CRISPR/Cas-induced targeted mutagenesis with Agrobacterium mediated protein delivery

Schmitz, D.J.

Chapter 4

CRISPR/Cas-induced mutagenesis in Nicotiana benthamiana through Cas9 protein translocation via the type IV secretion system of *Agrobacterium*

Daan J. Schmitz, Sylvia de Pater, Paul J.J. Hooykaas

Abstract

The RNA guided endonuclease based on the CRISPR/Cas system of *Streptococcus pyogenus* is a potent new tool for genome engineering in plants. Delivery of this system is usually done by transforming plants with *Agrobacterium tumefaciens* which transfers a T-DNA encoding the required genes through its Type IV Secretion System (T4SS). Instead, here we report the delivery of the Cas9 protein of the CRISPR/Cas system of *Streptococcus pyogenus* through the T4SS of *Agrobacterium*, simultaneously with transfer of a T-DNA encoding the sgRNA directed against the phytoene desaturase gene. Transfer of Cas9 was accomplished by fusion of a T4SS translocation peptide to the Cas9 protein. Infiltration of *Nicotiana benthamiana* leaves with an *Agrobacterium* suspension resulted in targeted mutations at the phytoene desaturase locus. Deep amplicon sequencing showed that translocation of NCas9F through the T4SS resulted in deletion and insertion frequencies that are eighteen and twelve fold lower, respectively, than after delivery of a T-DNA encoding both Cas9 and the sgRNA. Mutations induced by translocated NCas9F were found upstream of the PAM and were distributed in the same manner as mutations induced with Cas9 expressed from a T-DNA.

Introduction

Genome editing in plants can be achieved by the induction of site-specific double stranded breaks (DSBs) in the genome with several classes of nucleases. Meganucleases, Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and more recently the CRISPR/Cas system (for: clustered regularly interspaced palindromic repeats / CRISPR-associated) have become important tools for the targeted genetic modification of plant genomes. DSBs induced by these nucleases can be repaired through non-homologous end joining (NHEJ) or homologous recombination (HR). Repair via error-prone NHEJ can result in small insertions and deletions enabling reverse genetics. By providing an artificial repair template with homology to the target sequence, repair via HR can be used to replace or correct existing genes.

The CRISPR/Cas system, originating from prokaryotes, functions as an adaptive immune system that acts against invading bacteriophages and other invading nucleic acids [1]. The prototypical CRISPR/Cas endonuclease from the type II bacterial CRISPR/Cas system found in *Streptococcus pyogenus* can be programmed to create targeted double strand breaks (DSBs). The tracrRNA and crRNAs can be engineered into a single guide RNA (sgRNA) [2]. The Cas9 endonuclease can be directed to a specific target sequence by the incorporation of a 20 nucleotide sequence in the sgRNA that is complementary to the target sequence [2,3]. This 20 base pair (bp) target sequence has to be flanked by a 3 bp protospacer-associated motif that is required for Cas9 activity [4,5].

For targeted mutagenesis in plant cells, the genes encoding the RNA guided endonuclease system are usually introduced using *Agrobacterium tumefaciens*. Translocation of the T-DNA occurs via its Type IV Secretion System (T4SS), encoded by the *virB* genes and *virD4* gene on its Ti plasmid [6,7]. Independently of the T-DNA, several virulence proteins are transported alongside the T-DNA into the host cell [8]. A hydrophilic secretion signal with a net positive charge is responsible for the translocation of proteins through the T4SS [9]. Several heterologous proteins, fused to this secretion signal, have been translocated through the T4SS [8–11]. In this way the Cre recombinase and homing endonuclease I-SceI were translocated by *Agrobacterium* into host cells to effect DNA recombination in the genome of target cells [8–10].

In this study we show that delivery of the Cas9 protein through the T4SS of *Agrobacterium* can be used for targeted mutagenesis in *Nicotiana benthamiana*.

