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DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins

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Editing plant genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to genetically modified plants. We transfected preassembled complexes of purified Cas9 protein and guide RNA into plant protoplasts of *Arabidopsis thaliana*, tobacco, lettuce and rice and achieved targeted mutagenesis in regenerated plants at frequencies of up to 46%. The targeted sites contained germline-transmissible small insertions or deletions that are indistinguishable from naturally occurring genetic variation.

Programmable nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided endonucleases (RGENs), have been used for genome editing in multiple cells and species including plants[1–3], paving the way for novel applications in biomedical research, medicine and biotechnology[4]. CRISPR RGENs are rapidly superseding ZFNs and TALENs owing to their ease of use; RGENs that consist of the Cas9 protein derived from *Streptococcus pyogenes* and guide RNAs (gRNAs) can be customized by replacing only the RNA component, sidestepping the labor-intensive and time-consuming protein engineering needed to customize TALENs and ZFNs. Programmable nucleases, delivered into plant cells either by using *Agrobacterium tumefaciens* or by transfecting plasmids that encode them, cleave chromosomal target sites in a sequence-dependent manner, producing site-specific DNA double-strand breaks (DSBs). The repair of these DSBs by endogenous systems results in targeted genome modifications.

It remains unclear whether genome-edited plants will be regulated by genetically modified organism (GMO) legislation in the EU and other regions[5]. Programmable nucleases induce small insertions and deletions (indels) or substitutions at chromosomal target sites that are indistinguishable from naturally occurring genetic variation. However, mutated plants might be considered GMOs by regulatory authorities in certain countries, which could reduce the potentially widespread use of programmable nucleases in plant biotechnology and agriculture. For example, when *A. tumefaciens* is used as a delivery vector, the resulting genome-edited plants contain foreign DNA sequences, including those that encode the programmable nucleases, in the host genome. Removal of these *A. tumefaciens*–derived DNA sequences by breeding is not feasible in species such as grape, potato and banana that reproduce asexually.

Non-integrating plasmids could be transfected into plant cells to deliver programmable nucleases. However, transfected plasmids are degraded in cells by endogenous nucleases, and the resulting small DNA fragments are sometimes inserted at both on-target and off-target sites in host cells[6]; therefore, this approach might be unsuitable in plants if regulatory approval is required.

Delivery of preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs), rather than plasmids that encode these components, into plant cells could remove the likelihood of inserting recombinant DNA in the host genome[7]. Furthermore, as has been shown in cultured human cells[6], RGEN RNPs cleave chromosomal target sites immediately after transfection and are degraded rapidly by endogenous proteases in cells, which might reduce the frequency of mosaicism and off-target effects in regenerated whole plants. Because there is no need to optimize codon usage or find promoters that will express Cas9 and gRNAs when using protein-and-RNA-only systems, the use of preassembled RGEN RNPs could broaden the applicability of genome editing to all transformable plant species. In addition, using RGEN RNPs enables *in vitro* prescreening to guide the choice of highly active gRNAs[8] and genotyping of mutant clones via restriction fragment length polymorphism (RFLP) analysis[9]. To the best of our knowledge, RGEN RNPs have not been used in any plant species.

Here we report the delivery of RGEN RNPs into protoplasts of various plant species and the induction of targeted genome modifications in whole plants regenerated from them. Purified Cas9 protein was mixed with a two- to tenfold molar excess of gRNAs targeting four genes from three plant species *in vitro* to form preassembled RNPs. The RGEN RNPs were incubated with protoplasts derived from *A. thaliana*, tobacco (*Nicotiana attenuata*) and rice (*Oryza sativa*) in the presence of polyethylene glycol (PEG). We used both the T7 endonuclease I (T7E1)(ref. 10) assay and targeted deep sequencing to measure mutation frequencies in transfected cells (**Fig. 1a**,**b**). Indels were detected at the expected positions, that is, 3 nucleotides (nt) upstream of an NGG protospacer–adjacent motif (PAM), with frequencies that ranged from 8.4% to 44% (**Fig. 1a**).

We also co-transfected two gRNAs whose target sites were separated by 201 base pairs (bp) in the *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) gene in *Arabidopsis* to investigate whether the repair of two concurrent DSBs would result in targeted deletion of the intervening sequence, as has been seen in human cells[11]. Sanger sequencing showed that a 223-bp DNA sequence was deleted in protoplasts (**Fig. 1c**).

