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A Split *Staphylococcus aureus* Cas9 as a Compact Genome-Editing Tool in Plants

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Split-protein methods—where a protein is split into two inactive fragments that must re-assemble to form an active protein—can be used to regulate the activity of a given protein and reduce the size of gene transcription units. Here, we show that a *Staphylococcus aureus Cas9* (*SaCas9*) can be split, and that *split-SaCas9* expressed from *Agrobacterium* can induce targeted mutagenesis in *Nicotiana benthamiana*. Since *SaCas9* is smaller than the more commonly used *Cas9* derived from *Streptococcus pyogenes*, the split-SaCas9 provides the smallest tool yet for clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) plant genome editing. Both sets of split-SaCas9 (_430N/431C and _739N/740C) exhibited genome-editing activity, and the activity of split-SaCas9_739N/740C was almost the same as that of full-length SaCas9. This result indicates that split-SaCas9_739N/740C is suitable for use in targeted mutagenesis. We also show that the *split-SaCas9* fragment expressed from *Tomato mosaic virus* could induce targeted mutagenesis together with another fragment expressed from *Agrobacterium*, suggesting that a split-SaCas9 system using a plant virus vector is a promising tool for integration-free plant genome editing. Split-SaCas9 has the potential to regulate CRISPR/Cas9-mediated genome editing activity in plant cells both temporally and spatially.

Keywords: CRISPR/Cas9 • Split-SaCas9 • *Staphylococcus aureus* • *Tomato mosaic virus*.

Abbreviations: CAPS, cleaved amplified polymorphic sequence; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; sgRNA, single guide RNA; PAM, protospacer adjacent motif; *PDS*, *PHYTOENE DESATURASE*; Sa, *Staphylococcus aureus*; Sp, *Streptococcus pyogenes*; ToMV, *Tomato mosaic virus*.

Introduction

Since the recent discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system in bacteria, it has been widely used to edit both animal and plant genomes (Bhaya et al. 2011, Carroll 2014, Doudna and Charpentier 2014, Baltes and Voytas 2015, Belhaj et al. 2015, Bortesi and Fischer 2015, Jiang and Marraffini 2015, Osakabe and Osakabe 2015, Schaeffer and Nakata 2015). CRISPR/Cas9 is an adaptive immune system in bacteria, and each species has a similar but slightly different set of components. Cas9 from *Streptococcus pyogenes* (SpCas9) was the first RNA-guided DNA-cleaving enzyme to be used routinely in genome editing. Recently, it was reported that *Staphylococcus aureus* Cas9 (SaCas9) also has genome-editing activity in human (Ran et al. 2015), *Arabidopsis thaliana* (Steinert et al. 2015), *Nicotiana tabacum* and *Oryza sativa* (Kaya et al. 2016) cells. The protein size of SaCas9 (1,053 amino acids) is smaller than that of SpCas9 (1,386 amino acids). This property lowers the size of the gene cassette required to express *SaCas9* and the single guide RNA (sgRNA) and, given the packaging limit of the adeno-associated virus vector, SaCas9 has proved useful for therapeutic applications (Ran et al. 2015).

In plants, virus-mediated transient expression of Cas9 and *sgRNA* provides an integration-free genome editing method. To this end, *sgRNA* was expressed from plant virus vectors and successfully introduced by targeted mutagenesis with the aid of the *SpCas9* gene overexpressed from a stably integrated T-DNA (Ali et al. 2015, Yin et al. 2015). However, expression of Cas9 from a plant virus vector has not been successful thus far, possibly because of the size of SpCas9. The length of the foreign gene insert is known to correlate negatively with the stability of the plant virus vector (Avesani et al. 2007). Thus, expression of *Cas9* from a plant virus vector is one of the technical hurdles to be overcome in developing an integration-free genome-editing method.

Using a split-protein is an effective approach to regulate the activity of an enzyme or reduce the size of a gene transcription unit. In this method, a protein is split into two inactive fragments but assembles to form an active protein with or without the help of dimerization domains. Many split-proteins have been reported to date, and several are in general use, e.g. yellow fluorescent protein (Hu et al. 2002), ubiquitin protease (Johnsson and Varshavsky 1994) and β-galactosidase (Ullmann et al. 1967).

