Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Editing plant genomes with CRISPR/Cas9

Khaoula Belhaj[1], Angela Chaparro-Garcia[1], Sophien Kamoun, Nicola J Patron and Vladimir Nekrasov

CRISPR/Cas9 is a rapidly developing genome editing technology that has been successfully applied in many organisms, including model and crop plants. Cas9, an RNA-guided DNA endonuclease, can be targeted to specific genomic sequences by engineering a separately encoded guide RNA with which it forms a complex. As only a short RNA sequence must be synthesized to confer recognition of a new target, CRISPR/Cas9 is a relatively cheap and easy to implement technology that has proven to be extremely versatile. Remarkably, in some plant species, homozygous knockout mutants can be produced in a single generation. Together with other sequence-specific nucleases, CRISPR/Cas9 is a game-changing technology that is poised to revolutionise basic research and plant breeding.

Addresses
The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, UK

Corresponding authors: Kamoun, Sophien (sophien.kamoun@tsl.ac.uk), Patron, Nicola J (nicola.patron@tsl.ac.uk) and Nekrasov, Vladimir (vladimir.nekrasov@tsl.ac.uk)
[1] These authors contributed equally.

Current Opinion in Biotechnology 2015, **32**:76–84

This review comes from a themed issue on **Plant biotechnology**

Edited by **Inge Broer** and **George N Skaracis**

http://dx.doi.org/10.1016/j.copbio.2014.11.007

0958-1669/

Introduction

Creating genetic variation in plant crops is the key to sustainable agriculture [1]. Plant biotechnology is now entering a new phase where random mutagenesis methods, such as EMS mutagenesis and γ-radiation, are being superseded by genome editing technologies that enable precise manipulation of specific genomic sequences. Such technologies rely on sequence-specific nucleases (SSNs), molecular tools used to generate DNA double-strand breaks (DSBs) at a desired location within genome. DSBs are repaired by the cell's endogenous mechanisms, primarily non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ utilises DNA ligase IV to re-join separated ends. If bases have been deleted or the ends have been processed by nucleases, the repair may be imperfect. HDR, however, uses a template for repair and therefore repairs are likely to be perfect. In a natural situation the sister chromatid would be the template for repair, however templates to recode a target locus or to introduce a new element between flanking regions of homology can be delivered with an SSN [2]. In mammalian cells, DSBs were shown to stimulate homologous recombination at the break site [3]. In plants, transfer DNAs (T-DNA), delivered during Agrobacterium-mediated DNA transfer, were also found to be preferentially integrated into induced DSBs [4], a considerable advance over earlier experiments, in which insertions at a desired locus were observed to occur only at low frequencies if the T-DNA included regions of homology to the integration site of interest [5]. Despite this, HDR-mediated gene insertion remains challenging in plants. To date most genome editing has utilised the NHEJ pathway to knockout genes (e.g. via introducing a frameshift mutation or deleting a large fragment) and only a few instances of gene insertion by HDR have been reported [6,7]. The reasons may be that the tissues to which DNA is delivered to plants cells are often composed of determinate cells in which HDR is not the preferred repair mechanism [8]. Additionally, the delivered repair template must outcompete the sister chromatid. Also, delivery mechanisms used to deliver SSNs to plants, such as such as *Agrobacterium tumefaciens* and particle bombardment, may not deliver a sufficient amount of repair template.

In the last decade, several sequence-specific nucleases, including zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs), have been successfully used in plants, promising to revolutionise conventional plant breeding and genetic modification [9,10]. ZFNs and TALENs are artificial bipartite enzymes that consist of a modular DNA-binding domain and the FokI nuclease domain. In both cases the DNA-binding domain can be engineered to recognise a specific DNA sequence. However, the design and construction of large modular proteins is both laborious and expensive. In addition, there is a high rate of failure, at least for ZFNs, to recognise and cleave the intended DNA sequence [2]. The most rapidly emerging tool is a bacterial monomeric DNA endonuclease, known as Cas9 (CRISPR-associated protein 9), which can be targeted to a specific genomic sequence by an easily engineered 20 base pair (bp) RNA guide sequence that binds to its DNA target by Watson-Crick base-pairing [11•]. Target recognition is dependent on the so-called 'protospacer adjacent motif' (PAM), for

which the consensus sequence, NGG, is adjacent to the 3′ end of the 20 bp target [11•]. In bacterial genomes, the gene encoding Cas9 was found in close association with short, highly homologous sequences arranged in direct repeats interspaced with non-homologous spacer sequences [12] (Figure 1a). These regions, which became known as clustered regularly interspaced short palindromic repeats (CRISPR), were first observed in 1987 [13]. However, their role in prokaryotic adaptive immunity was only functionally demonstrated in 2007 when it was shown that the spacer sequences dictate the target of the endonuclease, with the spacers acquired from exogenous sources, such as an invading phage [14]. Several CRISPR/Cas immune systems have since been characterised in bacteria and archaea [15] with the type II system, typified by the Cas9 protein, being repurposed as an easily programmed tool for facile genome editing. In its natural state, a spacer-containing crRNA hybridises with a noncoding, trans-activating crRNA (tracrRNA) that facilitates DNA cleavage aided by RNase III [16] (Figure 1a). A fusion of these two RNA moieties into a 'single guide' RNA (sgRNA) was initially demonstrated to be functional *in vitro* [11•] and subsequently used to introduce double-strand breaks into specified regions of mammalian genomes [17,18] (Figure 1b). Because of their small size, multiple sgRNAs can be co-delivered with Cas9 to the cell making it possible to simultaneously edit more than one target at the same time in process called 'multiplex gene editing'. Because of their large size, and the requirement for a pair of proteins recognising antiparallel DNA strands to induce a DSB, ZFNs and TALENs are less suited to multiplex gene editing. Several other uses of the CRISPR/Cas9 system, including transcriptional regulation [19,20] and imaging of genetic loci [21] have also been demonstrated. In this review, we discuss the structure and mechanism of Cas9, the specificity of RNA-guided Cas9-mediated genome editing and its application in plants.

