed to provide protection against invading viruses when combined with Cas genes (Barrangou et al., 2007). The mechanism of this immune system based on RNA-mediated DNA targeting was demonstrated shortly thereafter (Brouns et al., 2008; Deltcheva et al., 2011; Garneau et al., 2010; Marraffini and Sontheimer, 2008).

The transition of the CRISPR/Cas system from biological phenomenon to genome engineering tool came about when it was shown that the target DNA sequence could be reprogrammed simply by changing 20 nucleotides in the crRNA and that the targeting specificity of the crRNA could be combined with the structural properties of the tracrRNA in a chimeric single guide RNA (gRNA), thus reducing the system from three to two components (Jinek et al., 2012; Fig. 2b). Shortly thereafter, five independent groups demonstrated that the two-component system was functional in eukaryotes (human, mouse and zebrafish), indicating that the other functions of the CRISPR locus genes were supported by endogenous eukaryotic enzymes (Cho et al., 2013; Cong et al., 2013; Hwang et al., 2013; Jinek et al., 2013; Mali et al., 2013). Importantly, it was also shown that multiple gRNAs with different sequences could be used to achieve high-efficiency multiplex genome engineering at different loci simultaneously (Cong et al., 2013; Mali et al., 2013). These milestones confirmed that the CRISPR/Cas9 system was a simple, inexpensive and versatile tool for genome editing, resulting in a groundswell of research based on the technique which has become known as the 'CRISPR craze' (Pennisi, 2013).

In August 2013, five reports were published discussing the first application of CRISPR/Cas9-based genome editing in plants (Feng et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013). This first group of studies already demonstrated the immense versatility of the technology in the field of plant biology by embracing the model species *Arabidopsis thaliana* and *Nicotiana benthamiana* as well as crops such as rice, by using a range of transformation platforms (protoplast transfection, agroinfiltration and the generation of stable transgenic plants), by targeting both endogenous genes and transgenes and by exploiting both NHEJ and HR to generate small deletions, targeted insertions and multiplex genome modifications. Subsequent work focused on additional crop species such as sorghum (Jiang et al., 2013b), wheat (Upadhyay et al., 2013; Wang et al., 2014b) and maize (Liang et al., 2014). These studies provided the first comparative data concerning aspects such as mutation efficiency, cleavage specificity, the resolution of locus structure and the potential to create large chromosomal deletions and also demonstrated that gRNAs can be expressed under the control of diverse promoters, including those recognized by RNA polymerase II and III (Fauser et al., 2014; Feng et al., 2014; Gao et al., 2014; Jiang et al., 2013b; Mao et al., 2013; Miao et al., 2013; Sugano et al., 2014; Upadhyay et al., 2013; Zhang et al., 2014; Zhou et al., 2014). The studies also confirmed that single chimeric gRNAs are more efficient than separate crRNA and tracrRNA components in plants, just as they are in other eukaryotes (Miao et al., 2013; Zhou et al., 2014). While early works described the CRISPR/Cas9-mediated insertion of short donor sequences (Li et al., 2013; Shan et al., 2013), Schiml et al. (2014) reported the integration of a 1.8 kb resistance cassette into the ADH1 locus of *A. thaliana* by HR. They exploited an *in planta* gene targeting strategy, in which both a targeting vector and targeting locus are activated simultaneously via DSB induction during plant development (Fauser et al., 2012). Most recently, the CRISPR/Cas9 system was shown to work in tomato hairy roots following transformation with *Agrobacterium rhizogenes* (Ron et al., 2014) and was the first genome editing platform used in the fruit crop sweet orange (Jia and Wang, 2014).

Interestingly, four independent groups have shown that the CRISPR/Cas9 system can introduce biallelic or homozygous mutations directly in the first generation of rice and tomato transformants, highlighting the exceptionally high efficiency of the system in these species (Brooks et al., 2014; Shan et al., 2013; Zhang et al., 2014; Zhou et al., 2014). It was also shown in Arabidopsis, rice and tomato that the genetic changes induced by Cas9/gRNA were present in the germ line and segregated normally in subsequent generations without further modifications (Brooks et al., 2014; Fauser et al., 2014; Feng et al., 2014; Jia et al., 2014; Schiml et al., 2014; Zhang et al., 2014; Zhou et al., 2014). An overview of publications reporting applications of the CRISPR/Cas9 system in plants is provided in Table 1–3.

A comparison of CRISPR/Cas9, ZFNs and TALENs

ZFNs and TALENs function as dimers and only protein components are required. Sequence specificity is conferred by the DNA-binding domain of each polypeptide and cleavage is carried out by the FokI nuclease domain. In contrast, the CRISPR/Cas9 system consists of a single monomeric protein and a chimeric RNA. Sequence specificity is conferred by a 20-nt sequence in the gRNA and cleavage is mediated by the Cas9 protein. The design of ZFNs is considered difficult due to the complex nature of the interaction between zinc fingers and DNA and further limitations imposed by context-dependent specificity (Sander et al., 2011). Commercially available ZFNs generally perform better than those designed using publicly available resources but they are much more expensive (Ramirez et al., 2008). TALENs are easier to design because there are one-to-one recognition rules between protein repeats and nucleotide sequences, and their construction has been simplified by efficient DNA assembly techniques such as Golden Gate cloning (Engler et al., 2008). However, TALENs are based on highly repetitive sequences which can promote homologous recombination *in vivo* (Holkers et al., 2013). In comparison, gRNA-based cleavage relies on a simple Watson–Crick base pairing with the target DNA sequence, so sophisticated protein engineering for each target is unnecessary, and only 20 nt in the gRNA need to be modified to recognize a different target.

ZFNs and TALENs both carry the catalytic domain of the restriction endonuclease FokI, which generates a DSB with cohesive overhangs varying in length depending on the linker and spacer. Cas9 has two cleavage domains known as RuvC and HNH (Fig. 2), which cleave the target DNA three nucleotides upstream of the PAM leaving blunt ends (Jinek et al., 2012). Occasionally, Cas9 produces overhangs of 1–2 nt *in vitro* (Gasiunas et al., 2012; Jinek et al., 2012). Defined overhangs are useful for the precise insertion of DNA molecules with compatible overhangs, which occurs by NHEJ-mediated ligation (Cristea et al., 2013; Maresca et al., 2013). As discussed below (Section 6), the CRISPR/Cas9 system can also be used to create such structures using the double-nickase approach (Ran et al., 2013).

