donor (**Fig. 2f**). Sanger sequencing further verified the anticipated creation of the *Avr*II site in the target sequence without additional modifications and indicated an HDR-mediated gene replacement at a frequency of 9.0% (**Fig. 2g**). In addition, we detected NHEJ-mediated targeted mutagenesis at the *NbPDS* locus with a frequency of 14.2% (**Supplementary Fig. 4**). As mesophyll protoplasts are isolated from differentiated leaves without active cell division, we tested the possibility of enhancing HDR by triggering ectopic cell division. Co-expression of *Arabidopsis CYCD3* (*CYCLIN D-TYPE 3*), a master activator of the cell cycle, hardly promoted the HDR in *N. benthamiana* protoplasts (**Fig. 2f**). Exploration of HDR in *Arabidopsis* protoplasts was unsuccessful, presumably owing to intrinsically low efficiency of HDR in *Arabidopsis*[18].

To facilitate genome-wide application of the sgRNA:pcoCas9 technology in *Arabidopsis*, we generated, using bioinformatics, a database containing a total of 1,466,718 unique sgRNA target sequences in *Arabidopsis* exons (**Supplementary Database**), which cover >99% (26,942 out of 27,206) of the nuclear protein-encoding genes defined by TAIR10 (The *Arabidopsis* Information Resource 10, http://arabidopsis.org/portals/genAnnotation/gene_structural_annotation/annotation_data.jsp/). Targeting efficacy and specificity of selected sgRNA target candidates from this database need to be experimentally determined each time during future implementation. We also introduced a facile method to manually design a shared sgRNA target site specific for multiple homologous target genes by aligning their coding sequences and carrying out a BLAST search to evaluate off-target possibilities (**Supplementary Fig. 3**). The sgRNA:pcoCas9 technology enables an easy reprogramming of DNA targeting specificity by changing the 20-nt guide sequence in the sgRNA without modifying the pcoCas9 protein. We have established a simple and rapid procedure to create a custom sgRNA through overlapping PCR (**Supplementary Fig. 5** and **Supplementary Table 1**). Thus, it is feasible to use single or tandemly expressed sgRNAs (**Fig. 2c**) to simultaneously target multigene families, which is not easily done with ZFNs and TALENs.

We have tested a total of seven target sequences in five target genes in *Arabidopsis* or *N. benthamiana*, and obtained targeted mutagenesis in all cases. The variation in mutagenesis efficiency among different genes in *Arabidopsis* may stem from distinct sgRNA binding strength to individual target sequences or distinct chromatin structure and epigenetic state at individual target loci, which requires future investigation. We have demonstrated that plant protoplasts provide a useful system to rapidly evaluate the efficiency of the sgRNA:pcoCas9-mediated genome editing at a specific genomic locus. Our data also suggest that targeting an *Arabidopsis* gene with multiple sgRNAs could improve the success rate of targeted mutagenesis and generate deletions to ensure gene knockout. Notably, sgRNA:pcoCas9 achieved high efficiency of HDR-mediated gene replacement in *N. benthamiana* protoplasts. The simplicity and versatility of the sgRNA:pcoCas9 technology demonstrated in this work promise marker gene–independent and antibiotic selection–free genome engineering with high precision in diverse plant species to advance basic science and biotech.

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AUTHOR CONTRIBUTIONS

J.-F.L. and J.S. designed experiments; J.-F.L. and D.Z. performed experiments; J.A., J.E.N., M.M. and G.M.C. conducted bioinformatics analyses; J.B. supplied plant materials; J.-F.L. and J.S. wrote the manuscript.