Results

Engineering the Cas9 expression constructs

In order to be able to test the translocation of the Cas9 protein of the type II class of CRISPR/Cas systems from bacteria to *N. benthamiana* cells a translational fusion between Cas9 and the last 37 amino acids of the *Agrobacterium* virulence protein VirF was created. These 37 amino acids are known to function as a recognition signal for the T4SS of *Agrobacterium* and can be used to target proteins for translocation [8–10]. Target recognition of the CRISPR/Cas system requires an RNA guide. In Chapter 3, it was shown that the sgRNA, when expressed in *Agrobacterium*, was not translocated via the T4SS together with the Cas9 protein to yeast cells. Therefore, a T-DNA vector was created encoding a sgRNA targeting the phytoene desaturase (*PDS*) gene, for expression of the sgRNA in the host cells. The protospacer of this sgRNA contains an GG motif at the 3' end of the target sequence, which has been shown to increase the frequency of targeted mutagenesis [12]. To compare the targeted mutagenesis frequency of the translocated NCas9F protein to constitutively expressed Cas9, a T-DNA vector was used expressing Cas9 under control of the ubiquitin promoter and the sgRNA under control of the small nuclear RNA promoter U6-26 [13].

Targeted mutagenesis in N. benthamiana leaves with translocated Cas9

To test the functionality of the translocated Cas9 in *N. benthamiana*, leaves were infiltrated with an *Agrobacterium* strain expressing the NCas9F protein and a T-DNA encoding the PDS sgRNA or a T-DNA expressing both Cas9 and the sgRNA. Ten days post infiltration, the tissue was harvested and the DNA was extracted. To easily detect mutations induced by the NCas9F nuclease we used the restriction enzyme loss method [14], as the target sequence of the sgRNA overlaps with a DdeI restriction site. To enrich for DNA molecules carrying mutations the genomic DNA of the infiltrated leaves was pre-digested with DdeI. A 490 bp fragment was then amplified by PCR with primers flanking the target sequence, and the resulting PCR products were again digested with DdeI. Restriction digestion resistant bands were cloned and analyzed by sequencing. Translocation of a T-DNA encoding both Cas9 and the sgRNA resulted in restriction digestion resistant PCR products in all of the nine infiltrated leaves (Fig. 1a). The resistent PCR products were isolated and cloned into a high-copy vector transformed into *E. coli* and individual plasmid clones were sequenced. Deletions ranging from 1 to 11 bp, single nucleotide insertions and small deletions combined with insertions were found (Fig. 1b). Translocation of the NCas9F protein together with a T-DNA encoding the PDS sgRNA resulted in restriction digestion resistant PCR products in seven out of nine infiltrated leaves (Fig. 1a). The sequences of the PCR products were very similar to those obtained by T-DNA encoded Cas9, including deletions ranging from 1 to 9 bp, single nucleotide insertions and combinations (Fig. 1c). The results showed that translocated NCas9F protein together with a sgRNA encoded by a T-DNA can bring about targeted mutagenesis in cells of *N. benthamiana* leaves.