ZFNs can theoretically target any sequence but in practice the choice of targets is limited by the availability of modules based on the context-dependent assembly platform (Sander et al., 2011). A functional ZFN pair can be prepared for every ~100 bp of DNA sequence on average using publicly available libraries (Kim et al., 2009). TALEN targets are limited by the need for a thymidine residue at the first position (Doyle et al., 2012), but not all TALENs work efficiently *in vivo* and some pairs therefore fail to generate the anticipated mutations, which means that each TALEN pair must be experimentally validated (Hwang et al., 2013). In contrast, the only theoretical requirement of the *S. pyogenes* CRISPR/Cas9 system is the presence of the NGG (or NAG) PAM motif downstream of the target sequence. However, imperfectly matched spacer sequences can result in cleavage at off-target positions, which means that gRNA sequences must be chosen carefully to avoid such artifacts thus reducing the number of targets that can be used in

Table 1
CRISPR-Cas9 mediated NHEJ in transient transfection experiments.

Species	Transformation method	Cas9 codon optimization	Promoters (Cas9, gRNA)	Target	Mutation frequency	Detection method	Off-target (no. of sites analyzed)	Detection method	Multiplex (deletion)	Reference
Arabidopsis thaliana	PEG-protoplast transfection	Arabidopsis (with intron)	CaMV35SPDK, AtU6	PDS3, FLS2	1.1–5.6%	PCR + sequencing				Li et al. (2013)
A. thaliana	Leaf agroinfiltration	Arabidopsis (with intron)	CaMV35SPDK, AtU6	PDS3	2.7%	PCR + sequencing			Yes (48 bp)	Li et al. (2013)
A. thaliana	PEG-protoplast transfection	Arabidopsis (with intron)	CaMV35SPDK, AtU6	RACK1b, RACK1c	2.5–2.7%	PCR + sequencing	No (1 site)	PCR + sequencing		Li et al. (2013)
A. thaliana	Leaf agroinfiltration	*C. reinhardtii*	CaMV35S, AtU6	Co-transfected GFP	n.a.	Pre-digested PCR + RE				Jiang et al. (2013a, 2013b)
Nicotiana benthamiana	PEG-protoplast transfection	Arabidopsis (with intron)	CaMV35SPDK, AtU6	PDS3	37.7–38.5%	PCR + sequencing				Li et al. (2013)
N. benthamiana	Leaf agroinfiltration	Arabidopsis (with intron)	CaMV35SPDK, AtU6	PDS3	4.8%	PCR + sequencing				Li et al. (2013)
N. benthamiana	Leaf agroinfiltration	Human	CaMV35S, AtU6	PDS	1.8–2.4%	PCR + RE	No (18 sites)	PCR + RE		Nekrasov et al. (2013)
N. benthamiana	Leaf agroinfiltration	*C. reinhardtii*	CaMV35S, AtU6	Co-transfected GFP	n.a.	pre-digested PCR + RE				Jiang et al. (2013a, 2013b)
N. benthamiana	Leaf agroinfiltration	Human	CaMV35S, CaMV35S	PDS	12.7–13.8%					Upadhyay et al. (2013)
Nicotiana tabacum	PEG-protoplast transfection	Tobacco	2xCaMV35S, AtU6	PDS, PDR6	16.27–20.3%	PCR + RE			Yes (1.8 kb)	Gao et al. (2014)
Oryza sativa	PEG-protoplast transfection	Rice	2xCaMV35S, OsU3	PDS, BADH2, MPK2, Os02g23823	14.5–38.0%	PCR + RE	No[a] (3 sites)	PCR + RE		Shan et al. (2013)
O. sativa	PEG-protoplast transfection	Human	CaMV35S, OsU3 or OsU6	MPK5	3–8%	RE + qPCR and T7E1 assay	No (2 sites), Yes (1 site with a mismatch at position 12)	RE + PCR		Xie and Yang (2013)
O. sativa	PEG-protoplast transfection	Rice	CaMV35S, OsU6	SWEET14	n.a.	pre-digested PCR + RE				Jiang et al. (2013a, 2013b)
O. sativa	PEG-protoplast transfection	Rice	ZmUbi, OsU6	KO1, KOL5; CPS4, CYP99A2; CYP76M5, CYP76M6	n.a.	PCR + sequencing			Yes (115, 170, 245 kb)	Zhou et al. (2014)
Triticum aestivum	PEG-protoplast transfection	Rice	2xCaMV35S,TaU6	MLO	28.5%	PCR + RE				Shan et al. (2013)
T. aestivum	PEG-protoplast transfection	Plant	ZmUbi,TaU6	MLO-A1	36%	T7E1				Wang et al. (2014a, 2014b)
T. aestivum	Agrotransfection of cells from immature embryos	Human	CaMV35S, CaMV35S	PDS, INOX	18–22%	PCR + sequencing				Upadhyay et al. (2013)
T. aestivum	Agrotransfection of cells from immature embryos	Human	CaMV35S, CaMV35S	INOX		PCR + sequencing	No*	PCR + RE	Yes (53 bp)	Upadhyay et al. (2013)
Zea mays	PEG-protoplast transfection	Rice	2xCaMV35S, ZmU3	IPK	16.4–19.1%	PCR + RE				Liang et al. (2014)
Citrus sinensis	Leaf agroinfiltration	Human	CaMv35S, CaMV35S	PDS	3.2–3.9%	PCR + RE	No (8 sites)	PCR + RE		Jia et al. (2014)

PEG = polyethylenglycol; CaMV35S = promoter of the 35S gene of the *cauliflower mosaic virus*; 2x35SCaMV = 35S promoter with duplicated enhancer; CaMV35SPDK = hybrid promoter, 35S enhancer fused to the maize C4PPDK basal promoter; AtU6 = *A. thaliana* U6 promoter; OsU3, U6 = *O. sativa* U3 or U6 promoters; ZmUbi, U3 = *Z. mays* ubiquitin or U3 promoters; TaU6 = *T. aestivum* U6 promoter; PCR = polymerase chain reaction; RE = restriction enzyme digestion.

[a] If at least 1 mismatch in the 12 nt seed sequence.

Table 2
CRISPR-Cas9 mediated NHEJ in stable transformants.