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SpCas9 comprises a nuclease lobe and an α-helical lobe (Nishimasu et al. 2014). In the primary structure, the nuclease lobe is interrupted by the α-helical lobe. Wright et al. (2015) split SpCas9 into two lobes, keeping the structure of the nuclease lobe as native as possible by linking the N- and C-terminal nuclease lobe with a three amino acid linker (GSS). The two lobes interact via the sgRNA, and the complex can induce targeted mutagenesis in human cells. Zetsche et al. (2015) showed that SpCas9 can be split into two fragments (N- and C-terminal pieces) in a different way. The two fragments were fused to FK506-binding protein (FKBP) and the FKBP12–rapamycin-binding (FRB) domain, respectively. Rapamycin promotes reassembly of split-SpCas9 *via* FKBP and FRB, and the reassembled split-SpCas9 can induce targeted mutagenesis in human cells. Nguyen et al. (2016) reported a chemically controlled split-SpCas9 in which the two fragments are fused to ligand-binding domains of nuclear receptors, and assembly is ligand dependent. Truong et al. (2015) developed an intein-mediated split-SpCas9 system in human cells, while Nihongaki et al. (2015) also developed a photoactivatable Cas9 using split-SpCas9. As in SpCas9, structural analysis of SaCas9 has revealed several flexible regions that could serve as potential split sites, and two type of split-SaCas9 (430N/431C and 739N/740C) have been shown to exhibit genome editing activity in human cells (Nishimasu et al. 2015).

Here, we show that split-SaCas9, with the two parts expressed transiently from *Agrobacterium* and the plant virus vector, respectively, is able to induce targeted mutagenesis in plant cells. We also propose that the spatiotemporal control of *split-SaCas9* gene expression will deliver a highly regulatable platform for targeted mutagenesis in plant cells.

Results and Discussion

Transient expression of split-SaCas9 in *N. benthamiana* leaves via *Agrobacterium* infiltration

Applying the transient expression system in *N. benthamiana* leaves via *Agrobacterium* infiltration, we examined the activity of two sets of split-SaCas9 (split-SaCas9_430N/431C and split-SaCas9_739N/740C) in plant cells (**Fig. 1A**). A 3×FLAG tag and 3×nuclear localization signal (NLS) were fused to each fragment (430N, 431C, 739N and 740C) of split-SaCas9 at their N-terminus for their detection and delivery into the nucleus, respectively (**Fig. 1B**). Expression of *split-SaCas9* fragments was controlled by the *Cauliflower mosaic virus* 35S promoter (35S-pro) (**Fig. 1B**). The *sgRNA*, including the target sequence and scaffold sequence for SaCas9, was expressed under the control of the *U6-26* promoter (Li et al. 2007) derived from *A. thaliana* (**Fig. 1B**). We used *sgPDS1* and *sgPDS2* corresponding to the endogenous *PHYTOENE DESATURASE* (*PDS*) gene of *N. benthamiana* (**Fig. 1C**). A mixture of four *Agrobacterium* cultures harboring *split-SaCas9_430N* (or *_739N*), *split-SaCas9_431C* (or *_740C*), *sgPDS1* and *sgPDS2* was infiltrated into an *N. benthamiana* leaf to allow co-expression in the same cells (**Fig. 1D**). At 7 d after infiltration, leaves were harvested and protein extracts from each infiltrated leaf were subjected to Western blot analysis. The FLAG-tagged proteins of the split-SaCas9_430N and _431C, or the split-SaCas9_739N and _740C, were detected at their predicted size (**Fig. 1E**), indicating that each set of split-SaCas9 proteins (430N/431C or 739N/740C) was expressed correctly in the infiltrated leaves.