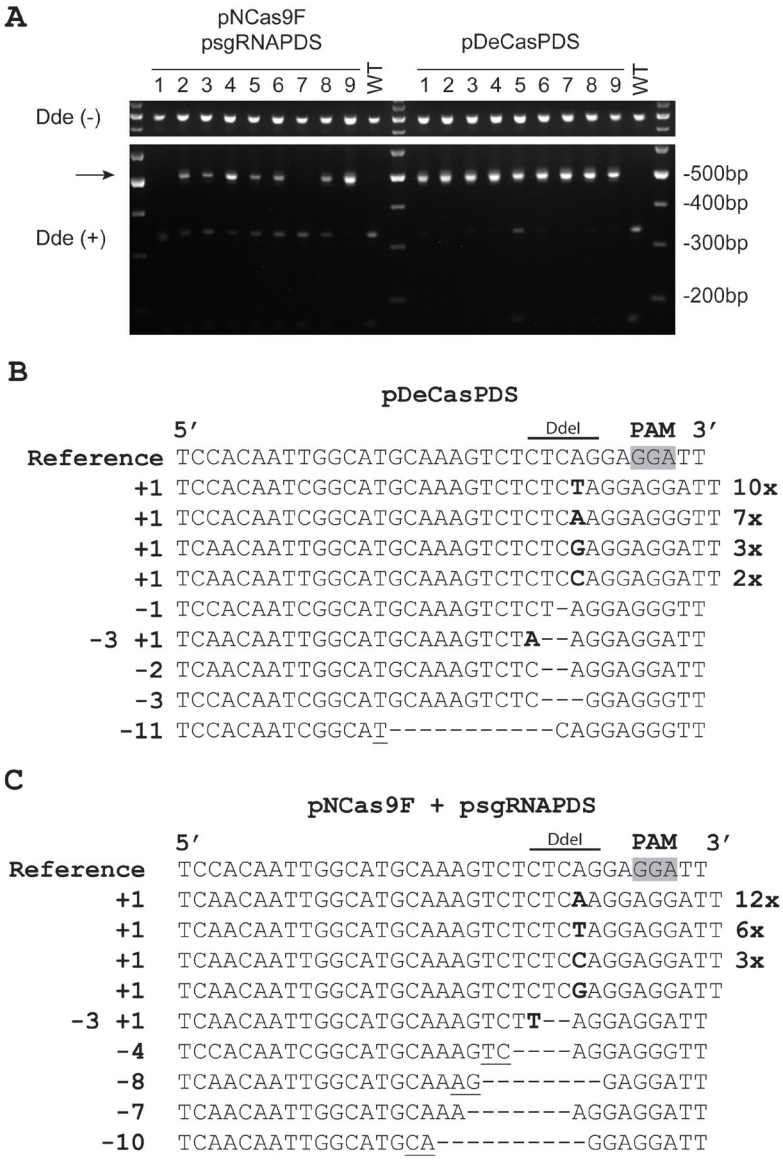

Figure 1. CRISPR/Cas9 endonuclease-induced mutagenesis. The *PDS* target site was amplified using genomic DNA that was pre-digested with DdeI. The resulting 490bp PCR products were digested with DdeI. (**A**) DdeI resistant 490bp bands (arrow) obtained from tissue co-transformed by NCas9F protein and T-DNA encoding the sgRNA and by pDeCasPDS containing genes encoding Cas9 and sgRNA. (**B**) Sequence analysis of mutations obtained after NCas9F protein translocation and T-DNA encoding the sgRNA. (**C**) Sequence analysis of mutations obtained after transfer of T-DNA encoding Cas9 and sgRNA. The DdeI target site and PAM are indicated. Insertions are shown in bold, deletions with dashes and microhomologies are underlined. Length of insertions and deletions are shown at the left. Numbers of multiple clones with the same sequence are indicated at the right.

Efficiency of mutagenesis by translocated Cas9 protein in comparison with Cas9 expressed from a T-DNA

In order to detect mutations, enrichment by restriction enzyme digestion was applied in the experiments described in the previous paragraph. In order to compare the efficiency of targeted mutagenesis after NCas9F protein delivery with that after T-DNA transfer directly, amplicon deep sequencing was performed. Amplicons were generated by PCR from DNA isolated from nine independently infiltrated leaves, 10 days after inoculation. These PCR amplicons covering the protospacer and PAM sequences were sequenced using the illumina HiSeq 2500 platform. Figure 2 depicts the results as relative numbers of mutated reads per position. Most deletions detected were found 3 to 13 bp upstream of the PAM sequence irrespective of how Cas9 was delivered (Fig. 2a). Deletions in cells expressing Cas9 and sgRNA from a single T-DNA were likewise found 3 to 13 bp upstream of the PAM, but at a higher frequency than in cells into which the NCas9F protein had been translocated (Fig. 2a). Almost all insertions found with both translocated NCas9F and Cas9 and sgRNA expressed from a single T-DNA were located 3 to 4 bp upstream of the PAM (Fig. 2b). Such single bp insertions were not detected in sequenced amplicons obtained from non-infiltrated *N. benthamiana* tissue. The percentage of sequences with a deletion obtained from tissue with translocated NCas9F was found to be 0.24%, but the percentage seen after expression of Cas9 and sgRNA expressed from a single T-DNA was found to be eighteen fold higher at 4.31%. Similarly, the insertion frequency with translocated NCas9F was found to be 1.55% and 12 fold higher at 19.8% after expression of Cas9 and sgRNA from a single T-DNA. Further analysis revealed that around ~80% of these single bp insertions are adenosine or thymidine insertions.