Species	Transformation method	Cas9 codon optimization	Promoters (Cas9, gRNA)	Target	Mutation frequency	Detection method	Off-target/detection method	Multiplex (deletion)	Transmission to progeny	Reference
Arabidopsis thaliana	Agro-transformation by floral dip	Human	AtUBQ1, AtU6	Co-transfected *GUUS*	80% (35/44)	Surveyor assay				Mao et al. (2013)
A. thaliana	Agro-transformation by floral dip	Human	AtUBQ1, AtU6	*TT4, CHLI1, CHL12*	38–89%	Phenotype, surveyor assay, sequencing		Yes (230 bp)		Mao et al. (2013)
A. thaliana	Agro-transformation by floral dip	Arabidopsis (Nickase)	PcUBI4-2	*ADH1, TT4, RTEL1*	0.04%	PCR + deep sequencing				Fauser et al. (2014)
A. thaliana	Agro-transformation by floral dip	Arabidopsis	PcUBI4-2	*ADH1, TT4, RTEL1*	26.7%	PCR + deep sequencing			Yes	Fauser et al. (2014)
A. thaliana	Agro-transformation by floral dip	Arabidopsis (Nickase)	PcUBI4-2	*ADH1*	42.8% (Paired Nickases)	PCR + deep sequencing			Yes	Schiml et al. (2014)
A. thaliana	Agro-transformation by floral dip	*C. reinhardtii*	CaMV35S, AtU6	co-transfected *GFP*	58% (35/60)	GFP fluorescence			Yes	Jia et al. (2014)
A. thaliana	Agro-transformation by floral dip	Human	2xCaMV35S, AtU6	*BRI1, GAI, JAZ1*	30% (6/20)–84%(16/19)	PCR + RE			Yes	Feng et al. (2013)
Oryza sativa	Particle bombardment of callus	Rice	2xCaMV35S, OsU3	*OsPDS, OsBADH2*	7.1% (9/97)–9.4% (7/98)	PCR + RE				Shan et al. (2013)
O. sativa	Agro-transformation of callus	Human	OsUBQ, OsU6	*OsMYB1*	50% (10/20)	Surveyor assay				Mao et al. (2013)
O. sativa	Agro-transformation of callus	Rice	ZmUbi, OsU3	*CAO1, LAZY1*	83.3% (25/30)–91.6%(11/12)	PCR + sequencing				Miao et al. (2013)
O. sativa	Agro-transformation of callus	Human	CaMV35S, OsU6	*ROC5, SPP, YSA*	5% (1/21)–75% (24/32)	PCR + RE				Feng et al. (2013)
O. sativa	Agro-transformation of callus	Rice	2xCaMV35S, ZmU3	*PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP, YSA*	21.1–66.7%	PCR + sequencing	No/whole-genome sequencing	Yes	Yes	Zhang et al.(2014)
O. sativa	Agro-transformation of callus	Plant	2xCaMV35S, AtU6	*BAL*	2% (2/94)–16% (14/90)	PCR + sequencing	No (3 sites)/PCR + sequencing			Xu et al. (2014)
O. sativa	Agro-transformation of callus	Human	2xCaMV35S, AtU6	*BAL*	0% (0/96)–2% (2/96)	PCR + sequencing				Xu et al. (2014)
O. sativa	Agro-transformation of callus	Rice	ZmUbi, OsU6	*SWEET1a-1b-11-13*	12.5% (2/16)–100% (8/8)	T7E1 assay + sequencing	No (6 sites)/PCR + sequencing		Yes	Zhou et al. (2014)
Sorghum bicolor	Agro-transformation of immature embryos	Monocot	OsActin1, OsU6	Co-transfected *DsRed*	28% (5/18)	DsRed fluorescence				Jia et al. (2014)
Solanum lycopersicum	Hairy root transformation by *A. rhizogenes*	*Nicotiana*	CaMV35S, AtU6	*GFP, SHR*	n.d.	RE + PCR; phenotype				Ron et al. (2014)
S. lycopersicum	Agro-transformation of cotyledons	Human	2xCaMV35S, AtU6	*SlAGO7, Solyc08g041770, Solyc07g021170, Solyc12g044760*	75 (6/8)–100% (29/29, 8/8)	phenotype, PCR + sequencing		Yes (90–140 bp)	Yes	Brooks et al. (2014)
N. tabacum	Agro-transformation of leaf discs	Tobacco	2xCaMV35S, AtU6	*PDS, PDR6*	82% (9/11)–88%(14/16)	Phenotype, PCR + RE sequencing	No (1 site)/PCR + RE		Yes	Gao et al. (2014)
T. aestivum	Particle bombardment of embryos	Plant	ZmUbi, TaU6	*MLO-A1*	5.6% (4/72)	T7E1			Yes	Brooks et al. (2014)
Marchantia polymorpha	Agro-transformation of sporelings	Human	CaMV35S or MpEF, MpU6	*ARF1*	11% (5/45)	Phenotype, PCR + sequencing				Sugano et al. (2014)

AtUBQ1, U6 = *A. thaliana* ubiquitin1 or U6 promoter; PcUBI4-2 = *P. crispum* polyubiquitin promoter; CaMV35S = promoter of the 35S gene of the *cauliflower mosaic virus*; 2x35SCaMV = 35S promoter with duplicated enhancer; OsUBQ, Actin1, U3, U6 = *O. sativa* ubiquitin, actin 1, U3 or U6 promoters; ZmUbi, U3 = *Z. mays* ubiquitin or U3 promoters; TaU6 = *T. aestivum* U6 promoter; MpEF = *M. polymorpha* elongation factor 1α promoter; MpU6 = *M. polymorpha* U6 promoter; PCR = polymerase chain reaction; RE = restriction enzyme digestion; T7E1 = T7 endonuclease I assay; n.d. = not determined.

Table 3
Homologous recombination using the CRISPR system.

Species	Transfection method	Cas9 codon optimization	Promoters (Cas9, gRNA)	Target	Exogenous HR template	HR frequency	detection method	Reference
Arabidopsis thaliana	Stable Agro-transformation	Human	AtUBQ1, AtU6	*GUUS*	None (SSA)	11.4%	GUS staining	Mao et al. (2013)
A. thaliana	Stable Agro-transformation	Arabidopsis	PcUBI4-2, AtU6	*GUUS, UGUS*	None (SSA, SDSA)	n.d.	GUS staining	Fauser et al. (2014)
A. thaliana	Stable Agro-transformation	Arabidopsis (nickase)	PcUBI4-2, AtU6	*GUUS, UGUS*	None (SSA, SDSA)	n.d.	GUS staining	Fauser et al. (2014)
A. thaliana	Transient PEG-protoplast transfection	Human	2xCaMV35S, AtU6	*YFFP*	None (SSA)	18.8%	YFP fluorescence	Feng et al. (2013)
A. thaliana	Stable Agro-transformation	Arabidopsis	PcUBI4-2, AtU6	*ADH1*	*In planta* released ds DNA, 1.8 kb +674 and 673 bp homology arms	0.14%.	PCR, phenotype, sequencing	Schiml et al. (2014)
Nicotiana benthamiana	Transient PEG-protoplast transfection	Arabidopsis (with intron)	CaMV35SPDK, AtU6	*NbPDS3*	ds DNA, 533 + 114 bp homology arms	10.7%	PCR + RE	Li et al., 2013
Oryza sativa	Transient PEG-protoplast transfection	Rice	2xCaMV35S, OsU3	*OsPDS, OsBADH2*	ss oligo DNA, 72b	7%	RE + PCR + RE	Shan et al. (2013)
O. sativa	Transient particle bombardment of callus	Rice	ZmUbi, OsU3	*GUUS*	None (SSA)	n.d.	GUS staining	Miao et al. (2013)