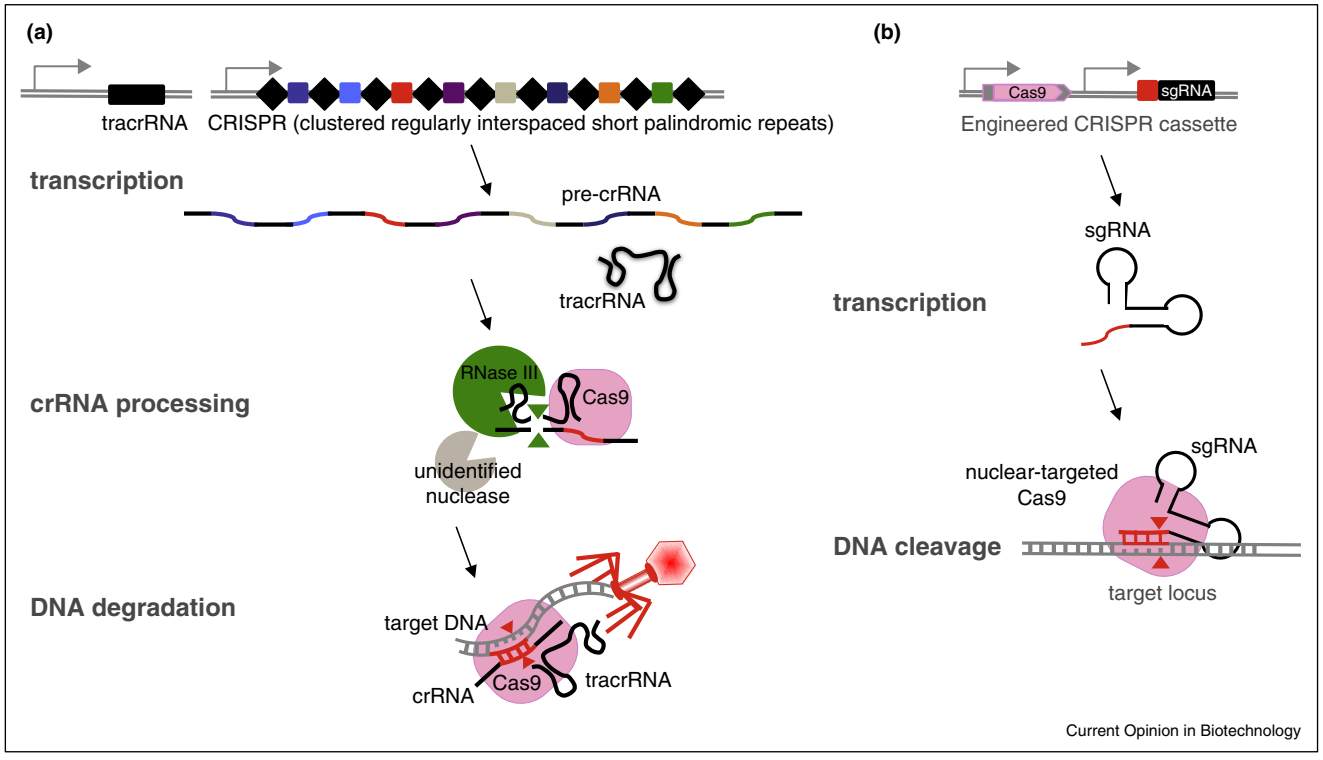
Cas9 nuclease: structure and mechanism of function