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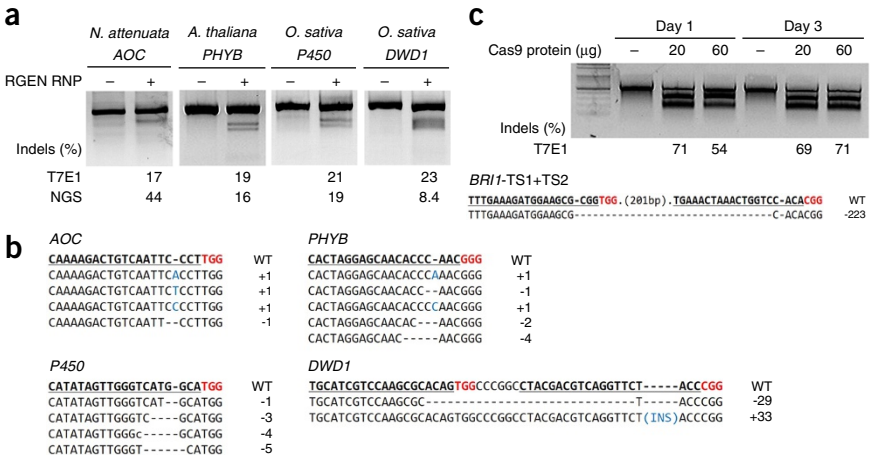

Figure 1 RGEN RNP-mediated gene disruption in plant protoplasts *of Nicotiana attenuata, Arabidopsis thaliana* and *Oryza sativa.* (**a**) Mutation frequencies measured by the T7E1 assay and targeted deep sequencing. "–" indicates controls without RGEN RNP. (**b**) Mutant DNA sequences induced by RGEN RNPs in plant cells. The PAM sequences are shown in red and inserted nucleotides in blue. WT, wild-type. (**c**) A time-course analysis of genome editing of the *BRI1* gene in *A. thaliana* protoplasts. Top, the T7E1 assay ("–" indicates controls without Cas9 protein). Bottom, DNA sequences of the wild-type (WT) and mutant sequences.

Notably, RGEN-induced mutations were detected 24 h after transfection, suggesting that RGENs cut target sites immediately after transfection and induce mutation before a full cycle of cell division is completed.

Next, we investigated whether RGEN RNPs induce off-target mutations at sites highly homologous to on-target sites. We searched for potential off-target sites of the *PHYTOCHROME B* (*PHYB*) and *BRI1* gene–specific sgRNAs in the *Arabidopsis* genome using the Cas-OFFinder program[12] and used targeted deep sequencing to measure mutation frequencies (**Supplementary Fig. 1**). Indels were not detected at any sites that differed from on-target sites by 2–5 nt, in line with previous findings in human cells[13,14].

Finally, we transfected an RGEN RNP to disrupt the lettuce (*Lactuca sativa*) homolog of the *A. thaliana BRASSINOSTEROID INSENSITIVE 2* (*BIN2*) gene (**Supplementary Fig. 2**), which encodes a negative regulator in a brassinosteroid (BR) signaling pathway[15], into lettuce protoplasts, and obtained microcalli regenerated from the RNP-transfected cells (**Fig. 2** and **Supplementary Fig. 3**). We used the same RGEN RNP in an RFLP analysis to genotype the lettuce microcalli. Unlike the T7E1 assay, this analysis distinguishes monoallelic mutant clones (50% cleavage) from heterozygous biallelic mutant clones (no cleavage) and homozygous biallelic mutant clones (no cleavage) from wild-type clones (100% cleavage)[9]. Furthermore, the RGEN-RFLP assay is not limited by sequence polymorphisms near the nuclease target site that may exist in the lettuce genome. This assay showed that 2 of 35 (5.7%) calli contained monoallelic mutations and 14 of 35 (40%) calli contained biallelic mutations at the target site (**Fig. 2b**), demonstrating that RGEN-induced mutations were maintained after regeneration. The overall mutation frequency in lettuce calli was 46%. We used targeted deep sequencing to confirm genotypes in the 16 mutant calli. The number of base pairs deleted or inserted at the target