AtUBQ1, U6 = *A. thaliana* ubiquitin1 or U6 promoter; PcUBI4-2 = *P. crispum* polyubiquitin promoter; 2x35SCaMV = promoter of the 35S gene of the *cauliflower mosaic virus* with duplicated enhancer; CaMV35SPDK = hybrid promoter, 35S enhancer fused to the maize C4PPDK basal promoter; OsU3 = *O. sativa* U3 promoter; ZmUbi = *Z. mays* ubiquitin 3 promoters; PCR = polymerase chain reaction; RE = restriction enzyme digestion; n.d. = not determined; SSA = single-strand annealing repair pathway; SDSA = synthesis-dependent strand annealing repair pathway.

practice. An extensive *in silico* analysis of nuclear genome sequences from eight representative plant species (Arabidopsis, *Medicago truncatula*, soybean, tomato, *Brachypodium distachyon*, rice, sorghum and maize) has been used to predict specific gRNA spacers with minimal off-target cleavage using data from mammalian systems (Xie et al., 2014). This identified 5–12 NGG-PAMs for every 100 bp of genomic DNA. The total number of PAMs correlated with genome size, but a greater number of specific gRNAs was predicted in monocot genomes compared to dicot genomes when differences in genome size were taken into account. For all species except maize, it was possible to design specific gRNAs to target 85–99% of currently annotated transcript units, with 68–96% of these transcription units containing at least 10 different targetable NGG-PAM sites. In maize, which has the largest genome and greatest number of annotated genes among the eight species that were analyzed, only 30% of the transcription units could be targeted by specific gRNAs. The lack of specific gRNAs for so many maize genes probably reflects the genome complexity (duplication events) and genomic sequence context. It is therefore anticipated that wheat and barley, with even larger genomes than maize, may present similar challenges for CRISPR/Cas9-mediated genome editing (Xie et al., 2014).

In the future, the range of plant genomic sequences amenable to the CRISPR/Cas system could be expanded by using Cas9 homologs with different PAM requirements. Furthermore, the use of standard full-length gRNAs can be limiting because they are usually transcribed by RNA polymerase III under the control of either the U6 or U3 promoters, which respectively require guanidine and adenosine residues at the 5′ end for optimal transcription. Because the system can tolerate mismatches at the 5′ end of the gRNA, this limitation can be circumvented by choosing targets without considering the identity of the first base or by appending the required nucleotide to the 5′ end of the gRNA thus producing transcripts that are one nucleotide longer (Cho et al., 2014; Hwang et al., 2013; Ran et al., 2013; Shan et al., 2013). Gao and Zhao (2014) used an artificial gene containing ribozyme sequences, which can be transcribed from any promoter, generating gRNAs by self-catalyzed cleavage of the primary transcript in yeast. The production of gRNAs using an RNA polymerase II promoter and terminator has also been reported in plants in two independent studies, eliminating all restrictions at the 5′ end of the transcripts (Jia and Wang, 2014; Upadhyay et al., 2013, Table 1). The ability to use RNA polymerase II regulatory elements for the production of gRNAs offers many intriguing possibilities, such as the simultaneous differential expression of multiple gRNAs using well-characterized tissue-specific, developmental stage-specific and inducible promoters.

In terms of comparative performance, the first studies using the CRISPR/Cas9 system for genome editing in mammalian cell lines and zebrafish embryos showed that the technology was at least as efficient as ZFNs and TALENs targeting the same sites (Cong et al., 2013; Hwang et al., 2013) and in some cases even higher (Ding et al., 2013). Although the CRISPR/Cas9 system is generally efficient, some gRNAs achieve higher mutation rates than others, and this does not always depend on the local accessibility of the nuclease complex. Some guidelines are emerging to predict the efficiency of gRNAs. For example, Wang et al. (2014a) compared several gRNAs targeting the same gene in a human cell line and looked for trends associated with targeting efficiency. They found that gRNAs with an unusually high or low GC content tended to be less effective than those with an average GC content, and that gRNAs targeting the transcribed strand were less effective than those targeting the non-transcribed strand. They also found that Cas9 preferentially binds to gRNAs containing purine residues in the last four positions of the spacer sequence, and that the efficiency of cleavage is influenced by the affinity between the gRNA and Cas9. Similar conclusions were drawn during the development of a web tool to design gRNAs for the effective targeting of mouse and human genes (Doench et al., 2014).

As in mammals, the CRISPR/Cas9 system has been shown to achieve high mutation rates in plants, matching or exceeding those obtained with ZFNs and TALENs (Lozano-Juste and Cutler, 2014). For example, a comparison of TALENs and CRISPR/Cas9 targeting the same sites in maize showed 13.1% efficiency with CRISPR and 9.1% with TALENs (Liang et al., 2014). However, it should be noted that the mutation rate can be influenced by differences in the target sequences, the gRNA structure/sequence, the version of Cas9 (codon-optimized for different species) and the gRNA expression strategy. Furthermore, the reported mutation rate can depend on the sensitivity of the analytical method (e.g., T7 endonuclease I or Surveyor assay, PCR/restriction analysis or PCR/direct sequencing). Therefore, it is not surprising that reported mutation rates vary even within the same species. For example, PEG-mediated transfection of rice protoplasts resulted in a mutation efficiency of up to 38% (targeting the *PDS* gene, Shan et al., 2013) or 8% (targeting the *MPK5* gene, Xie and Yang, 2013). Finally, CRISPR/Cas9 activity is greatly dependent on the cell type and delivery method, as is the case for other nucleases. For example, PEG-mediated

transfection of *N. benthamiana* mesophyll protoplasts resulted in a mutation rate of 37.7% in the *PDS* gene (Li et al., 2013), whereas the same constructs delivered by agroinfiltration into whole leaves achieved a mutation rate of 4.8% in the same gene. It is unclear whether this ~10-fold change represents differences in transfection efficiency, gRNA/Cas9 expression levels or DNA repair mechanisms in the distinct cell types.

Advantages of the CRISPR/Cas9 system

Everything that can be achieved with the CRISPR/Cas9 system can in principle also be achieved using either ZFNs or TALENs. Nevertheless, the appearance of such a large number of publications based on the CRISPR/Cas9 technology in such a short time, including virgin reports of genome editing in species such as sweet orange (Jia and Wang, 2014), highlights the clear advantages of CRISPR/Cas9 in terms of simplicity, accessibility, cost and versatility.

Unlike its predecessors, the CRISPR/Cas9 system does not require any protein engineering steps, making it much more straightforward to test multiple gRNAs for each target gene. Furthermore, only 20 nt in the gRNA sequence need to be changed to confer a different target specificity, which means that cloning is also unnecessary. Any number of gRNAs can be produced by *in vitro* transcription using two complementary annealed oligonucleotides (Cho et al., 2013). This allows the inexpensive assembly of large gRNA libraries so that the CRISPR/Cas9 system can be used for high-throughput functional genomics applications, bringing genome editing within the budget of any molecular biology laboratory.