The crystal structures of Cas9 endonucleases of different sub-types revealed a conserved core and a bi-lobed architecture with adjacent active sites and two nucleic acid binding grooves (Figure 2) [22]. The two lobes include a large globular recognition (REC) lobe connected to a small nuclease (NUC) lobe. The REC lobe is a Cas9-specific functional domain and is composed of two domains, REC1 and REC2, and a long α-helical arginine-rich domain, referred to as the Bridge Helix. The NUC lobe accommodates two nuclease domains, RuvC and HNH, and a PAM-interacting domain (PI domain) (Figure 2a) [22–24]. Two nucleic acid binding grooves, a wide major groove and a narrow minor groove are located within the REC and NUC lobes, respectively [22,23]. Cas9 is a flexible protein that operates alone to bind and cleave the DNA target in a sequence-dependent manner [22,23,25,26•]. Single particle electron microscopy revealed that Cas9 is maintained in an auto-inhibited conformation in the absence of nucleic acid ligands and switches to an active form upon guide RNA loading (Figure 2b) [22]. This triggers conformational rearrangement of both REC and NUC lobes to form a central channel where the RNA-DNA heteroduplex will later be positioned [22]. The guide RNA interacts primarily with the REC lobe to form a binary Cas9-sgRNA complex [23]. Next, the complex interrogates the DNA double helix for canonical PAM motifs on the non-complementary DNA strand (Figure 2c) [22,25]. As Cas9 has no energy-dependent helicase activity, PAM recognition has been suggested to destabilize the adjacent sequence triggering R-loop formation [23,25]. The mechanism of PAM-dependent DNA recognition and unwinding was recently elucidated. Initial crosslinking experiments suggested that two unstructured tryptophan-containing flexible loops within the PI domain were involved in PAM recognition [22]. The crystal structure of Cas9 in a complex with a partially duplexed target DNA containing the canonical PAM motif and an sgRNA shed light on the molecular events underlying PAM recognition and DNA strand separation [26•]. The authors unraveled a major-groove base-recognition code for PAM binding where two arginine residues (R^{1333} and R^{1335}) read out the PAM GG dinucleotides on the non-complementary strand. Furthermore, interactions of two other minor-groove lysine and serine residues (K^{1107} and S^{1109}) with the PAM duplex create a K^{1107}-S^{1109} loop (a 'phosphate lock' loop) that connects with the phosphate group of the PAM sequence (position +1) in the complimentary strand (Figure 2d). This allows the phosphate group orient the complimentary DNA strand for base pairing and hybridization with the guide RNA, leading to separation of DNA strands (Figure 2e). The Cas9-sgRNA complex then probes the flanking DNA for potential guide RNA complementarity [25]. Base pairing of matching nucleotides at the seed region (8–12 bp) allows step-by-step destabilization of the target DNA and guide RNA-DNA heteroduplex formation (Figure 2f). The latter is buried within the NUC and REC lobe of Cas9 to form a four-way junction that mounts the arginine-rich Bridge Helix [26•]. As a result, both nuclease domains of the NUC lobe become ready for target cleavage. The mobile HNH domain approaches and cleaves the complementary strand in the tertiary complex, whereas the RuvC nicks the non-complementary strand (Figure 2f) [23]. How Cas9 dissociates from the sgRNA and recycles is still unknown [25]. The structural studies on Cas9 have provided an insight into how Cas9 may be engineered to create variants with novel PAM specificities.

CRISPR/Cas9 applications in plants

The CRISPR/Cas9 system has been successfully applied in model plants, including *Nicotiana benthamiana* [27–30],