Split-SaCas9 induces targeted mutagenesis in *N. benthamiana*

To test whether transiently expressed full-length *SaCas9* can induce mutation at targeted loci, we infiltrated a mixture of three *Agrobacterium* cultures, harboring full-length *SaCas9*, *sgPDS1* and *sgPDS2*, respectively, into *N. benthamiana* leaves. At 7 d after infiltration, genomic DNA was extracted from infiltrated leaves and subjected to cleaved amplified polymorphic sequence (CAPS) analysis. Undigested bands at the *Bst*NI or *Pst*I restriction site near the protospacer adjacent motif (PAM) were detected in *sgPDS1* and *sgPDS2* targeted loci, respectively (**Fig. 2A**), indicating that transiently expressed *SaCas9* with two *sgRNA* genes had induced targeted mutagenesis in *N. benthamiana*. Next, we infiltrated a mixture of four Agrobacterium harboring *split-SaCas9_430N* (or*_739N*), *split-SaCas9_431C* (or*_740C*), *sgPDS1* and *sgPDS2* into *N. benthamiana* leaves, and again examined genome editing activity by CAPS analysis. In each leaf transiently expressing *split-SaCas9_430N/431C* or *split-SaCas9_739N/740C*, undigested bands representing the mutagenized allele were detected in both *sgPDS1* and *sgPDS2* targeted loci. These data demonstrate that, just like full-length SaCas9, the split-SaCas9_430N/431C and _739N/740C each form an enzyme complex with activity to induce a mutation in the target sequence. We estimated the efficiency of targeted mutagenesis by counting the number of cloned PCR products with and without a mutation in the *sgPDS1* targeted locus. The mutation efficiency induced by split-SaCas9_430N/431C (5.4%, 10.8%) is a little lower than that induced by split-SaCas9_739N/740C (15.2%, 14.7%) in *N. benthamiana* (**Fig. 2B**). A similar result was obtained in human cells: the mutation efficiency of split-SaCas9_430N/431C and split-SaCas9_739N/740C is 10% and 17%, respectively (Nishimasu et al. 2015), suggesting that the reassembly activity of split-SaCas9 430N/431C in vivo is less efficient than that of split-SaCas9_739N/740C. The efficiency of mutagenesis induced by split-SaCas9_739N/740C was similar to that of the full-length SaCas9 in our *N. benthamiana* transient expression system. We also made transgenic *N. tabacum* plants expressing *split-SaCas9_739N/740C* and confirmed that they had expected mutations in the *sgPDS1* targeted locus (Supplementary Fig. S1).

Split-SaCas9 transiently expressed separately from ToMV and *Agrobacterium* can induce mutation in the *N. benthamiana* genome

We examined whether full-length SaCas9 or the split-SaCas9 expressed from a plant virus vector can induce targeted mutagenesis. The coat protein gene of a *Tomato mosaic virus* (ToMV) infectious cDNA clone was substituted with full-length *SaCas9* or each of the four *split-SaCas9* fragments (**Fig. 3A**). We speculated that it is very difficult to express