These results show that mutations created with translocated NCas9F protein have a similar distribution of both deletions and insertions as those seen after expression from a single T-DNA, albeit at 12-18 fold lower frequencies.

Regeneration of plants from mutated leaves

As previous experiments showed that translocated NCas9F was effective in creating targeted mutations in the leaf cells of *N. benthamiana* we tested if plants could be regenerated from such cells. Leaf discs were excised from *N. benthamiana* leaves infiltrated with an *Agrobacterium* strain expressing the NCas9F protein and a T-DNA encoding the sgRNA and were placed on selective medium containing kanamycin to select for the presence of the T-DNA. This resulted in the formation of 150 resistant calli from which shoots were regenerated on shoot-induction medium. Total DNA was isolated from these regenerated shoots from which the target locus was amplified by PCR. The amplified bands were treated with the restriction enzyme DdeI as loss of the restriction site was taken as indicator for the presence of a mutation. Undigested bands were not found, which indicated that none of the regenerated shoots had homozygous or heterozygous mutation at the desaturase target locus.

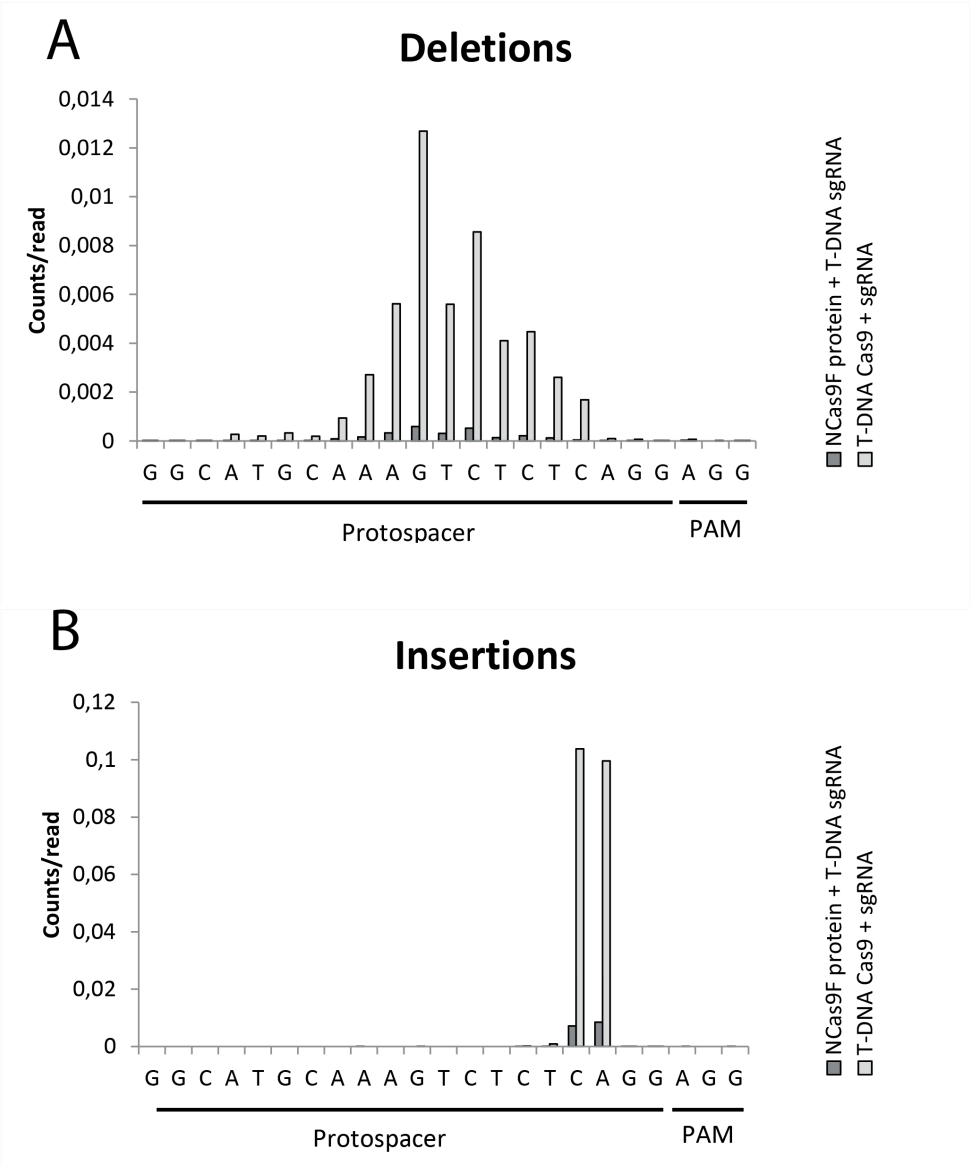

Figure 2. Deletion frequency (**A**) and insertion frequency (**B**) for each nucleotide of the *PDS* target. Deep sequencing analysis of the target sequence obtained from tissue infiltrated with an *Agrobacterium* transferring NCas9F protein combined with a T-DNA expressing the sgRNA and tissue infiltrated with *Agrobacterium* transferring a T-DNA expressing both Cas9 and sgRNA. Relative numbers of mutated reads are depicted as a fraction of the total number of reads numbers per position.

Discussion