Figure 1

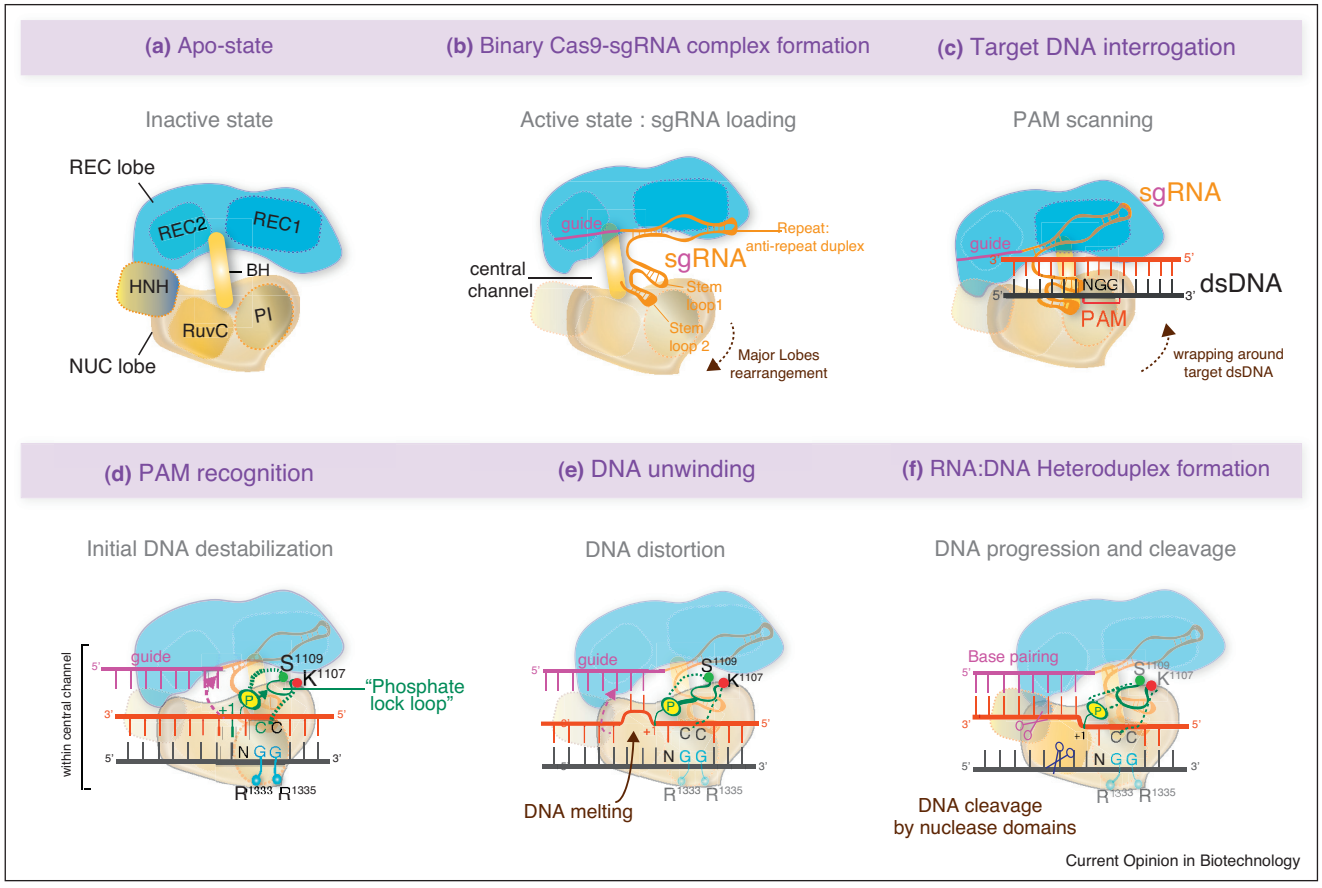


CRISPR/Cas9: from bacterial immunity to a powerful genome editing tool. **(a)** In bacterial type II CRISPR/Cas immune systems Cas9, RNaseIII and a trans-activating crRNA (tracrRNA) are required for maturation of the pre-crRNA, which is transcribed from the CRISPR region. The tracrRNA base-pairs with the repeat region of the pre-crRNA and is subsequently cleaved by RNaseIII. Cas9 is directed by the crRNA to previously recognised protospacer sequences in invading DNA and induces a double-strand break. **(b)** The re-purposed CRISPR/Cas9 system where the crRNA and tracrRNA are engineered into a single guide RNA (sgRNA) into which a target within the specific genome locus to be modified is encoded. A nuclear localisation signal is added to the Cas9 to enable import into the eukaryotic nuclear compartment.

Nicotiana tabacum [31] and Arabidopsis [27,29,32–34,35••,36••], and crops, such as wheat [37,38•,39], maize [40], rice [32,33,39,41–46], sorghum [29], tomato [47•,48] and sweet orange [49]. A detailed protocol of targeted mutagenesis in rice and wheat using the CRISPR/Cas9 system has also recently been published [50]. The process of creating a genetically edited plant carrying a mutation in the gene of interest using CRISPR/Cas9 is illustrated in Figure 3. Mutation frequencies are often described as the percentage of regenerated plants containing a CRISPR/Cas9 transgene, in which mutations can be detected at the locus, or loci, of interest. High efficiency (over 90%) has been reported in both Arabidopsis [35••] and rice [41]. If a mutagenesis event occurs early in plant regeneration, that is, before the first embryogenic cell divides, a diploid plant may be: first, heterozygous, if the locus on only one of the two sister chromatids was mutagenized, second, homozygous, if both alleles were mutagenized and the breaks were repaired with the same mutation, or third, biallelic, if both alleles were mutagenized but repair resulted in different alleles. In many cases, the mutation would occur later in development and independently in different tissues resulting in a chimeric plant consisting of cells with different genotypes, including wild type, heterozygous, homozygous or biallelic. CRISPR/Cas9-induced homozygous and biallelic mutations in first-generation transgenics have been reported in Arabidopsis [32,33], rice [44–46] and tomato [47•] allowing early gene-function studies. If homozygous or biallelic mutants are not generated as primary transformants, they must be progressed to the next generation for loss-of-function phenotype analysis. A number of studies have demonstrated Mendelian heritability of CRISPR/Cas9-induced mutations in Arabidopsis [34,35••,36••], rice [44,45] and tomato [47•].

CRISPR/Cas9 also allows multiplex gene editing by the simultaneous expression of two or more sgRNAs. This has been reported in Arabidopsis [27,33], rice [44] and tomato [47•]. Multiple sgRNAs have also been used to create chromosomal deletions, from tens to thousands of DNA base pairs in *N. benthamiana* [30], Arabidopsis [27,33], rice (up to 245 kb) [45] and tomato [47•]. Deleting chromosomal fragments allows deletions of entire clusters of genes [45].