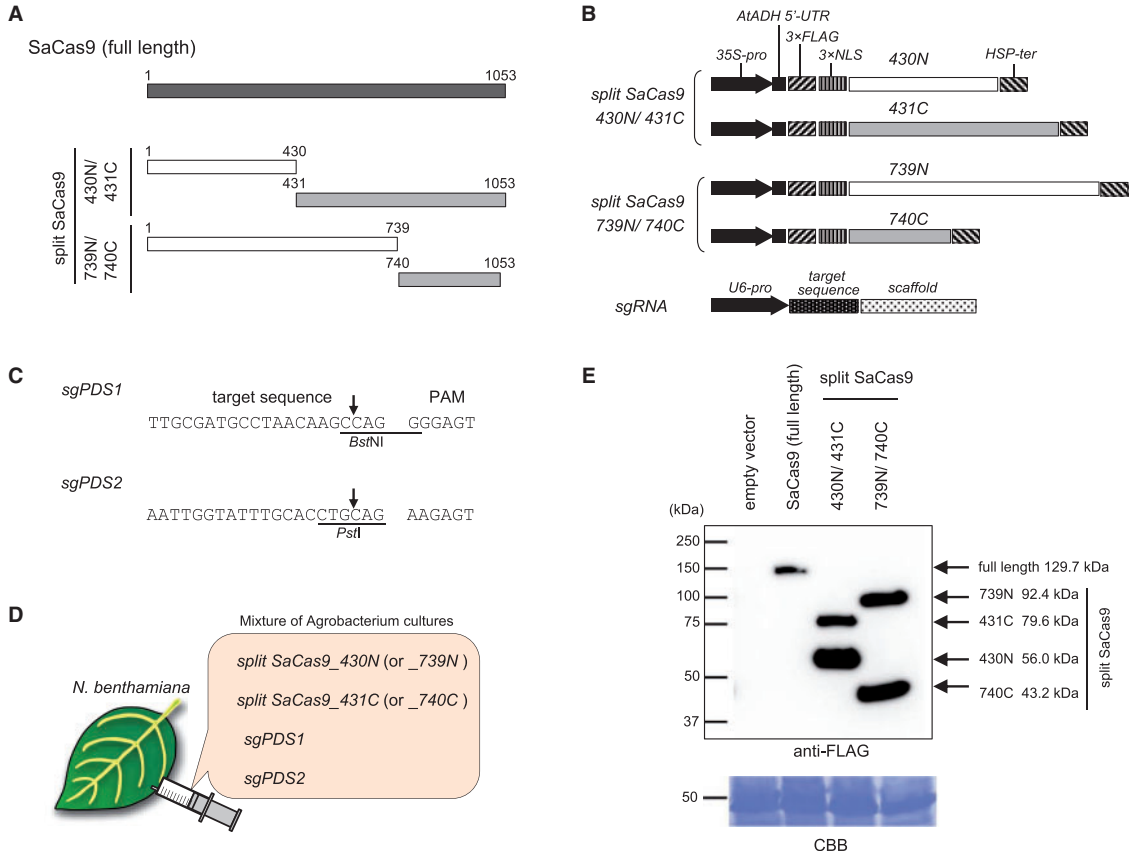

Fig. 1 Transient expression of split-SaCas9 in *N. benthamiana*. (A) Primary structures of SaCas9 protein and two sets of split-SaCas9 proteins. (B) Gene structures for expression of *split-SaCas9* and *sgRNA*. *AtADH 5′-UTR*: 5′-untranslated region of the *A. thaliana ALCOHOL DEHYDROGENASE* gene. *HSP-ter*: the terminator region of *A. thaliana HEAT SHOCK PROTEIN 18.2* gene. (C) Target sequences (21 nucleotides) of *sgPDS1* and *sgPDS2* for the *N. benthamiana PDS* gene. PAM: protospacer adjacent motif (5′-NNGRRT-3′) for SaCas9. Underlines indicate restriction enzyme sites used for CAPS analysis. Arrows indicate the SaCas9 cleavage site. (D) Experimental scheme of *Agrobacterium*-mediated genome editing. Mixtures of *Agrobacterium* cultures were infiltrated into an *N. benthamiana* leaf with a syringe without a needle. (E) Protein expression of SaCas9 (full length) or split-SaCas9 in infiltrated leaves was detected by Western blotting with anti-FLAG antibodies (upper panel). The membrane with transferred protein was stained with Coomassie Brilliant Blue (CBB) as a loading control (lower panel).