In this study we have shown that the NCas9F protein of the type II bacterial CRISPR system can be translocated from *Agrobacterium* to *N. benthamiana* and combined with a T-DNA expressing a sgRNA induced targeted DSBs that after imperfect repair via NHEJ resulted in targeted mutations.

Mutations induced by translocated NCas9F were distributed similarly as mutations induced with Cas9 expressed from a T-DNA. The deletion and insertion frequencies were however about eighteen and twelve fold lower, respectively, with translocated NCas9F protein than with Cas9 expressed from a T-DNA. These lower frequencies could be due to limiting levels of translocated NCas9F, the short presence of NCas9F in the host after translocation, lower activity of NCas9F protein compared to Cas9 protein, or because transfer of T-DNA and NCas9F does not always occur simultaneously.

High levels of small insertions several basepair upstream of the PAM were detected in our experiments using amplicon sequencing. Using the restriction enzyme loss method, the most common mutations found were also small insertions although the frequency was slightly lower than in our experiments using amplicon sequencing. Other reports about targeted mutagenesis in *N. benthamiana* with CRISPR/Cas using the enzyme loss method to detect mutations do not mention these high levels of small insertions [15–18]. High levels of small insertions were however reported in *Arabidopsis* after DSB induction with CRISPR/Cas using next-generation sequencing [13]. This suggests that the outcome of repair of DSBs induced with CRISPR/Cas in plants results in a low frequency of small deletions and a high frequency of single bp insertions upstream of the PAM. These single bp insertions mainly consist of adenosine or thymidine insertions which is consistent with the 'A-rule' which states that polymerases are known to preferentially incorporate deoxyadenosine-monophosphate (dAMP) when template base coding is not available [19].