Figure 2



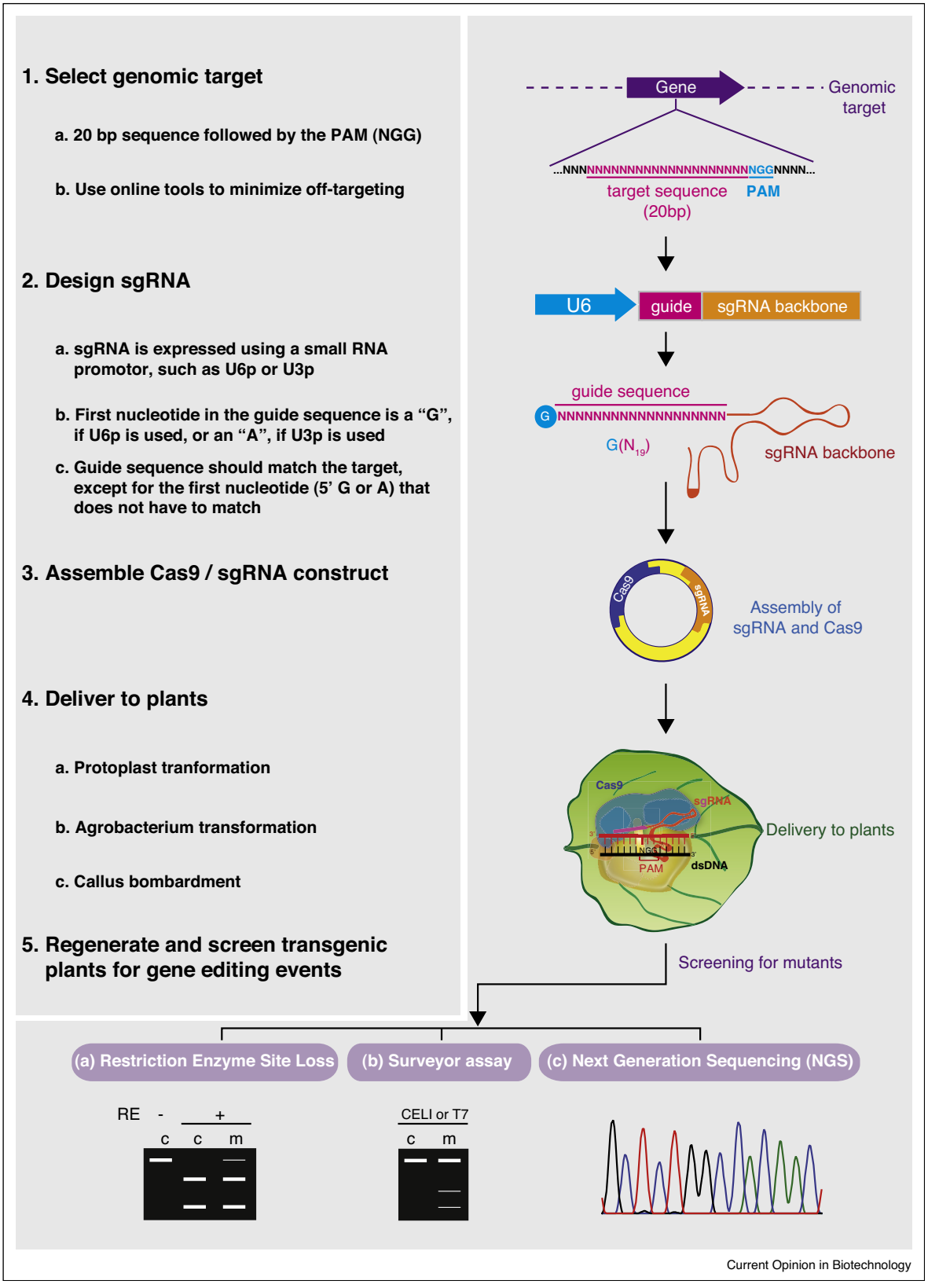
The mechanism of RNA guided DNA cleavage mediated by Cas9. The cartoon highlights important steps in double-stranded DNA cleavage by Cas9 when guided by an sgRNA. **(a)** Cas9 has a bi-lobed architecture and opts for an auto-inhibited conformation in the inactive state. The REC and NUC domains are shown in blue and yellow, respectively. **(b)** sgRNA loading activates Cas9 to create a binary Cas9-sgRNA complex. Major rearrangements of the two lobes leads to the formation of a central channel where the RNA-DNA heteroduplex will be positioned. **(c)** The NUC domain is reoriented. Cas9 wraps around the double stranded DNA and interrogates it for a PAM motif. **(d)** Upon recognition of a PAM by the PI domain, two major groove arginine residues (R^{1333} and R^{1335}) read out the GG dinucleotide of the PAM, while two other minor groove lysine and serine residues (K^{1107} and S^{1109}) interact with the PAM duplex on the complementary strand to create the phosphate lock loop and destabilize the DNA. **(e)** The phosphate group (+1 position) connects with the phosphate lock loop. This orients the DNA for base pairing with the seed region (8–12 bp) of an sgRNA triggering local DNA melting immediately upstream (1–2 bp) of the PAM. **(f)** The RNA-DNA heteroduplex is formed. The seed region pairs up with the complementary DNA allowing Cas9 to further separate the DNA in a stepwise manner. The HNH and RuvC nuclease domains cleave both complementary and non-complementary DNA strands, respectively, at +3 position away from the PAM. The sgRNA is coloured in orange with the guide sequence highlighted in pink. The complementary DNA strand is coloured in red and the non-complementary DNA stand in black. REC lobe, recognition lobe; NUC lobe, nuclease lobe; REC1, recognition domain 1; REC2, recognition domain2; BH, Bridge Helix; HNH, HNH nuclease domain; RuvC, RuvC nuclease domain; PAM, protospacer adjacent motif; PI, PAM-interacting domain; P, phosphate group; S, serine; K, lysine; R, arginine. The figure is modified from [22,26•].

Genome editing applications that utilise the cell's homology-directed repair (HDR) of CRISPR/Cas created DSBs are also of great interest to the plant research community. HDR offers the promise of 'knocking-in' DNA fragments, such as tags or new domains, as well as allele replacements and recoded genes. CRISPR/Cas9 mediated HDR has been demonstrated [27,32,35••,36••,39]. In most cases a reporter genes has been used, however Schiml *et al.* recently reported the successful insertion of the neomycin transferase (*nptII*) selectable marker gene into the endogenous *ALS* gene in Arabidopsis [7]. One of the challenges for HDR-mediated genome editing is simultaneous delivery of sufficient quantities of the SSN and the DNA repair template. The use of a nuclear-replicating DNA virus to produce multiple copies of the delivered repair template within the plant call has been proposed as a solution for this [51••].

Choosing the right CRISPR/Cas9 target: issues of specificity and off-targeting

Off-target activity is of considerable concern for the implementation of genome editing technologies, particularly for

Figure 3



The pipeline of generating a CRISPR/Cas9-mutagenised plant line. c, control; m, mutagenized; RE, restriction enzyme. CELI and T7 are DNA endonucleases used in the surveyor assay.

human gene therapy. In plants, off-target mutations are less problematic as, in most species, background mutations can be eliminated by back-crossing. The specificity of CRISPR/Cas9 is determined by the complement of the guide sequence of the sgRNA and the DNA target. A perfect match between the last 8–12 bases of the guide sequence, referred to as the 'seed sequence', and the equivalent region of the DNA target (i.e. the region proximal to the 5′ end of the PAM) is particularly important for efficient target cleavage by Cas9 [11•,17,43]. However, mismatches within the PAM-distal region of the target are generally tolerated [11•,17,43].