Unlike ZFNs and TALENs, the CRISPR/Cas9 system can cleave methylated DNA in human cells (Hsu et al., 2013), allowing genomic modifications that are beyond the reach of the other nucleases (Ding et al., 2013). Although this aspect has not been specifically explored in plants, it is reasonable to assume that the ability to cleave methylated DNA is intrinsic to the CRISPR/Cas9 system and not dependent on the target genome. Approximately 70% of CpG/CpNpG sites are methylated in plants, particularly the CpG islands found in promoters and proximal exons (Vanyushin and Ashapkin, 2011). The CRISPR/Cas9 technology is therefore more versatile for genome editing in plants generally but particularly suitable for monocots with high genomic GC content such as rice (Miao et al., 2013). Conventional TALENs cannot cleave DNA containing 5-methylcytosine but methylated cytosine is indistinguishable from thymidine in the major groove. Therefore, the repeat that recognizes cytosine can be replaced with a repeat which recognizes thymidine, generating TALENs that can cleave methylated DNA albeit at the expense of target specificity (Deng et al., 2012; Valton et al., 2012).

The main practical advantage of CRISPR/Cas9 compared to ZFNs and TALENs is the ease of multiplexing. The simultaneous introduction of DSBs at multiple sites can be used to edit several genes at the same time (Li et al., 2013; Mao et al., 2013) and can be particularly useful to knock out redundant genes or parallel pathways. The same strategy can also be used to engineer large genomic deletions or inversions by targeting two widely spaced cleavage sites on the same chromosome (Li et al., 2013; Upadhyay et al., 2013; Zhou et al., 2014). Multiplex editing with the CRISPR/Cas9 system simply requires the monomeric Cas9 protein and any number of different sequence-specific gRNAs. In contrast, multiplex editing with ZFNs or TALENs requires separate dimeric proteins specific for each target site.

Finally, the open access policy of the CRISPR research community has promoted the widespread uptake and use of this technology in contrast, for example, to the proprietary nature of the ZFN platform. The community provides access to plasmids (e.g., via the non-profit repository Addgene), web tools for selecting gRNA sequences and predicting specificity (e.g., for plant genomes: http://cbi.hzau.edu.cn/cgi-bin/CRISPR; http://www.genome.arizona.edu/crispr/; and http://www.rgenome.net/cas-offinder, http://www.e-crisp.org/E-CRISP/index.html) and hosts active discussion groups (e.g.: https://groups.google.com/forum/#!forum/crispr). These facilities have encouraged newcomers to adopt the technology and contributed to the rapid progress in our understanding of the system and its practical applications.

The specificity of CRISPR/Cas9

One of the few criticisms of the CRISPR/Cas9 technology is the relatively high frequency of off-target mutations reported in some of the earlier studies (Cong et al., 2013; Fu et al., 2013; Hsu et al., 2013; Jiang et al., 2013a; Mali et al., 2013; Pattanayak et al., 2013). Although a 20 nt sequence in the gRNA was initially considered necessary to determine specificity, it was later shown that only the 8–12 nt at the 3′ end (the seed sequence) is needed for target site recognition and cleavage (Cong et al., 2013; Jiang et al., 2013a; Jinek et al., 2012), whereas multiple mismatches in the PAM-distal region can be tolerated, depending on the total number and arrangement (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). DNA sequences that contain an extra base (DNA bulge) or a missing base (gRNA bulge) at various locations along the corresponding gRNA sequence have also been shown to induce off-target cleavage (Lin et al., 2014). The relaxed specificity of the CRISPR/Cas9 complex at non-seed positions in the crRNA spacer appears to be an intrinsic property that reduces the likelihood of immune system evasion by viruses with point mutations (Semenova et al., 2011).

Several strategies have been developed to reduce off-target genome editing, the most important of which is the considered design of the gRNA. In contrast to ZFNs and TALENs, whose target specificity is determined by protein–DNA interactions that are often context-dependent and unpredictable, the CRISPR/Cas9 system recognizes target sites by Watson–Crick base pairing allowing off-target sites to be predicted more reliably by sequence analysis (Cho et al., 2014). The CRISPR/Cas9 system is also easy to reprogram, so gRNAs can be tested for off-target effects rapidly and inexpensively. For example, Hsu et al. (2013) tested over 700 sgRNA variants in parallel to gain insight into the issue of specificity. Based on such comparative studies, a number of guidelines and online tools have been developed to facilitate the selection of unique target sites in well-characterized organisms including several plants (see above). Optimizing nuclease expression is another way to control specificity because high concentrations of gRNA and Cas9 can promote off-target effects (Fujii et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013).

The FokI nuclease domain of ZFNs and TALENs functions only as a dimer, with each catalytic monomer (nickase) cleaving a single DNA strand to create a staggered DSB with overhangs. Similarly, a mutated version of Cas9 has been produced with a D10A mutation in the RuvC nuclease domain that converts it into a nickase. The use of two Cas9 nickases can therefore generate offset single-strand nicks that produce a staggered DSB (Fig. 3a). This strategy increases the number of bases specifically recognized for target cleavage, thereby reducing the likelihood of homologous sequences being present elsewhere in the genome. This strategy can yield precise genome modifications with efficiencies comparable to the unmodified enzyme, but with 50- to 1500-fold greater specificity in human and mouse cells (Cho et al., 2014; Duda et al., 2014; Mali et al., 2013; Ran et al., 2013; Shen et al., 2014) and Arabidopsis (Fauser et al., 2014; Schiml et al., 2014). One caveat to this approach is that two equally efficient gRNAs are needed to make an efficient nickase pair, potentially limiting the number of target sites given that not all gRNAs are equivalent in terms of activity (Cho et al., 2014). Furthermore, each component of a paired nickase system remains catalytically active, and although nicks are generally repaired with high fidelity, it is not possible to exclude the possibility of additional off-target mutations.