We attempted to obtain *N. benthamiana* shoots with targeted mutations induced by translocated NCas9F. A total of a 150 shoots were regenerated from leaf discs taken from infiltrated leaves and analyzed for targeted mutations. With a combined insertion and deletion frequency of 1.79% a few mutated shoots were expected. However we did not find a single line with targeted mutations. This suggests that either we were very unlucky (chance less than 7%) or that the frequency is in fact lower, for instance because cells with mutations mostly have both alleles mutated, which would reduce the percentage of mutated cells to 0.9%.

In summary the experimental data discussed above show that it is possible to use *Agrobacterium* for the translocation of the Cas9 protein into plant cells to create targeted mutations, but that the frequency is low. Therefore, it will be laborious to identify and regenerate the mutated cells into complete mutated plants.

Material & Methods

Plasmid construction

For the construction of NCas9F see materials & methods of Chapter 2.

To create the sgRNA expression vectors oligo's DS196/DS197 were phosphorylated, annealed and subsequently cloned into the BbsI site of pEn-Chimera [13]. A correct clone was used as entry vector for a Gateway reaction with destination vector pDe-CAS9 [13] resulting in pDECas9PDS and destination vector pMDC100 [20] resulting in psgRNAPDS.

Leaf infiltration

Seeds of *N. benthamiana* were germinated and grown in controlled climate chambers at 24°C with a 16 h light /8 h dark photoperiod with 75 % humidity for a period of three weeks before infiltration. *Agrobacterium* strain AGL1 with pNCasF and psgRNAPDS or pDECasPDS were grown overnight at 29°C shaking at 180 RPM in a 10 ml culture of LB (5 g/l NaCl) supplemented with appropriate antibiotics. The next day *Agrobacterium* cells were re-suspended in induction medium [21] to an OD600 of 1.2 and were then kept at room temperature for 3 hours without shaking. The *Agrobacterium* cells were then introduced into the two youngest leaves of 3 weeks old *N. benthamiana* plants using needleless 1 ml syringes.

Footprint detection

Leaf discs were harvested 4 days post infiltration from which genomic DNA was isolated using CTAB DNA extraction [22]. Genomic DNA (500ng) was pre-digested with DdeI (Thermo Scientific Inc.) before the target sequence was amplified using primers DS192 and DS193. Amplified products were digested again with DdeI (Thermo Scientific Inc.) after which resistant bands were cloned into pJET1.2 (Thermo Scientific Inc.). Individual clones were sent for Sanger sequencing (Macrogen Inc.).

Shoot regeneration

Infiltrated leaves were surface sterilized by immersion into 70% ethanol for 1 minute followed by immersion into 1% fresh sodium hypochlorite with 0.1% Tween 20 for 30 minutes. From these leaves discs (with a diameter of 1 cm) were excised and put on selection medium (1X Murashige and Skoog basal salt mixture, 1x Gamborg's B5 vitamins, 3% Sucrose, 0,59 g/l MES, 1.0 mg/l BAP, 0.1 mg/l NAA, 0.4% Gelrite pH 5.7, 100 µg/ml kanamycin and 320 µg/ml timentin). Leaf discs were transferred onto fresh medium every 10 days until shoots appeared. Shoots were transferred to 1/2 MS medium (100 µg/ml kanamycin and 320 µg/ml timentin).

Amplicon deep sequencing

Leaves of three weeks old *N. benthamiana* plants were infiltrated and tissue was collected 10-days post infiltration. DNA was isolated using CTAB DNA isolation; 100ng of this DNA was used for amplification with the primers listed in Table 1 with Illumina adapter sequences using Phusion® High-Fidelity DNA Polymerase. Amplicons were purified from gel using the Zymoclean™ Gel DNA Recovery Kit. Paired-end sequencing was performed by BaseClear B.V. on the Illumina HiSeq 2500 platform. Data analysis was performed using the integrative genome viewer [23,24] and the Galaxy webserver [25–27]. Deletion and insertion frequencies were determined by dividing the number of reads containing insertions or deletions by the

total number of reads. Number of reads containing insertions or deletions was determined using a custom script that sorted and counted unique reads.