Reporter assays and deep sequencing studies in human cells revealed that Cas9-induced mutations of some potential off-target sites are below the threshold of the negative control (<0.01%) [52,53]. However, at off-target sites that have high identity with the intended target the mutation rate can be much higher (1.6–43%) [52]. Other studies have reported even higher off-target activity [54,55] but the discrepancy may be due to differences in cell types and sgRNA architecture. It is acknowledged that off-target activity seems to be locus-dependent and, until wider evidence is available, generalization of the reported evidence should be avoided [56].

Whole genome sequencing analysis in plants has uncovered almost negligible mutations at off-target sites [35••,44]. A study in Arabidopsis of highly homologous putative alternative target sites, followed by a PAM motif, showed no activity [35••]. In addition, plants, in which homozygous or biallelic mutations were identified did not acquire further mutations in subsequent generations [35••] indicating that once the target is modified, Cas9 can no longer bind to it.

A number of approaches have been suggested for the reduction of off-target activity. Lowering the level of Cas9 and/or sgRNA expression can reduce off-target activity, though this may also result in reduced efficacy at the intended target [54,56]. The use of truncated guide RNAs (17–18 bp), which are more sensitive to nucleotide mismatches, were also shown to reduce undesired off-target mutations [57]. Another approach has been suggested by the development of Cas9 mutants [58,59]. Cas9 specificity was increased 100–1500 fold by the use of a pair of Cas9 nickase variants (Cas9-D10A or Cas9-H840A), which were directed to targets on opposite DNA strands up to 100 bp apart. The pair of induced nicks, one on each strand, resulted in a DSB whereas potential off-target sites were unlikely to be sufficiently close to each other to induce more than individual nicks, which are generally perfectly repaired [58,59]. Off-target mutation rates have been reduced up to 5000-fold, by fusing a catalytically dead Cas9 (dCas9) to the FokI nuclease [60,61]. The resulting Cas9 variant, dCas9-FokI, was used to target DNA sites with two Cas9 targets spaced 15–25 bp and located on opposite DNA strands. Again, the requirement of two, correctly positioned 20 bp DNA targets is responsible for the increased specificity [60,61]. The ability to select unique targets in the genome eliminates the need for sgRNA/Cas9 modifications described above. To this end, a number of bioinformatics tools have been developed to scan genomes for unique targets, several being available online (Table 1).

As is the case with specificity, the efficiency of CRISPR/Cas9 may be influenced by the sequence, location and context of the target. Epigenetic factors, such as DNA methylation or histone modification are known to limit

Table 1

Available bioinformatic tools for selecting optimal CRISPR/Cas9 target sites and predicting off-targets

Site	Purpose	Reference
http://www.genome-engineering.org Directly at: http://crispr.mit.edu/	CRISPR/Cas9 design tool to find target sites within an input sequence. *A. thaliana* genome is available.	[56,58]
http://eendb.zfgenetics.org/casot	Open-sourced tool that is used locally, designed to identify potential off-target sites in any user-specified genome.	[66]
http://plants.ensembl.org/info/website/ftp/index.html	Download link to access 38 plant genomes.	
http://www.e-crisp.org/E-CRISP/designcrispr.html	Web-based tool to design sgRNA sequences for genome-library projects or individual sequences. Target site homology is also evaluated to predict off-targets. 5 plant genomes are available.	[67]
www.genome.arizona.edu/crispr	8 representative plant genomes are available to predict sgRNAs with low chance of off-target sites.	[68]
https://chopchop.rc.fas.harvard.edu/	Online tool for accurate target sequence selection and prediction of off-target binding of sgRNAs. Includes the design of target-specific primers for PCR genotyping. The only plant genome available is *A. thaliana*.	[69]
http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design	Online tool for designing highly active sgRNAs.	[64•]

some DNA-binding proteins. However, *in vitro* and *in vivo* assays have both demonstrated that Cas9 efficiently cleaves methylated DNA [56]. Chromatin immunoprecipitation (ChIP-seq) analysis has shown that sgRNA-guided Cas9 preferentially binds to open chromatin regions including off-target sites [62,63].

Interestingly, Hsu *et al.* discovered that the NAG sequence can serve as a non-canonical PAM, although in this case the efficiency of target recognition by Cas9 is reduced by 80 per cent [56]. Within the PAM motif, a cytosine as the variable nucleotide strongly enhances Cas9 specificity [64•]. In addition, optimal Cas9 activity is obtained when the PAM sequence does not have a guanine adjacent to its 3′ end (i.e. the PAM consensus sequence has now been extended to four nucleotides (CGGH)) [64•]. Recent reports also demonstrated that purine bases at positions 17 to 20 within the DNA target increase Cas9 efficiency, while pyrimidine bases decrease it [64•,65]. Whether these observations, made mostly in mammalian cells, will be relevant to targets in plants is not yet known.

Conclusions

The CRISPR/Cas9 system is the most recent addition to the toolbox of sequence-specific nucleases that includes ZFNs and TALENs. The simplicity and robustness of this system makes it as an attractive genome editing tool for plant biology. To date, the primary application has been the creations of gene knockouts. As multiple sgRNAs can be easily assembled into a single delivery vector, for example using Golden Gate cloning [30,47•], one can foresee application of the CRISPR/Cas9 for knocking-out whole gene families. Large chromosomal deletions (up to 245 kb) generated with CRISPR/Cas9 have also been reported [45] opening up opportunities for deleting gene clusters. Harnessing homologous recombination for gene-addition remains an important challenge for plant genome editing. In plants, the degree to which off-target mutations take place still needs to be systematically addressed. Nevertheless, a few published studies reported low to negligible off-target activity compared to animal systems. With the increased toolbox of Cas9 variants and publicly available bioinformatic tools to enable high-specificity (Table 1), this technology positions itself at the forefront of genome editing methods. Being an easy and affordable tool, CRISPR/Cas9 promises to revolutionise basic and applied plant research.