To address this problem, fusions of catalytically inactive Cas9 and FokI nuclease have been generated, and these show comparable efficiency to the nickases but substantially higher (>140-fold) specificity than the wild-type enzyme (Guilinger et al., 2014; Tsai et al., 2014)

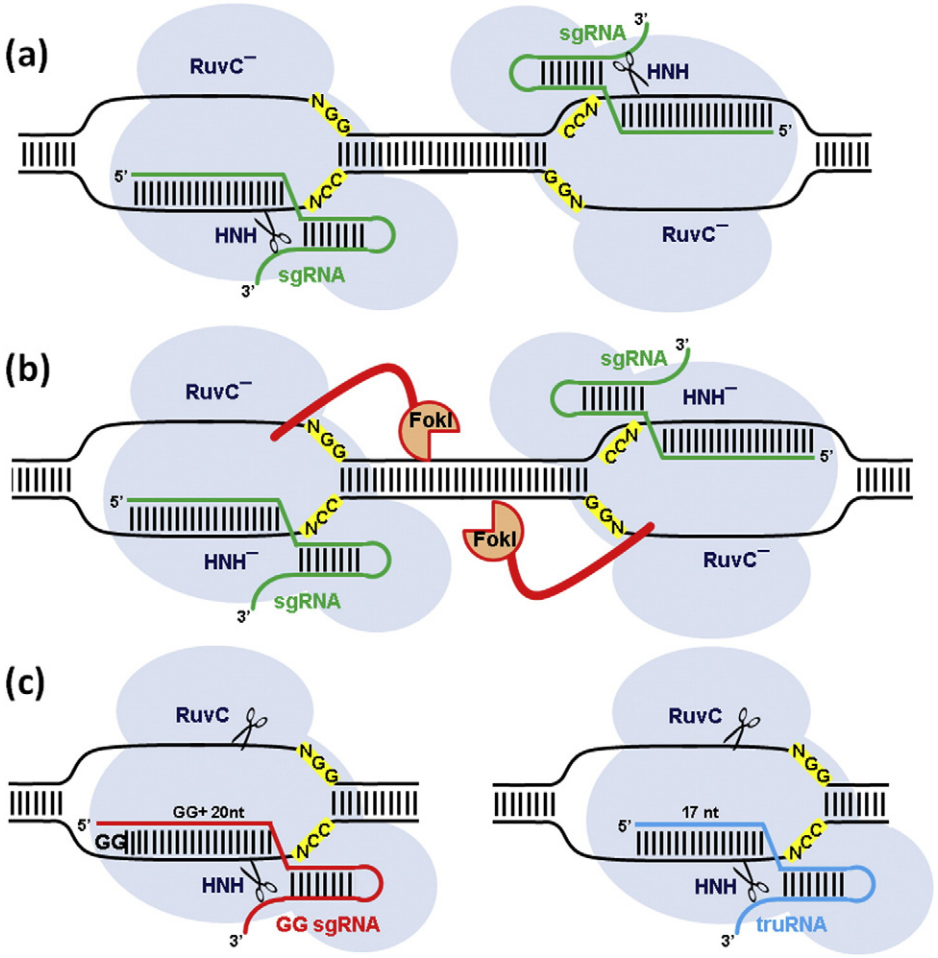

Fig. 3. Strategies to increase CRISPR/Cas9 target specificity. The most important strategy to avoid off-target effects is the design of a specific gRNA by checking for the presence of homologous sequences in the genome. Further strategies can then be employed to reduce the risk of off-target cleavage further. (a) A pair of offset Cas9 nickases. The D10A mutation inactivates the RuvC endonuclease domain so that Cas9 can cleave only the DNA strand complementary to the gRNA. The simultaneous use of two Cas9 nickases binding to sequences on opposite DNA strands generates a staggered DSB with overhangs. (b) Cas9-FokI fusion proteins. A catalytically inactive Cas9 variant, carrying a mutation in both endonuclease domains (RuvC⁻ HNH⁻), can be fused to the FokI nuclease domain. DNA cleavage by FokI is dependent on dimerization and the enzyme must bind two precisely disposed half-sites on the genome, greatly reducing the number of possible off-target sequences. (c) Altering the length of the gRNA. Both extending the gRNA by adding two guanidine residues at the 5′ end (left), or shortening it to a truncated gRNA (truRNA) of only 17 nt (right) can reduce off-target effects.

(Fig. 3b). The rationale behind this strategy is that the engineered version of Cas9 must form dimers to cleave DNA (like ZFNs and TALENs) and must therefore bind two precisely disposed half-sites, greatly reducing the number of potential off-target sequences.

Altering the length of the gRNA can also minimize non-target modifications (Fig. 3c). Guide RNAs with two additional guanidine residues at the 5′ end were able to avoid off-target sites more efficiently than normal gRNAs but were also slightly less active at on-target sites (Cho et al., 2014). In contrast, truncated chimeric single guide RNAs (tru-sgRNAs) 17 nt in length reduced off-target mutations by orders of magnitude without affecting on-target mutation efficiency (Fu et al., 2014). Truncation may render the RNA-DNA complex more sensitive to mismatches, perhaps by reducing binding energy at the gRNA-DNA interface. The tru-sgRNAs and Cas9 nickase approaches can also be combined, potentially increasing specificity even further (Fu et al., 2014).

Off-target effects vary even among different cell types in the same species. For example, much lower off-target mutation rates were observed in human pluripotent stem cells compared to cancer cell lines based on whole-genome sequencing data (Smith et al., 2014; Veres et al., 2014). The limited data available thus far also suggest that off-target effects are rare in plants. In rice, Xie and Yang (2013) reported a 1.6% off-target mutation rate (five times lower than the on-target mutation rate) in a single off-target sequence carrying a mismatch at position 11 upstream from the PAM. In contrast, no off-target mutations were found in Arabidopsis, *N. benthamiana*, wheat and sweet orange, or in an independent study involving rice, when potential off-target sites with at least a conserved 12-nt seed sequence were investigated (Feng et al., 2014; Jia and Wang, 2014; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Upadhyay et al., 2013; Zhou et al., 2014) even using whole-genome sequencing (Feng et al., 2014). Although more data are required before firm conclusions can be drawn about the off-target mutation rates in plants, these results already indicate that the careful selection of specific gRNA sequences should minimize the risk of unwanted genome modifications.

Applications and implications in plant breeding

Plants provide us with food, animal feed, medicines, chemicals, renewable materials and biofuels. The domestication of plants has involved the development of strategies to improve the performance of crops and tailor their properties. Conventional breeding relies on existing natural genetic variation and extensive back-crossing programs are necessary to introgress the selected traits into an elite background. The availability of beneficial alleles in nature therefore limits what can be achieved using this approach. New alleles can be introduced by random mutagenesis, but this must be followed by the time-consuming screening of large populations to identify mutants with

desirable properties. Genome editing can therefore accelerate plant breeding by allowing the introduction of precise and predictable modifications directly in an elite background, and the CRISPR/Cas9 system is particularly beneficial because multiple traits can be modified simultaneously.

NHEJ-mediated gene knockouts are the simplest form of targeted modification, and these could be used e.g., to eliminate genes that negatively affect food quality, to confer susceptibility to pathogens or to divert metabolic flux away from valuable end-products. For example, Wang et al. (2014b) used both TALEN and CRISPR/Cas9 technologies to target the genes of the mildew-resistance locus (MLO) in wheat and successfully knocked out all three *MLO* homoeoalleles, generating plants resistant to powdery mildew disease. Precise nucleotide exchanges using oligonucleotide donor sequences could be used to modify the regulatory sequences upstream of genes that determine agricultural performance therefore improving crop yields. The insertion of large sequences by NHEJ or HR would allow the introduction of transgenes at defined loci that promote high-level transcription and do not interfere with the activity of endogenous genes.