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Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease

To the Editor:

Sustainable intensification of crop production is essential to ensure food demand is matched by supply as the human population continues to increase[1]. This will require high-yielding crop varieties that can be grown sustainably with fewer inputs on less land. Both plant breeding and genetic modification (GM) methods make valuable contributions to varietal improvement, but targeted genome engineering promises to be critical to elevating future yields. Most such methods require targeting DNA breaks to defined locations followed by either nonhomologous end joining (NHEJ) or homologous recombination[2]. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) can be engineered to create such breaks, but these systems require two different DNA binding proteins flanking a sequence of interest, each with a C-terminal FokI nuclease module. We report here that the bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) system, comprising a CRISPR-associated (Cas)9 protein and an engineered single guide RNA (sgRNA) that specifies a targeted nucleic acid sequence[3], is applicable to plants to induce mutations at defined loci.

To test the potential of the Cas9 system to induce gene knockouts in plants, we took advantage of *Agrobacterium tumefaciens*–mediated transient expression assays (agroinfiltration) to co-express a Cas9 variant

with a eukaryotic nuclear localization signal and an sgRNA in the model plant *Nicotiana benthamiana*[4]. First, we constructed a green fluorescent protein (GFP)-tagged version of Cas9 using a previously described clone[5]. We expressed GFP-Cas9 in *N. benthamiana* leaf tissue using standard agroinfiltration protocols[6] and observed a clear nuclear localization (**Supplementary Fig. 1**) consistent with the nuclear localization previously observed in human cells[7]. We then generated an sgRNA with the guide sequence matching a 20-bp region within the phytoene desaturase (*PDS*) gene in *Nicotiana benthamiana* (**Fig. 1a**). The sgRNA was placed under an *Arabidopsis U6* promoter (**Supplementary Fig. 2**). Both GFP-Cas9 and sgRNA were co-expressed in *N. benthamiana* leaf tissue using *A. tumefaciens* as a vector. The tissue was harvested 2 days later and DNA was extracted. To easily detect sgRNA-guided, Cas9-induced mutations at the *PDS* locus, we used the restriction enzyme site loss method[2]; as the target sequence within the *PDS* gene overlaps with an MlyI restriction site, we digested the genomic DNA with MlyI and then performed a polymerase chain reaction (PCR) with primers flanking the target site (**Supplementary Table 1**). By doing so, we greatly reduced unaltered wild-type DNA in the sample and enriched for DNA molecules carrying mutations that remove the MlyI site.

The presence of both Cas9 and the sgRNA resulted in increased levels of the PCR product (**Fig. 1b**, lane 1) compared with negative control treatments (**Fig. 1b**, lanes 2 and 3). Nondigested *N. benthamiana* genomic DNA was used as a positive control (**Fig. 1b**, lane 4). The assay was robust and reproducible because we detected MlyI-resistant amplicons in three additional independent experiments using different plants (**Supplementary Figs. 3** and **4**). The PCR products from **Figure 1b**, lanes 1 and 4 were cloned into a high-copy vector and individual clones sequenced. Sequence analysis of 20 clones derived from the PCR product in lane 1 revealed the presence of indels in 17 of them. The indels can be grouped into nine different types ranging from 1- to 9-bp deletions to 1-bp insertions (**Fig. 1c** and **Supplementary Fig. 5**). All recovered indels abolish the MlyI restriction site within the target region. With regard to 1-bp indels, we cannot totally rule out the possibility that these mutations were introduced by the DNA polymerase during the PCR amplification step. Sequences of the eight clones derived from the control PCR product shown in lane 4 were all wild type.

To estimate the efficiency of targeted mutagenesis, we amplified nondigested genomic DNA from negative controls and *N. benthamiana* leaves expressing both Cas9 and sgRNA, digested the amplicons with MlyI and subjected them to gel electrophoresis. We then measured the intensity of the uncut band relative to the intensity of all detectable bands in a gel lane as described previously[8] (**Fig. 2a** and **Supplementary Fig. 6**). We estimated the mutation rate to be in the range of 1.8% to 2.4% (2.1% average) based on four independent experiments.