Acknowledgements

We thank The Gatsby Charitable Foundation, European Research Council (ERC) and Biotechnology and Biological Sciences Research Council (BBSRC) for funding. We are grateful to Kavod Quilate for inspiration.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Griggs D, Stafford-Smith M, Gaffney O, Rockstrom J, Ohman MC, Shyamsundar P, Steffen W, Glaser G, Kanie N, Noble I: **Policy: sustainable development goals for people and planet**. *Nature* 2013, **495**:305-307.
2. Voytas DF: **Plant genome engineering with sequence-specific nucleases**. *Annu Rev Plant Biol* 2013, **64**:327-350.
3. Brenner DA, Smigocki AC, Camerini-Otero RD: **Double-strand gap repair results in homologous recombination in mouse L cells**. *Proc Natl Acad Sci U S A* 1986, **83**:1762-1766.
4. Salomon S, Puchta H: **Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells**. *EMBO J* 1998, **17**:6086-6095.
5. Paszkowski J, Baur M, Bogucki A, Potrykus I: **Gene targeting in plants**. *EMBO J* 1988, **7**:4021-4026.
6. Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X *et al.*: **Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases**. *Nature* 2009, **459**:437-441.
7. Schiml S, Fauser F, Puchta H: **The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny**. *Plant J* 2014 http://dx.doi.org/10.1111/tpj.12704.
8. Knoll A, Fauser F, Puchta H: **DNA recombination in somatic plant cells: mechanisms and evolutionary consequences**. *Chromosome Res* 2014, **22**:191-201.
9. Podevin N, Davies HV, Hartung F, Nogue F, Casacuberta JM: **Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding**. *Trends Biotechnol* 2013, **31**:375-383.
10. Voytas DF, Gao C: **Precision genome engineering and agriculture: opportunities and regulatory challenges**. *PLoS Biol* 2014, **12**:e1001877.
11. • Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**:816-821.

In this report Jinek *et al.* demonstrated for the first time that the Cas9 nuclease can be programmed to cleave a DNA sequence of interest (*in vitro*) by engineering the guide RNA. The authors foresaw the use of CRISPR/Cas9 as a tool for genome engineering.

12. Jansen R, Embden JD, Gaastra W, Schouls LM: **Identification of genes that are associated with DNA repeats in prokaryotes**. *Mol Microbiol* 2002, **43**:1565-1575.
13. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A: **Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product**. *J Bacteriol* 1987, **169**:5429-5433.
14. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P: **CRISPR provides acquired resistance against viruses in prokaryotes**. *Science* 2007, **315**:1709-1712.
15. Bhaya D, Davison M, Barrangou R: **CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation**. *Annu Rev Genet* 2011, **45**:273-297.
16. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E: **CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III**. *Nature* 2011, **471**:602-607.
17. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA *et al.*: **Multiplex genome engineering using CRISPR/Cas systems**. *Science* 2013, **339**:819-823.
18. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM: **RNA-guided human genome engineering via Cas9**. *Science* 2013, **339**:823-826.

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

20. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK: **CRISPR RNA-guided activation of endogenous human genes**. *Nat Methods* 2013, **10**:977-979.

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

22. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S *et al.*: **Structures of Cas9 endonucleases reveal RNA-mediated conformational activation**. *Science* 2014, **343**:1247997.

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

24. Hsu PD, Lander ES, Zhang F: **Development and applications of CRISPR-Cas9 for genome engineering**. *Cell* 2014, **157**:1262-1278.

25. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA: **DNA interrogation by the CRISPR RNA-guided endonuclease Cas9**. *Nature* 2014, **507**:62-67.

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

A crystal structure of Cas9 in a complex with an sgRNA and a double-stranded target DNA carrying the canonical PAM motif was reported. The report provided an insight into how Cas9 can be engineered to create variants with novel PAM specificities.

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

28. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S: **Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease**. *Nat Biotechnol* 2013, **31**:691-693.

29. Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP: **Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice**. *Nucleic Acids Res* 2013, **41**:e188.

30. Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V: **Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system**. *Plant Methods* 2013, **9**:39.

31. Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, Wu Y, Zhao P, Xia Q: **CRISPR/Cas9-mediated targeted mutagenesis in *Nicotiana tabacum***. *Plant Mol Biol* 2014 http://dx.doi.org/10.1007/s11103-014-0263-0.

32. Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P, Cao F, Zhu S, Zhang F, Mao Y *et al.*: **Efficient genome editing in plants using a CRISPR/Cas system**. *Cell Res* 2013, **23**:1229-1232.

33. Mao Y, Zhang H, Xu N, Zhang B, Gao F, Zhu JK: **Application of the CRISPR-Cas system for efficient genome engineering in plants**. *Mol Plant* 2013, **6**:2008-2011.

34. Jiang W, Yang B, Weeks DP: **Efficient CRISPR/Cas9-mediated gene editing in *Arabidopsis thaliana* and inheritance of modified genes in the T2 and T3 generations**. *PLOS ONE* 2014, **9**:e99225.

35. •• Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL, Wang Z, Zhang Z, Zheng R, Yang L *et al.*: **Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis**. *Proc Natl Acad Sci U S A* 2014, **111**:4632-4637.

This is the first report where CRISPR/Cas9-induced genome modifications were shown to be stably inherited in Mendelian fashion in Arabidopsis. Inheritance for genome modifications introduced via both NHEJ and HR DNA repair pathways was demonstrated.