Table 1. Overview of primers used in this study

Primer	Sequence
DS190 FW Nb target GGNGG	ATTGGGCATGCAAAGTCTCTCAGG
DS191 RV Nb target GGNGG	AAACCCTGAGAGACTTTGCATGCC
DS192 FW GGNGG Restric	GGTGTGCCTGATAGGGTGAC
DS193 RV GGNGG Restric	GGCAAACACAAAAGCATCTCC
DS217 Cas9-F FW 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAAGGCCACCTTTTGACTC AATATG
DS218 Cas9-F RV 1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAAAATCTGCAGGAGAAA CATGG
DS219 Cas9-F FW 2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACGGCCACCTTTTGACTC AATATG
DS220 Cas9-F RV 2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACAATCTGCAGGAGAAA CATGG
DS221 Cas9-F FW 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGGGCCACCTTTTGACTC AATATG
DS222 Cas9-F RV 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGAATCTGCAGGAGAAA CATGG
DS223 Cas9-F FW 4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAATGGCCACCTTTTGACTCA ATATG
DS224 Cas9-F RV 4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAATAATCTGCAGGAGAAA CATGG
DS225 Cas9-F FW 5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACAGGCCACCTTTTGACTCA ATATG
DS226 Cas9-F RV 5	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACAAATCTGCAGGAGAAA CATGG
DS227 Cas9-F FW 6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCGGCCACCTTTTGACTCA ATATG
DS228 Cas9-F RV 6	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACCAATCTGCAGGAGAAA CATGG
DS229 Cas9-F FW 7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACGGGCCACCTTTTGACTC AATATG
DS230 Cas9-F RV 7	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGAATCTGCAGGAGAAA CATGG
DS231 Cas9-F FW 8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTGGCCACCTTTTGACTCA ATATG
DS232 Cas9-F RV 8	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTAATCTGCAGGAGAAA CATGG
DS233 Cas9-F FW 9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGGCCACCTTTTGACTC AATATG
DS234 Cas9-F RV 9	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAAATCTGCAGGAGAAA CATGG
DS253 WT FW	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATGGCCACCTTTTGACTCA ATATG
DS254 WT RV	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCATAATCTGCAGGAGAAAC ATGG
DS235 T-DNA Cas9 FW 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGGCCACCTTTTGACTCA ATATG

DS236 T-DNA Cas9 RV 1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCAATCTGCAGGAGAAA CATGG
DS237 T-DNA Cas9 FW 2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGGGCCACCTTTTGACTCA ATATG
DS238 T-DNA Cas9 RV 2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGGAATCTGCAGGAGAAA CATGG
DS239 T-DNA Cas9 FW 3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGTGGCCACCTTTTGACTCA ATATG
DS240 T-DNA Cas9 RV 3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGTAATCTGCAGGAGAAA CATGG
DS241 T-DNA Cas9 FW 4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATAGGCCACCTTTTGACTCA ATATG
DS242 T-DNA Cas9 RV 4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATAAATCTGCAGGAGAAA CATGG
DS243 T-DNA Cas9 FW 5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATCGGCCACCTTTTGACTCA ATATG
DS244 T-DNA Cas9 RV 5	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATCAATCTGCAGGAGAAA CATGG
DS245 T-DNA Cas9 FW 6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGGGCCACCTTTTGACTCA ATATG
DS246 T-DNA Cas9 RV 6	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGAATCTGCAGGAGAAA CATGG
DS247 T-DNA Cas9 FW 7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTGGCCACCTTTTGACTCA ATATG
DS248 T-DNA Cas9 RV 7	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTAATCTGCAGGAGAAA CATGG
DS249 T-DNA Cas9 FW 8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAAGGCCACCTTTTGACTC AATATG
DS250 T-DNA Cas9 RV 8	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAAAATCTGCAGGAGAAA CATGG
DS251 T-DNA Cas9 FW 9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCACGGCCACCTTTTGACTCA ATATG
DS252 T-DNA Cas9 RV 9	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCACAATCTGCAGGAGAAA CATGG

Acknowledgements

This work was supported by TTI-Green Genetics (project number TTI GG CORE 4CC057RP).