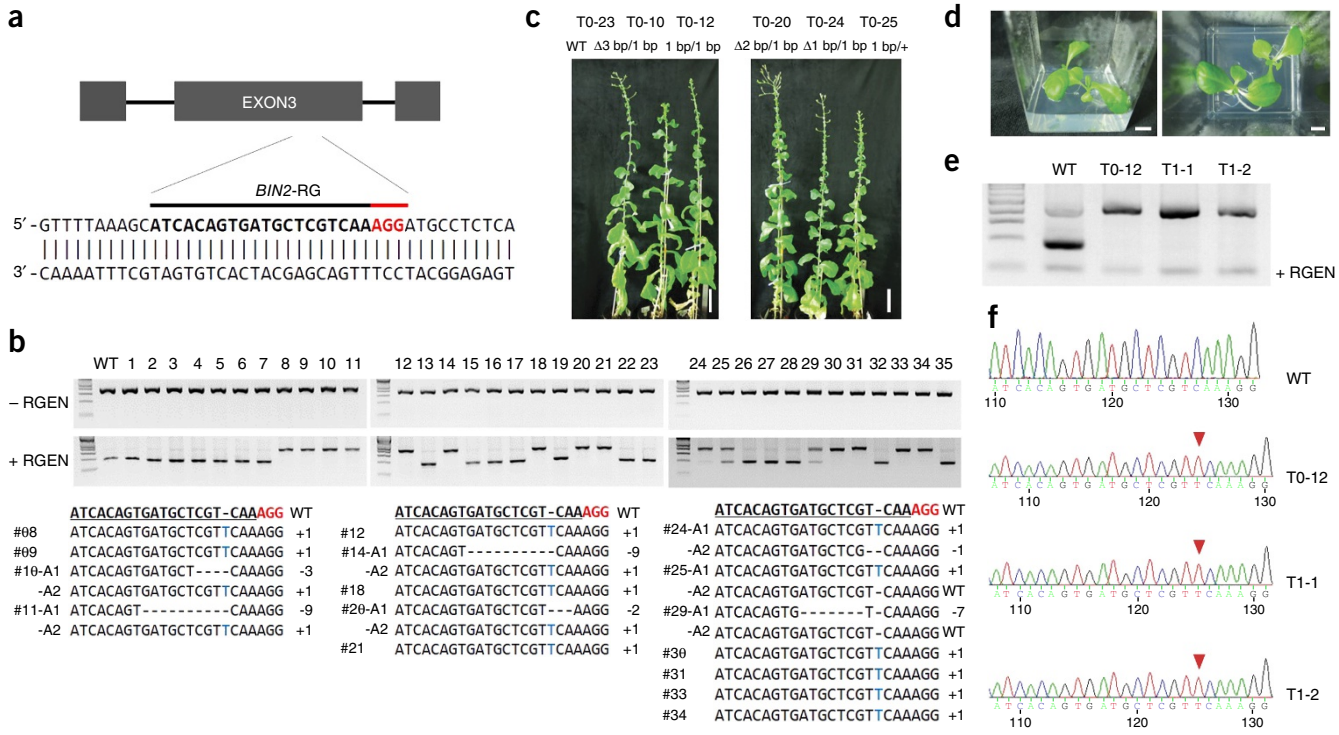

Figure 2 Targeted gene knockout in lettuce using an RGEN RNP. (**a**) The target sequence in the *BIN2* gene. The PAM sequence is shown in red. (**b**) Genotyping of microcalli. Top, RGEN-RFLP analysis. Bottom, mutant DNA sequences in microcalli. (**c**) Whole plants regenerated from RGEN RNP-transfected protoplasts. Scale bars, 10 cm. (**d**) T1 plantlets obtained from a homozygous biallelic mutant termed T0-12. Scale bars, 1 cm. (**e**) RGEN-RFLP analysis for genotyping T1 plantlets. (**f**) DNA sequences of the wild type, the T0-12 mutant, and T1 mutants derived from the T0-12 line. Red triangles indicate an inserted nucleotide.

site ranged from −9 to +1, consistent with mutagenic patterns observed in human cells[14]. No apparent mosaicism was detected in these clones (**Supplementary Fig. 4**), suggesting that the RGEN RNP cleaved the target site immediately after transfection and induced indels before cell division was completed.

We next evaluated whether the *BIN2*-specific RGEN induced off target mutations in the lettuce genome using high-throughput sequencing. No off-target mutations were detected at 91 homologous sites that differed by 1–5 nucleotides from the on-target site in three *BIN2*-mutated plantlets (**Supplementary Tables 1** and **2**), consistent with our findings in human cells that off-target mutations induced by CRISPR RGENs are rarely found in a single cell-derived clone[16].