Site-specific nucleases also allow targeted molecular trait stacking, i.e., the addition of several genes in close vicinity to an existing transgenic locus. This makes it feasible to introduce multiple traits into crops with a low risk of segregation, which is difficult to achieve by classical breeding or even conventional genetic engineering (Ainley et al., 2013). Once stacking has been achieved, the entire array of transgenes can be mobilized into other germplasm by crossing because it behaves as a single locus. It is possible to achieve these aims using site-specific recombination, but targeted integration using programmable nucleases combined with precise NHEJ or HR does not leave behind any footprints associated with the integration method, such as *lox*P or *att*B sequences.

Although the European regulatory framework for genetically modified crops focuses on the process and not the product (hence two identical plants produced by conventional mutagenesis and genetic engineering would be regulated differently under the current guidelines), there is hope and confidence that plants altered by the excision of a few nucleotides using genome editing tools such as CRISPR/Cas9 would not be classified as genetically modified organisms (Hartung and Schiemann, 2014; Li et al., 2012; Podevin et al., 2013). There are several ways to create transgene-free mutated plants using programmable nucleases, including the transient expression of the nuclease components using agroinfiltration or viral vectors, the delivery of the components directly as functional gRNA and Cas9 protein or the incorporation of the gRNA and Cas9 transgenes on a separate chromosome to the targeted locus so that they can be removed by segregation. Although the specificity of the CRISPR/Cas9 technology remains to be investigated in detail, it is already clear that the frequency of off-target mutations is well below that caused by chemical and physical mutagenesis techniques (Podevin et al., 2013). Indeed, the use of site-specific nucleases could remove much of the regulatory burden associated with transgenic plants by addressing one of the main causes of concern, namely, the random integration of transgenes and the resulting potential for unintended effects such as disrupting host metabolism and/or producing toxic or allergenic compounds. The complex regulatory process and the requirement for time-consuming and expensive safety analysis have resulted in a *de facto* moratorium on the development and commercial release of transgenic plants with the exception of large companies that have the resources to fund long development programs (Podevin et al., 2013). The potential to introduce transgenes at a specific and predetermined chromosomal position using site-specific nucleases should all but eliminate the risk of such unpredictable events.

One further application of CRISPR/Cas9 that is likely to expand in the future is the targeted insertion of transgenes in the fields of metabolic engineering and molecular farming, where plants or plant cells are used as factories for the production of specific metabolites or proteins. Currently, both applications rely on random transgene insertion such that populations of primary transformants must be screened so that high-performance clones can be selected. This reflects the impact of genomic position effects (where regulatory elements and chromatin structure surrounding the transgene integration site influence transgene expression) and other features of the transgenic locus (e.g., transgene copy number, the presence of inverted repeats and truncated sequences), all of which affect the likelihood of silencing. The establishment of a generic recipient line with a predetermined and characterized 'safe harbor locus' promoting the strong expression of any transgene and thus a high yield of the corresponding product, would accelerate the development and possibly the approval of new plant-based production lines.

Beyond genome editing

The CRISPR/Cas9 system can be used for several purposes in addition to genome editing, e.g., the ectopic regulation of gene expression, which can provide useful information about gene functions and can also be used to engineer novel genetic regulatory circuits for synthetic biology applications. The external control of gene expression typically relies on the use of inducible or repressible promoters, requiring the introduction of a new promoter and a particular treatment (physical or chemical) for promoter activation or repression. Disabled nucleases can be used to regulate gene expression because they can still bind to their target DNA sequence. This is the case with the catalytically inactive version of Cas9 which is known as dead Cas9 (dCas9). This protein is unable to cut DNA (Fig. 4a), but it can still be recruited to specific DNA sequences by gRNAs. If it is expressed as a fusion protein with the transactivation or transrepression domain of a transcription factor, the precise and reversible transcriptional control of target genes becomes possible (Gilbert et al., 2013; Maeder et al., 2013b). Piatek et al. (2014) modulated the transcription of both a reporter construct and the endogenous PDS gene in *N. benthamiana*, fusing the dCas9 C-terminus to the EDLL domain and the TAL activation domain to generate transcriptional activators, and to the SRDX domain from the ERF transcription factor to generate a repressor. They observed that transcriptional activity was influenced by the position of the gRNA with respect to the transcriptional start site, as well as the nature of the target strand (sense or antisense). Even naked dCas9 without any effector domains has been shown to repress both synthetic and endogenous genes through the steric blocking of transcription initiation and elongation, although the degree of repression was modest in mammalian cells (Qi et al., 2013). The use of dCas9 for specific gene regulation provides an alternative approach in species that currently lack controllable expression systems. Multiple gRNAs targeting the same promoter also demonstrate synergistic effects, indicating that tuning the level of transcriptional control is possible using this approach (Bikard and Marraffini, 2013; Piatek et al., 2014; Qi et al., 2013). Furthermore, multiple gRNAs targeting different promoters allow the simultaneous inducible regulation of different genes (Qi et al., 2013). Two independent research groups have already extended this approach by layering CRISPR regulatory devices based on either transcriptional activators (Nissim et al., 2014) or repressors (Kiani et al., 2014) to create functional cascaded circuits. In this context, another peculiar feature of the CRISPR/Cas9 system is the ability to use orthogonal Cas9 proteins to separately and simultaneously carry out genome editing and gene regulation in the same cell (Esvelt et al., 2013).

The inactive enzyme dCas9 can also be used to deliver specific cargos to targeted genomic locations (Fig. 4b). For example, dCas9 has been fused with a fluorescent protein to visualize selected loci in living cells, providing a useful tool for studying chromosome structure and dynamics (Anton et al., 2014; Chen et al., 2013). The same approach has been used to target proteins involved in histone modification and