We also examined whether plants could be regenerated from cells modified using the Cas9 system. *N. benthamiana* leaf sections expressing Cas9 and the sgRNA were excised and placed on selective medium to regenerate plantlets (**Supplementary Methods**). DNA extracted from leaf tissue of the regenerated plants was used to detect sgRNA-guided, Cas9-induced mutations with the restriction enzyme site loss method described above. Increases of MlyI-resistant PCR product were observed in 2 out of 30 plants regenerated from the sgRNA:Cas9-expressing tissue but not in the negative control treatments (**Fig. 2b**). To determine which mutations are present in the *PDS* locus of transgenic plants 2 and 3, we cloned DNA fragments from PCR products amplified using MlyI-digested genomic DNA (**Fig. 2b**). In the case of plant 2, only one type of mutation was found, whereas sequencing reads from plant 3 revealed four different mutations (**Supplementary Fig. 7**). Both plants appear to carry the wild-type *PDS* locus given that the PCR products amplified using the nondigested genomic DNA were partially cut by MlyI (**Fig. 2b**). Therefore, plant 3 is clearly mosaic with multiple mutations in addition to the wild-

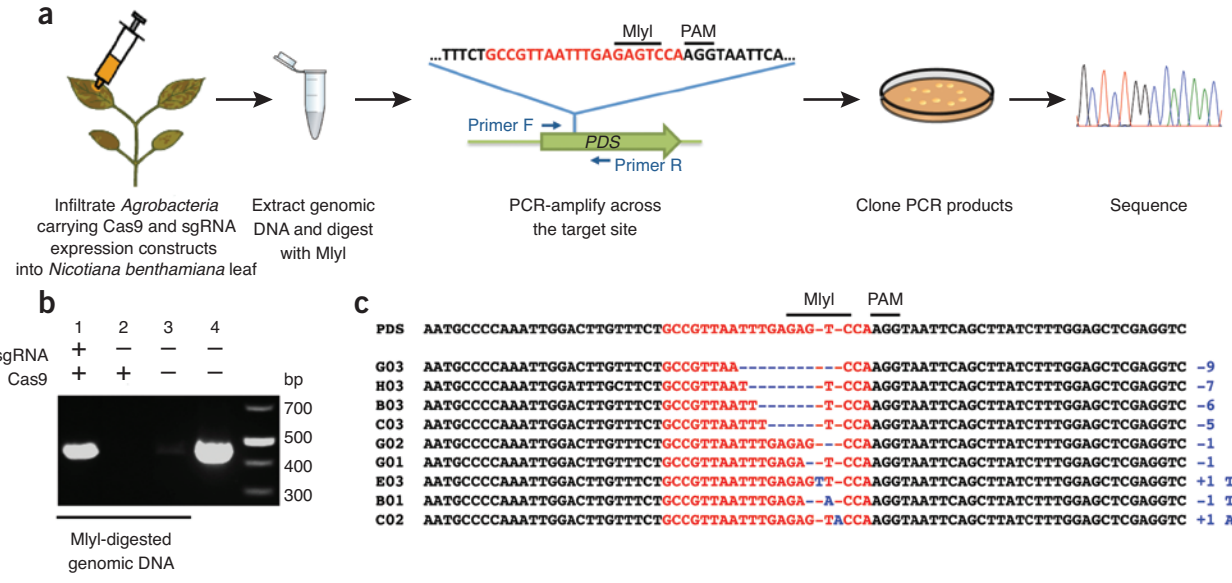

Figure 1 Targeted mutagenesis *in planta* using the Cas9 RNA-guided endonuclease. (**a**) Assay scheme. (**b**) DNA gel with PCR bands obtained upon amplification using primers flanking the target site within the *PDS* gene of *N. benthamiana*. In lanes 1–3 the template genomic DNA was digested with MlyI, whereas in lane 4 nondigested genomic DNA was used. (**c**) Alignment of reads with Cas9-induced indels in *PDS* obtained from lane 1 of **b**. The wild-type sequence is shown at the top. The sequence targeted by the synthetic sgRNA is shown in red whereas the mutations are shown in blue. PAM, the protospacer-adjacent motif, was selected to follow the consensus sequence NGG. The changes in length and sequence are shown to the right. Three additional replicates of this experiment are presented in **Supplementary Figure 3.**