both fragments of the split-SaCas9 in the same cell using ToMV vector, because viral cross-protection, where plant viruses cannot infect tissue that has already been infected with the same or related virus, is known to occur in plants (Sherwood and Fulton 1982). Then one fragment of split-SaCas9 was expressed from ToMV and another fragment was expressed from *Agrobacterium*. An *in vitro* transcribed RNA from these clones was inoculated onto *N. benthamiana* leaves. At 3–4 d after virus inoculation, *Agrobacterium* expressing *sgPDS1* was infiltrated into the same leaves (**Fig. 3B**). At the same time, *Agrobacterium* expressing the counterpart fragment of the *split-SaCas9* was also infiltrated. Genomic DNA was extracted from the leaves for CAPS analysis at 7 d after *Agrobacterium* infiltration. *Agrobacterium* expressing full-length *SaCas9* was used as a control (**Fig. 3C**, lane 2). In a leaf inoculated with ToMV expressing full-length *SaCas9*, no undigested bands were detected by CAPS analysis (**Fig. 3C**, lane 3) and no SaCas9 protein band was detected by Western blotting (Supplementary Fig. S2, lane 2). Thus, expression of full-length *SaCas9* from ToMV is not an effective strategy to induce targeted mutagenesis in *N. benthamiana*. As often reported when using plant viral vectors (Scholthof et al. 1996), the full-length *SaCas9* gene might be too large to be maintained in the ToMV genome during propagation. In contrast, when the *split-SaCas9_739N* was expressed from ToMV and the *split-SaCas9_740C* was expressed from *Agrobacterium*, undigested DNA bands were observed (**Fig. 3C**, lane 7). The same result was obtained in the leaf expressing the *split-SaCas9_740C* from ToMV and the *split-SaCas9_739N* from *Agrobacterium* (**Fig. 3C**, lane 6). On the other hand, when SaCas9 split into two fragments at position 430/431 (*split-SaCas9_430N* or *_431C*) was expressed from ToMV, undigested DNA bands were hardly detectable in CAPS analysis (**Fig. 3C**, lanes 4 and 5). The lower genome editing efficiency of *split-SaCas9_430N/431C* compared with that of *split-SaCas9_739N/740C* (**Fig. 2B**) might be due to insufficient expression of *SaCas9_430N* or *_431C* from ToMV vector (Supplementary Fig. S2) in addition to their low reassembly efficiency. Whatever the underlying reason for the lack of success with full-length SaCas9 and *split-SaCas9_430N/431C* when expressed by ToMV, our results show that *split-SaCas9_739N/740C* is suitable for virus-mediated expression in the *split-SaCas9* approach.