Subsequently, whole plants were successfully regenerated from these genome-edited calli and grown in soil (**Fig. 2c** and **Supplementary Fig. 5**). Seeds were obtained and germinated from a fully grown homozygous biallelic mutant. As expected, the mutant allele was transmitted to the next generation (**Fig. 2d–f**). Further studies are needed to test whether the *BIN2*-disrupted lettuce has the predicted phenotype of increased BR signaling.

In conclusion, we have shown that RGEN RNPs can be used to induce targeted genome modifications in six genes in four different plant species. RGEN-induced mutations were stably maintained in whole plants that were regenerated from the protoplasts and were transmitted to the germline. Because no recombinant DNA is used in this process, the resulting genome-edited plants might be exempt from current GMO regulations[17], paving the way for the widespread use of RNA-guided genome editing in plant biotechnology and agriculture.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. NCBI Sequence Read Archive: SRX1240083.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.-S.K. and S.C. supervised the research. J.W.W., S.I.K. and C.C. carried out plant regeneration. J.K., S.W.C. H.K., S.-G.K. and S.-T.K. performed mutation analysis.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the online version of the paper.

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ONLINE METHODS

Cas9 protein and guide RNAs. Cas9 protein tagged with a nuclear localization signal was purchased from ToolGen, Inc. (South Korea). Templates for guide RNA transcription were generated by oligo-extension using Phusion polymerase (**Supplementary Table 3**). Guide RNAs were *in vitro* transcribed through runoff reactions using the T7 RNA polymerase (New England BioLabs) according to the manufacturer's protocol. The reaction mixture was treated with DNase I (New England BioLabs) in 1× DNase I reaction buffer. Transcribed sgRNAs were resolved on an 8% denaturing urea-polyacrylamide gel with SYBR gold staining (Invitrogen) for quality control. Transcribed sgRNAs were purified with MG PCR Product Purification SV (Macrogen) and quantified by spectrometry.

Protoplast culture. Protoplasts were isolated as previously described from *Arabidopsis*[18], rice[19] and lettuce[20]. Initially, *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia-0, rice (*Oryza sativa* L.) cv. Dongjin, and lettuce (*Lactuca sativa* L.) cv Cheongchima seeds were sterilized in a 70% ethanol, 0.4% hypochlorite solution for 15 min, washed three times in distilled water, and sown on 0.5× Murashige and Skoog solid medium supplemented with 2% sucrose. The seedlings were grown under a 16 h light (150 μmol m^{-2} s^{-1}) and 8 h dark cycle at 25 °C in a growth room. For protoplast isolation, the leaves of 14 d *Arabidopsis* seedlings, the stem and sheath of 14 d rice seedlings, and the cotyledons of 7 d lettuce seedlings were digested with enzyme solution (1.0% cellulase R10, 0.5% macerozyme R10, 0.45 M mannitol, 20 mM MES [pH 5.7], CPW solution[21]) during incubation with shaking (40 r.p.m.) for 12 h at 25 °C in darkness and then diluted with an equal volume of W5 solution[22]. The mixture was filtered before protoplasts were collected by centrifugation at 100*g* in a round-bottomed tube for 5 min. Re-suspended protoplasts were purified by floating on a CPW 21S (21% [w/v] sucrose in CPW solution, pH 5.8) followed by centrifugation at 80*g* for 7 min. The purified protoplasts were washed with W5 solution and pelleted by centrifugation at 70*g* for 5 min. Finally, protoplasts were re-suspended in W5 solution and counted under the microscope using a hemocytometer. Protoplasts were diluted to a density of 1×10^6 protoplasts/ml of MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES [pH 5.7]). In the case of tobacco protoplasts, 3-week-old *Nicotiana attenuata* leaves grown in B5 media were digested with enzymes (1% cellulose R10, 0.25% macerozyme R10, 0.5 M Mannitol, 8 mM $CaCl_2$, 5 mM MES [pH 5.7], 0.1% BSA) for 5 h at 25 °C in darkness. Subsequently, protoplasts were washed with an equal volume of W5 solution twice. To obtain intact protoplasts, *N. attenuata* protoplasts in W5 solution were applied to an equal volume of 21% sucrose gradient followed by swing-out centrifugation at 50*g* for 5 min. The intact protoplasts were re-suspended in W5 solution and stabilized at least for 1 h at 4 °C before PEG-mediated transfection.