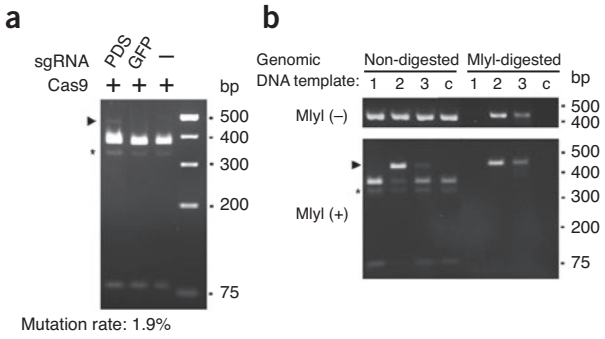

Figure 2 Measurement of the mutation rate induced by Cas9 RNA-guided endonuclease and transgenic *N. benthamiana* plants carrying mutations in the *PDS* gene. (**a**) The *PDS* locus was amplified using nondigested genomic DNA from leaf tissue expressing Cas9 and sgRNA targeting *PDS* as well as from negative controls (Cas9 plus an sgRNA targeting GFP, and Cas9 on its own). To measure the mutation rate, we divided the intensity of the uncut band by the intensity of all bands in the lane. Three additional replicates of this experiment are presented in **Supplementary Figure 6.** (**b**) *N. benthamiana* plants were transformed using *A. tumefaciens* carrying Cas9 and an sgRNA targeting the *PDS* locus as described in the **Supplementary Methods**. The two plantlets carrying mutations in the *PDS* gene were analyzed alongside negative controls. The *PDS* locus was amplified using either nondigested or MlyI-digested genomic DNA as a template. The resulting amplicons were then digested with MlyI. Plant 1 does not carry mutations in the *PDS* locus, whereas plants 2 and 3 do. Lane 'c' corresponds to nontransformed *N. benthamiana*. The arrowhead indicates the MlyI-resistant band; the asterisk indicates the band resulting from star (nonspecific DNA cleavage) activity of MlyI.

type sequence, whereas plant 2 could be either mosaic or heterozygous. Overall, these results suggest that Cas9 and the sgRNA are not toxic and that the induced mutations can be transferred to whole plants.

Given that the target sequence is 20 bp, the sgRNA:Cas9 system may not be as specific as TALEN-induced mutagenesis, which can be tailored to target longer sequences[9]. We identified a total of 98 potential off-target sequences by searching the *N. benthamiana* genome database against the 20-bp target sequence within the *PDS* locus using the BLASTN tool (**Supplementary Table 2**). We managed to assay 18 of the identified off-target sites using the restriction enzyme site loss method described above (**Supplementary Methods**). These sites have 14- to 17-bp out of 20-bp identity to the targeted *PDS* sequence. None of 18 amplicons showed evidence of sgRNA-guided, Cas9-induced MlyI restriction site loss as observed with the *PDS* target sequence (**Supplementary Table 3** and **Supplementary Fig. 8**). We therefore did not detect any Cas9 activity with the subset of off-target sequences tested. Nevertheless, more comprehensive analyses of off-target activity are required to address this issue further, especially considering recent findings[9].

These data clearly indicate that Cas9 and an engineered sgRNA can direct DNA breaks at defined chromosomal locations in plants. The rapid and robust transient assay we have developed will enable plant-specific optimization of the Cas9 system. Relative to other methods of plant genome engineering, the CRISPR-Cas9 system has the potential to simplify the process of plant genome engineering and editing because only a short fragment in the sgRNA needs to be designed to target a new locus. This creates a valuable new tool for plant biologists and breeders, and it hastens the prospects of achieving routine targeted genome engineering for basic and applied science.

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AUTHOR CONTRIBUTIONS

V.N. performed the experiments. V.N. and J.D.G.J. designed the constructs. V.N., J.D.G.J. and S.K. wrote the manuscript. V.N., B.S., D.W., J.D.G.J. and S.K. contributed to the design of the study.

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