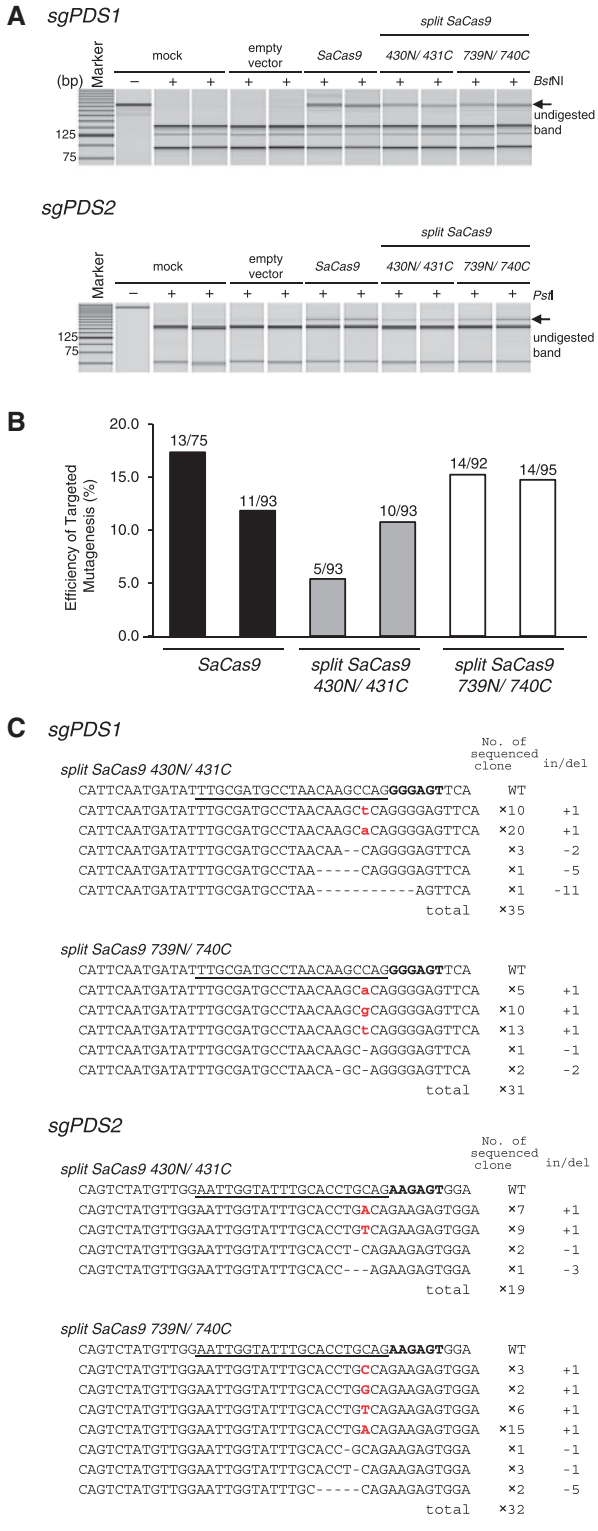

Fig. 2 A split-SaCas9 transiently expressed by Agroinfiltration-induced targeted mutagenesis in *N. benthamiana*. (A) CAPS analysis of two loci in the *PDS* gene in leaves transiently expressing *split-SaCas9*. –, Non-digested PCR products; +, *Bst*NI- (*sgPDS1*) or *Pst*I (*sgPDS2*)-digested PCR products. Agroinfiltration, DNA extraction, PCRs and restriction enzyme digestion were performed independently in duplicate. Mock, infiltration buffer; empty vector, *pRI201-AN*; marker, a 25 bp size marker. Size of *Bst*NI-digested bands: 151 and 96 bp in wild type (*sgPDS1*). Size of *Pst*I-digested bands: 184, 177, 53 and 4 bp in wild-type (*sgPDS2*). The size of the undigested band expected upon changes to nucleotide sequences at the target sequence is around 247 bp (*sgPDS1*) and 237 (184 + 53) bp (*sgPDS2*). (B) Mutation frequency (%) in the *sgPDS1* locus. Fractions above the histogram bars indicate the number of cloned DNAs with mutations (numerator) and the number of total analyzed cloned DNAs from PCR products (denominator). The results of two independent experiments are shown. (C) Mutations detected in *sgPDS1* and *sgPDS2* loci. The wild-type sequence is shown at the top with the target sequence underlined and the PAM (NNGRRT) in bold. DNA deletions and insertions are shown as dashes and lower case red letters. The net change in length and the number of clones are noted to the right of each sequence (+, insertion; −, deletion; x, number of clones). The total number of clones means the number of clones with mutation(s) in the target sequence.

Possible applications in spatiotemporal regulation of genome editing using split-SaCas9

Our findings demonstrate that split-SaCas9 can be used for targeted mutagenesis in plants, and suggest that the split-SaCas9 system using a plant virus vector is a promising tool for integration-free plant genome editing. In addition, we propose that split-SaCas9 could be useful for the temporal and spatial regulation of CRISPR/Cas9-mediated genome editing in plants. In plants constitutively and ectopically expressing full-length *Cas9*, Cas9 is active in all tissues and during all developmental stages. On the other hand, the *split-SaCas9* system has the advantage of controlling the activity of SaCas9. One example is a mosaic analysis using the split-SaCas9. When plants stably or transiently expressing the *split-SaCas9* fragment are inoculated with ToMV or *Agrobacterium* expressing the partner *split-SaCas9* fragment in leaf, root or other tissues, targeted mutagenesis will be induced in that tissue only. If the target gene of interest is critical for development or fertilization, using the split-SaCas9 may be especially effective. Another is limiting the time of Cas9 activity using the split-SaCas9. Prolonged Cas9 expression seems to increase the frequency of off-target mutagenesis (Yee 2016). The split-SaCas9 fragment can then be expressed under the control of an inducible promoter, activated by, for example, heat or chemical treatment, with the partner *split-SaCas9* fragment being expressed under the control of a tissue- or developmental stage-specific promoter such as the *APETALA 1* gene (Gao et al. 2015) and *INCURVATA2* (Hyun et al. 2015). Thus, by limiting when and where Cas9 is active, transient genome editing mediated by the split-SaCas9 introduced here would also be advantageous to decrease off-target effects.

