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

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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. 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 fluorescence protein (Hu et al. 2002), ubiquitin protease (Johnsson and Varshavsky 1994) and β-galactosidase (Ullmann et al. 1967).

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; Sp, Staphylococcus aureus; ToMV, Tomato mosaic virus.
SaCas9 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 pieces 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 intermediately 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 types 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 PHOTTOGEN 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 BstNI or Psrl 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 mutated 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 _740C, were detected at their predicted size (Fig. 2B), indicating that transient expression of 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...
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. The same result was obtained in the leaf expressing the split-SaCas9 from ToMV and the split-SaCas9 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.
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 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/431C, 431C/1053, 739N/740C or 740C/1083 coding region of SaCas9 were cloned into the SmaI–SacI 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. benthamiana PDS gene was cloned into the SmaI–SacI site in the binary vector pRI-SaCas9 (Kaya et al. 2016) using the PCR product generated by the In-Fusion HD Cloning kit (TaKaRa). The synthesized target sequence corresponding to the N. benthamiana PDS gene was cloned into the SmaI–SacI site in the binary vector pRI-SaCas9 (Kaya et al. 2016) using the PCR product generated by the In-Fusion HD Cloning kit (TaKaRa). The synthesized target sequence corresponding to the N. benthamiana PDS gene was cloned into the SmaI–SacI site in the binary vector pRI-SaCas9 (Kaya et al. 2016) using the PCR product generated by the In-Fusion HD Cloning kit (TaKaRa).
**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 MluI, 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 borborundum (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).

**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.

References

Ali, Z., Abul-faraj, A., Li, L., Ghosh, N., Platek, M., Mahjoub, A., et al. (2015) Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol. Plant. 8: 1288–1291.

Avesani, L., Marconi, G., Morandini, F., Albertini, E., Bruschetta, M., Bortesi, L., et al. (2007) Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment. Transgenic Res. 16: 587–597.

Baltes, N.J. and Voytas, D.F. (2015) Enabling plant synthetic biology through genome engineering. Trends Biotechnol. 33: 120–131.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N.J. and Nekrasov, V. (2015) CRISPR–Cas9 editing and beyond. Trends Biotechnol. 33: 41–52.

Carroll, D. (2014) Genome engineering with targetable nucleases. Annu. Rev. Biochem. 83: 409–439.

Chen, W., Kim, H. and Hwang, S. (2015) CRISPR–Cas9 enable novel approaches for generating and manipulating plant genomes. Plant J. 82: 278–290.

Doudna, J.A. and Charpentier. E. (2014) Genome editing. The new frontier of genome engineering with CRISPR–Cas9. Science 346: 1258096.

Furusawa, A. and Sato, M. (2015) CRISPR–Cas9 for plant genome editing and beyond. Biotechnol. Adv. 33: 41–52.

Galpin, K. and Shuangdei, C. (2015) High sensitivity and specificity of CRISPR–Cas9-mediated indels in Nicotiana tabacum. Plant Mol. Biol. 87: 99–110.

Guilfoyle, T.J. (2014) CRISPR–Cas9 systems and their potential for plant genome engineering. Science 344: 1236203.

Huang, Y. and Belmonte, J.E. (2015) The CRISPR/Cas9 system for genome editing in plants. Annu. Rev. Genet. 49: 222–229.

Huang, Y., Xie, X. and Wu, X. (2015) CRISPR–Cas9 systems for genome engineering in Arabidopsis. Annu. Rev. Plant Biol. 66: 935–959.

Jacob, F. and Monod, J. (1962) Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 5: 127–145.

Kaya, H., Furusawa, A., Nagashii, M., Shu, F., Suzuki and R. Takahashi for general experimental technical support, and Helen Rothnie for comments on the manuscript.

Kaya, H., Nakajima, T., Araki, T., Miki, M., Ohki, K., Shigeyoshi, Y. and Osakabe, K. (2015) CRISPR–Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. Plant Sci. 240: 130–142.

Kornfeld, J.E., et al. (2015) Rational design of a split-Cas9 enzyme complex. Proc. Natl Acad. Sci. USA 112: 611–623.

Kuhn, K., Kuhn, R., Czesak, J., Engelhardt, S., Wurst, W., et al. (2015) Development of an intein-mediated split-Cas9 system for gene therapy. Nucleic Acids Res. 43: 6450–6458.

Wright, A.V., Sternberg, S.H., Taylor, D.W., Staal, B.T., Bardales, J.A., Kornfeld, J.E., et al. (2015) Rational design of a split-Cas9 enzyme complex. Proc. Natl Acad. Sci. USA 112: 2984–2989.

Yee, J.K. (2016) Off-target effects of engineered nucleases. FEBs J. 283: 3239–3248.
Yin, K., Han, T., Liu, G., Chen, T., Wang, Y., Yu, A.Y., et al. (2015) A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci. Rep. 5: 14926.

Zetsche, B., Volz, S.E. and Zhang, F. (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. Nat. Biotechnol. 33: 139–142.