Protoplast transfection. PEG-mediated RNP transfections were performed as previously described[18]. Briefly, to introduce DSBs using an RNP complex, 1×10^5 protoplast cells were transfected with Cas9 protein (10–60 μg) premixed with *in vitro*–transcribed sgRNA (20–120 μg). Prior to transfection, Cas9 protein in storage buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerol) was mixed with sgRNA in 1× NEB buffer 3 and incubated for 10 min at room temperature. A mixture of 1×10^5 protoplasts (or 5×10^5 protoplasts in the case of lettuce) re-suspended in 200 μl MMG solution was gently mixed with 5–20 μl of RNP complex and 210 μl of freshly prepared PEG solution (40% [w/v] PEG 4000; Sigma No. 95904, 0.2 M mannitol and 0.1 M $CaCl_2$), and then incubated at 25 °C for 10 min in darkness. After incubation, 950 μL W5 solution (2 mM MES [pH 5.7], 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl) was added slowly. The resulting solution was mixed well by inverting the tube. Protoplasts were pelleted by centrifugation at 100*g* for 3 min and re-suspended gently in 1 ml WI solution (0.5 M mannitol, 20 mM KCl and 4 mM MES (pH 5.7)). Finally, the protoplasts were transferred into multi-well plates and cultured under dark conditions at 25 °C for 24–48 h. Cells were analyzed one day after transfection.

Protoplast regeneration. RNP-transfected protoplasts were re-suspended in 0.5× B5 culture medium[23] supplemented with 375 mg/l $CaCl_2 \cdot 2H_2O$,18.35 mg/l NaFe-EDTA, 270 mg/l sodium succinate, 103 g/l sucrose, 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3 mg/l 6-benzylaminopurine (BAP) and 0.1 g/l MES. The protoplasts were mixed with a 1:1 solution of 0.5× B5 medium and 2.4% agarose to a culture density of 2.5×10^5 protoplasts/ml. The protoplasts embedded in agarose were plated onto 6-well plates, overlaid with 2 ml of liquid 0.5× B5 culture medium, and cultured at 25 °C in darkness. After 7 days, the liquid medium was replaced with fresh culture medium. The cultures were transferred to the light (16 h light [30 μmol m^{-2} s^{-1}] and 8 h darkness) and cultured at 25 °C. After 3 weeks of culture, micro-calli grown to a few millimeters in diameter were transferred to MS regeneration medium supplemented with 30 g/l sucrose, 0.6% plant agar, 0.1 mg/l α-naphthalaneacetic acid (NAA), 0.5 mg/l BAP. Induction of multiple lettuce shoots was observed after about 4 weeks on regeneration medium.

Rooting, transfer to soil and hardening of lettuce. To regenerate whole plants, proliferated and elongated adventitious shoots were transferred to a fresh regeneration medium and incubated for 4–6 weeks at 25 °C in the light (16 h light [150 μmol m^{-2} s^{-1}] and 8 h darkness). For root induction, approximately 3–5-cm-long plantlets were excised and transferred onto a solid hormone-free 0.5× MS medium in Magenta vessels. Plantlets developed from adventitious shoots were subjected to acclimation, transplanted to potting soil, and maintained in a growth chamber at 25 °C (under cool-white fluorescent lamps with a 16-h photoperiod).

T7E1 assay. Genomic DNA was isolated from protoplasts or calli using DNeasy Plant Mini Kit (Qiagen). The target DNA region was amplified and subjected to the T7E1 assay as described previously[10]. In brief, PCR products were denatured at 95 °C and cooled down to a room temperature slowly using a thermal cycler. Annealed PCR products were incubated with T7 endonuclease I (ToolGen, Inc.) at 37 °C for 20 min and analyzed via agarose gel electrophoresis.

RGEN-RFLP. The RGEN-RFLP assay was performed as previously described[9]. Briefly, PCR products (300–400 ng) were incubated in 1× NEB buffer 3 for 60 min at 37 °C with Cas9 protein (1 μg) and sgRNA (750 ng) in a reaction volume of 10 μl. RNase A (4 μg) was then added to the reaction mixture and incubated at 37 °C for 30 min to remove the sgRNA. The reaction was stopped by adding 6× stop solution (30% glycerol, 1.2% SDS, 250 mM EDTA). DNA products were electrophoresed using a 2.5% agarose gel.

Targeted deep sequencing. The on-target and potential off-target sites were amplified from genomic DNA. Indices and sequencing adaptors were added by additional PCR. High-throughput sequencing was performed using Illumina MiSeq (v2, 300-cycle).

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