Materials and Methods

Plasmid construction

We used a synthetic *SaCas9* gene optimized for *A. thaliana* codon usage (Kaya et al. 2016) for cloning of *split-SaCas9*. PCR fragments comprising the *430N* (1–430), *431C* (431–1,053), *739N* (1–739) or *740C* (740–1,083) coding region of *SaCas9* were cloned into the *Sma*I–*Sac*I site in the binary vector *pRI-SaCas9* (Kaya et al. 2016) using an In-Fusion HD Cloning kit (TaKaRa) (Supplementary Figs. S3–S7). The backbone of the *pRI-SaCas9* binary vector was *pRI201-AN* (TaKaRa). The synthesized target sequence corresponding to the *N.*

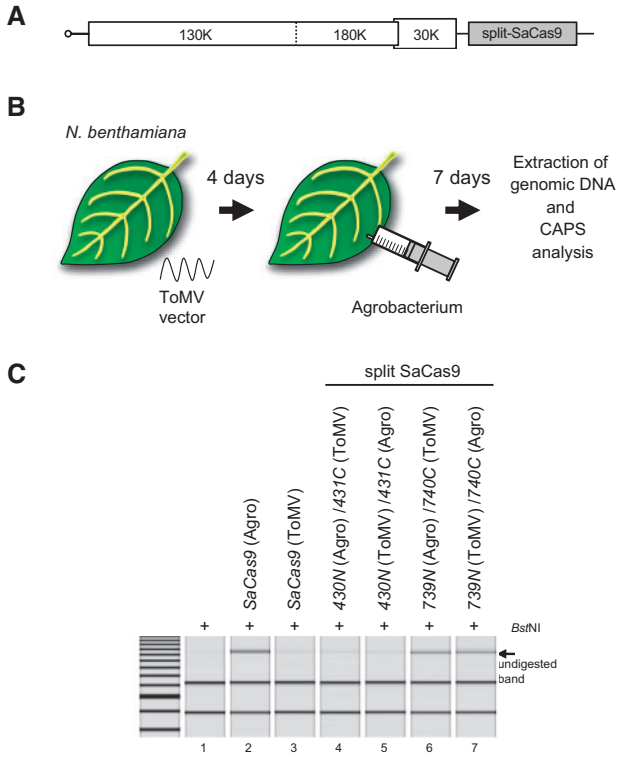

Fig. 3 Split-SaCas9 transiently expressed separately via ToMV inoculation and Agroinfiltration induced targeted mutagenesis in *N. benthamiana*. (A) Schematic representation of the ToMV vector expressing full-length *SaCas9* or *split-SaCas9*. A circle represents the cap structure, and rectangular boxes represent open reading frames. The 130K and 180K are involved in viral RNA replication. The 30K is involved in cell to cell movement. (B) Experimental scheme of ToMV infection and Agroinfiltration for genome editing. (C) Genomic DNA was extracted from leaves at 11 d after ToMV inoculation (7 d after Agroinfiltration). CAPS analysis of the *sgPDS1* locus was performed as in **Fig. 2A**. +: *Bst*NI-digested PCR products.