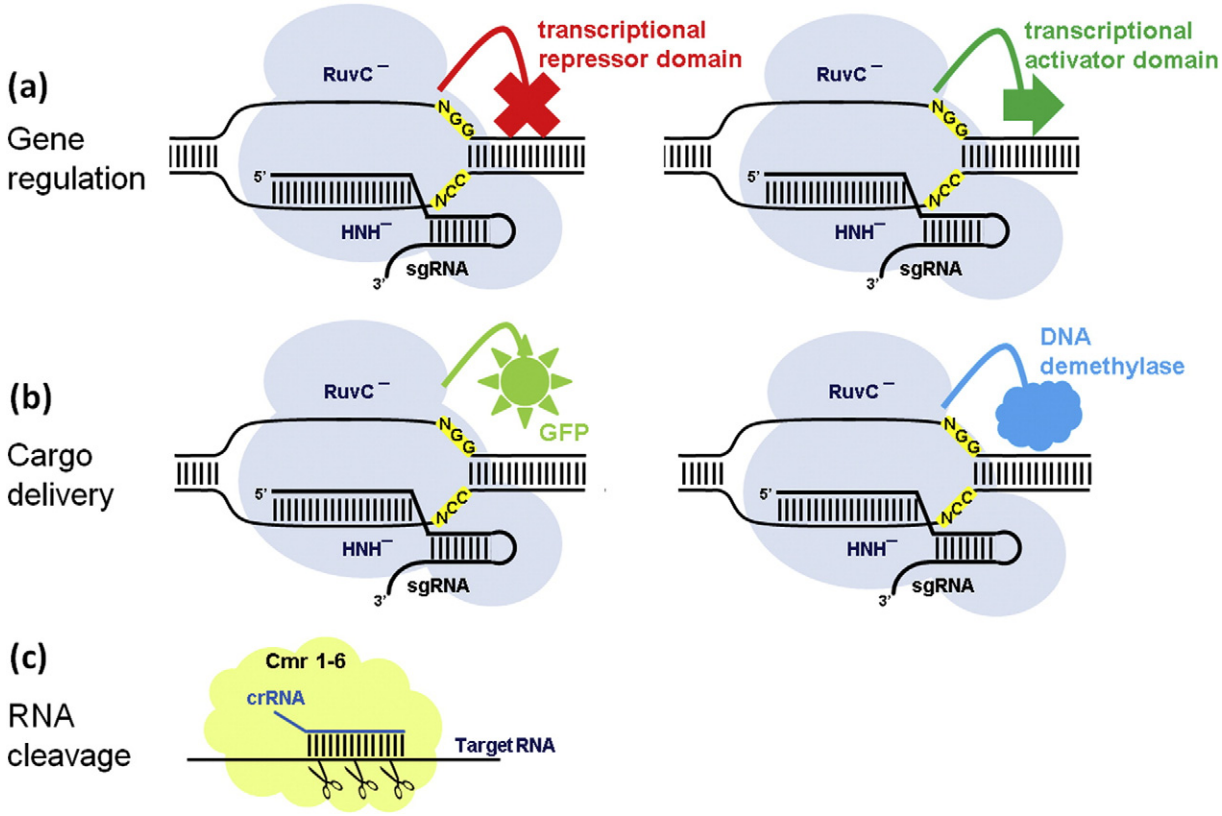

Fig. 4. Applications of the CRISPR/Cas system beyond genome editing. In addition to targeted genome editing, the CRISPR/Cas9 technology is suitable for other interesting applications. (a) Gene regulation. A catalytically inactive dead Cas9 (dCas9, in light blue) can be fused to either a transcriptional repressor (left) or activator (right). When the dCas9-repressor fusion is recruited by a gRNA that matches the promoter, 5′ untranslated region or coding sequence of an endogenous gene, it can block transcription initiation, elongation or the binding of transcription factors. When the dCas9-activator fusion is targeted to a promoter, the specific expression of the endogenous gene is induced. (b) Cargo delivery. The catalytically inactive dCas9 can also be exploited as a programmable DNA-binding protein to deliver diverse cargos to specific genomic locations. For example, fusion with a green fluorescent protein (left) provides a tool for visualizing chromosome structure or dynamics, and fusion with a demethylase (right) can be used for targeted epigenome editing. (c) RNA cleavage. The Type III-B CRISPR-Cas system (e.g., from *Pyrococcus furiosus*) is composed of nucleases that form the so-called Cmr complex (yellow) and represents a unique RNA silencing system. The Cmr-crRNA complex targets invading RNA in a PAM-independent process and is able to degrade complementary RNA sequences cleaving them at multiple sites.

DNA methylation, allowing the selective editing of the epigenome as already demonstrated with TAL repeats (Maeder et al., 2013a).

An important general feature of CRISPR/Cas systems that differentiates them from ZFNs and TALENs is their ability to differentially target either DNA or RNA. The Type III-B CRISPR-Cas system (e.g., from *Pyrococcus furiosus*) is a unique RNA silencing system involving the homology-dependent degradation of complementary RNA in the presence of engineered crRNA both *in vitro* and *in vivo* (Hale et al., 2009; Hale et al., 2012) (Fig. 4c). Such flexible post-transcriptional control of gene expression would compensate for the potential pitfalls of a solely DNA-targeting technology, when the binding of the complex to the target DNA is hindered either by chromatin structure or by the presence of other bound proteins, or when the elimination of only one of several splice variants from a single transcript is desired. Furthermore, such a system would be more specific than RNA interference because it would not rely on host factors such as Dicer or the components of the RNA-induced silencing complex and should therefore not cause the silencing of off-target genes.

Final remarks and outlook

The simplicity and accessibility of the CRISPR/Cas9 technology platform provides many advantages over other genome editing methods, and this means that loss-of-function screening is now feasible and affordable on a genomic scale (Shalem et al., 2014). The availability of the CRISPR/Cas9 technology will facilitate both forward and reverse genetics and will enhance basic research even in model species such as Arabidopsis, which already boast extensive (yet incomplete) mutant libraries. It will allow the growing amount of genomic and systems biology data to be exploited more comprehensively, speeding up both gene discovery and trait development in many plant species. Most information concerning the properties of the CRISPR/Cas9 system is currently derived from studies in mammals, and although it seems that many of the findings can be generalized it is still necessary to conduct similar studies in plants to ensure that system properties are translatable to different species. This certainly applies to extended applications such as orthogonal gene targeting, which have yet to be tested in plant systems.

Although the CRISPR/Cas9 system is an excellent tool for genome editing, the extent of off-target mutation needs to be investigated in more detail as well as the differences in cleavage efficiency among different but perfectly matched targets. The ability to produce and test multiple gRNAs in parallel and the availability of next-generation sequencing technologies will provide ample data for the comparison of the CRISPR/Cas9 system in many different species and cell types. Furthermore, the structural analysis of Cas9 and its interaction with gRNA and target DNA will facilitate the development of engineered nucleases with greater efficiency and specificity (Jinek et al., 2014; Sternberg et al., 2014). The development of Cas9 proteins requiring longer PAMs, which would occur less frequently in the genome, would be likely to reduce off-target effects even further.

Every evolutionary process involving host–pathogen interactions is an arms race featuring adaptations and counter-adaptations to overcome the opponent. Therefore, some viruses may well have evolved anti-CRISPR strategies to evade this bacterial immune system, and these as yet undiscovered regulators may provide additional tools to

modify and control the activity of the CRISPR/Cas9 system. Given the large number of researchers working with CRISPR/Cas9 technology and the speed at which it has developed since the first reports of genome editing only 2 years ago, further advances in our understanding and control of the system are likely to come rapidly, potentially leading to the design of a new generation of genome editing tools.

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