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

The authors demonstrated stable inheritance of CRISPR/Cas9-induced mutations by subsequent generations of Arabidopsis transgenic plants. In addition, they tested Cas9 nuclease versus Cas9 nickase for introducing genome modifications via NHEJ and HR pathways. The nickase was reported a great tool for HR-based genome engineering as it had a very low mutagenic activity.

37. Upadhyay SK, Kumar J, Alok A, Tuli R: **RNA-guided genome editing for target gene mutations in wheat**. *G3 (Bethesda)* 2013, **3**:2233-2238.

38. • Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu JL: **Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew**. *Nat Biotechnol* 2014, **32**:947-951.

The authors demonstrated how genome engineering can be efficiently applied for purposes of generating gene knock-outs to a polyploid crop, such as wheat. All six alleles of the *MLO* gene were knocked out and, as a result, a variety resistant to the powdery mildew fungus was produced. Although the targeted mutagenesis was performed using TALENs, CRISPR/Cas9 was also shown to generate mutations in stable transgenic lines.

39. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL *et al.*: **Targeted genome modification of crop plants using a CRISPR-Cas system**. *Nat Biotechnol* 2013, **31**:686-688.

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

41. Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X, Wan J, Gu H, Qu LJ: **Targeted mutagenesis in rice using CRISPR-Cas system**. *Cell Res* 2013, **23**:1233-1236.

42. Xie K, Yang Y: **RNA-guided genome editing in plants using a CRISPR-Cas system**. *Mol Plant* 2013, **6**:1975-1983.

43. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA: **RNA-guided editing of bacterial genomes using CRISPR-Cas systems**. *Nat Biotechnol* 2013, **31**:233-239.

44. Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N *et al.*: **The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation**. *Plant Biotechnol J* 2014, **12**:797-807.

45. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B: **Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice**. *Nucleic Acids Res* 2014, **42**:10903-10914.

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

47. • Brooks C, Nekrasov V, Lippman Z, Van Eck J: **Efficient gene editing in tomato in the first generation using the CRISPR/Cas9 system**. *Plant Physiol* 2014, **166**:1292-1297.

The CRISPR/Cas9 system was reported to be an efficient tool for targeted mutagenesis in tomato. CRISPR/Cas9-induced mutations were shown to be stably inherited. The mutagenesis was performed using the double-sgRNA targeting strategy that allows easy detection using the PCR band shift assay. CRISPR/Cas9 components were expressed from plasmids assembled using a highly flexible modular vector system.

48. Ron M, Kajala K, Pauluzzi G, Wang D, Reynoso MA, Zumstein K, Garcha J, Winte S, Masson H, Inagaki S *et al.*: **Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model**. *Plant Physiol* 2014, **166**:455-469.

49. Jia H, Wang N: **Targeted genome editing of sweet orange using Cas9/sgRNA**. *PLOS ONE* 2014, **9**:e93806.

50. Shan Q, Wang Y, Li J, Gao C: **Genome editing in rice and wheat using the CRISPR/Cas system**. *Nat Protoc* 2014, **9**:2395-2410.

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

In this manuscript the authors successfully used a nuclear-replicating DNA virus (geminivirus) to deliver sequence-specific nucleases (ZFNs,

TALENs or CRISPR/Cas9) together with a DNA repair template to plant cells. This strategy enabled them to achieve highly efficient plant genome engineering.

52. Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS: **Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases**. *Genome Res* 2014, **24**:132-141.

53. Cho SW, Kim S, Kim JM, Kim JS: **Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease**. *Nat Biotechnol* 2013, **31**:230-232.

54. Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR: **High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity**. *Nat Biotechnol* 2013, **31**:839-843.

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

56. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O *et al.*: **DNA targeting specificity of RNA-guided Cas9 nucleases**. *Nat Biotechnol* 2013, **31**:827-832.

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

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

59. Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, Hodgkins A, Iyer V, Huang X *et al.*: **Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects**. *Nat Methods* 2014, **11**:399-402.

60. Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, Goodwin MJ, Aryee MJ, Joung JK: **Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing**. *Nat Biotechnol* 2014, **32**:569-576.

61. Guilinger JP, Thompson DB, Liu DR: **Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification**. *Nat Biotechnol* 2014, **32**:577-582.

62. Wu X, Scott DA, Kriz AJ, Chiu AC, Hsu PD, Dadon DB, Cheng AW, Trevino AE, Konermann S, Chen S *et al.*: **Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells**. *Nat Biotechnol* 2014, **32**:670-676.

63. Kuscu C, Arslan S, Singh R, Thorpe J, Adli M: **Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease**. *Nat Biotechnol* 2014, **32**:677-683.

64. • Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE: **Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation**. *Nat Biotechnol* 2014 http://dx.doi.org/10.1038/nbt.3026.

The authors used an empirical approach to evaluate Cas9 targets within a number of endogenous loci in mouse and human cells in terms of efficiency of mutagenesis. A number of target sequence features and PAM motif features improving Cas9 activity were discovered. The report features an online tool for designing highly active sgRNAs.

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

66. Xiao A, Cheng Z, Kong L, Zhu Z, Lin S, Gao G, Zhang B: **CasOT: a genome-wide Cas9/gRNA off-target searching tool**. *Bioinformatics* 2014, **30**:1180-1182.

67. Heigwer F, Kerr G, Boutros M: **E-CRISP: fast CRISPR target site identification**. *Nat Methods* 2014, **11**:122-123.

68. Xie K, Zhang J, Yang Y: **Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops**. *Mol Plant* 2014, **7**:923-926.

69. Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E: **CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing**. *Nucleic Acids Res* 2014, **42**:W401-W407.