benthamiana PDS gene was cloned into the *Bbs*I site of an sgRNA expression vector for SaCas9 (backbone vector: *pUC19*) (Kaya et al. 2016) (Supplementary Fig. S8). The *Pac*I–*Asc*I fragment containing sgRNA from the sgRNA expression vector was subcloned into the *Pac*I–*Asc*I site of the binary vector *pRI-sgRNA* (without *SaCas9*), which excluded the *SaCas9* gene from *pRI-SaCas9*. The *N. benthamiana* codon-optimized *p19* gene was synthesized by GeneArt Gene Synthesis (Thermo Fisher Scientific). The cDNA of the *p19* gene was cloned into the *Nde*I–*Sac*I site of *pRI201-AN* using an In-Fusion HD Cloning kit (TAKARA). pTLW3—an infectious cDNA clone of ToMV (Kubota et al. 2003)—was modified to create pTL-MCS, which has multiple cloning sites for foreign gene expression, by disrupting the initiation codon of the coat protein gene [G5705 was changed to A of the ToMV genome (Ohno et al. 1984)], and replacing the nucleotides T5742–A5798 with 5′-accggtcctagggagctc-3′ to give *Age*I, *Avr*II and *Sac*I sites. Full-length SaCas9 and split-SaCas9 coding sequences were amplified by PCR to have an *Age*I site before the initiation codon and a *Sac*I site after the termination codon, and cloned into pTL-MCS (Supplementary Fig. S9).

Transient expression by Agroinfiltration in *N. benthamiana*

Nicotiana benthamiana plants were grown in soil in a greenhouse under a 18 h light/6 h dark cycle at 22°C. Transient expression in *N. benthamiana* leaves was performed as described in Kawarazaki et al. (2013) and Kimura et al. (2013). In brief, *Agrobacterium tumefaciens* strain GV3101 MP90 carrying an expression construct of interest was cultured in LB medium. Cells were then suspended in infiltration buffer (10 mM $MgCl_2$, 100 μg ml^{-1} acetosyringone). A mixture of *Agrobacterium* cultures was infiltrated into *N. benthamiana* leaves. *Agrobacterium* expressing the *p19* gene, which encodes the 19 kDa protein derived from *Tomato bushy stunt virus* that is able to enhance transient transgene expression (Qiu et al. 2002, Qu and Morris 2002), was added to all mixtures. Leaves were harvested 7 d after infiltration for subsequent protein and genomic DNA extraction.

Transformation of *N. tabacum*

Nicotiana tabacum L. cv. Petit Havana (SR-1) was transformed *via Agrobacterium*-mediated transformation as described in Taoka et al. (1999). Transgenic plants were regenerated from leaf disks on medium containing 50 mg l^{-1} kanamycin (Wako Pure Chemicals) and 25 mg l^{-1} meropenem.

Inoculation of ToMV vector to *N. benthamiana*

pTL-MCS-derived plasmids were linearized by *Mlu*I, and used as a template for in vitro transcription using AmpliCap-Max T7 High Yield Message Maker Kit (CELLSCRIPT) according to the manufacturer's instructions at 37°C for 2 h. The transcription mixture was then diluted 5-fold in water. The diluted mixture and abrasive carborundum (600 mesh; Nacalai Tesque) were dusted onto the fifth or sixth true leaves. The leaves were then rubbed gently by hand to inoculate ToMV mechanically.

Protein extraction and Western blot analysis

Total proteins were extracted from *Agrobacterium*-infiltrated leaves by grinding in extraction buffer [100 mM Tris–HCl pH 6.8, 4.0% (w/v) SDS, 20% glycerol, 10% (v/v) 2-mercaptoethanol, 100 mM dithiothreitol (DTT)]. Western blot analysis was performed using the anti-FLAG antibody M2 (Sigma-Aldrich) described in Kaya et al. (2014).

CAPS analysis

Genomic DNA was extracted from *Agrobacterium*-infiltrated leaves using Agencourt Chloropure (BECKMAN COULTER), and target loci were amplified by PCR. The PCR products were digested with restriction enzymes. The digested PCR products were analyzed using MCE-202 MultiNA with a DNA-500 kit (SHIMADZU).

Calculation of targeted mutagenesis efficiency

PCR products containing target loci were cloned into pCR-BluntII-TOPO vector (Thermo Fisher Scientific). Ninety-six colonies harboring pCR-BluntII-TOPO vector were independently subjected to colony PCR. The colony PCR products were digested with restriction enzymes and then analyzed using MCE-202 MultiNA with a DNA-500 kit (SHIMADZU). The efficiency of targeted mutagenesis was calculated by determining the ratio of the number of clones with undigested PCR bands to colonies yielding digested bands.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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