References

1. van der Oost J, Jore MM, Westra ER, Lundgren M, Brouns SJJ. CRISPR-based adaptive and heritable immunity in prokaryotes. Trends Biochem. Sci. 2009;8:401–7.

2. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA–guided DNA endonuclease in adaptice bacterial immunity. Science. 2012;337:816–22.

3. 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;45:273–97.

4. Deveau H, Barrangou R, Garneau JE, Labonté J, Fremaux C, Boyaval P, et al. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J. Bacteriol. 2008;190:1390–400.

5. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology. 2009;155:733–40.

6. Christie PJ, Cascales E. Structural and dynamic properties of bacterial type IV secretion systems (review). Mol. Membr. Biol. 2005;22:51–61.

7. Alvarez-Martinez CE, Christie PJ. Biological diversity of prokaryotic type IV secretion systems. Microbiol. Mol. Biol. Rev. 2009;73:775–808.

8. Vergunst AC, Schrammeijer B, den Dulk-Ras A, Vlaam de CMT, Regensburg-Tuïnk TJ, Hooykaas PJJ. VirB/D4-Dependent Protein Translocation from Agrobacterium into Plant Cells. Science. 2000;290:979–82.

9. Vergunst AC, van Lier MCM, den Dulk-Ras A, Stüve T a G, Ouwehand A, Hooykaas PJJ. Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of Agrobacterium. Proc. Natl. Acad. Sci. USA. 2005;102:832–7.

10. Rolloos M, Hooykaas PJJ, van der Zaal BJ. Enhanced targeted integration mediated by translocated I-SceI during the Agrobacterium mediated transformation of yeast. Sci. Rep. 2015;5:8345.

11. Schrammeijer B, den Dulk-Ras A, Vergunst A, Jurado Jácome E, Hooykaas PJJ. Analysis of Vir protein translocation from Agrobacterium tumefaciens using Saccharomyces cerevisiae as a model: evidence for transport of a novel effector protein VirE3. Nucleic Acids Res. 2003;31:860–8.

12. Farboud B, Meyer BJ. Dramatic enhancement of genome editing by CRISPR/cas9 through improved guide RNA design. Genetics. 2015;199:959–71.

13. 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;79:348–59.

14. Voytas DF. Plant genome engineering with sequence-specific nucleases. Annu. Rev. Plant Biol. 2013;64:327–50.

15. Lowder LG, Zhang D, Baltes NJ, Paul JW, Tang X, Zheng X, et al. A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation. Plant Physiol. 2015;169:971–85.

16. Ali Z, Abul-Faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, et al. Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. Mol. Plant. 2015;8:1288–91.

17. Yin K, Han T, Liu G, Chen T, Wang Y, Yu AYL, et al. A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci. Rep. 2015;5:14926.

18. Nekrasov V, Stakawicz B, Weigel D, Gones JDG, Kamoun S. Targeted mutagenesis in the model plant nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat. Biotechnol. 2013;31:688–91.

19. Strauss BS. The "A" rule revisited: Polymerases as determinants of mutational specificity. DNA Repair (Amst). 2002;1:125–35.

20. Curtis MD, Grossniklaus U. A Gateway cloning vector set for high-throughput functional analysis of genes in planta. Breakthr. Technol. 2003;133:462–9.

21. Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJ. Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. EMBO J. 1995;14:3206–14.

22. de Pater S, Neuteboom LW, Pinas JE, Hooykaas PJJ, van der Zaal BJ. ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation. Plant Biotechnol. J. 2009;7:821–35.

23. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): High-performance genomics data visualization and exploration. Brief. Bioinform. 2013;14:178–92.

24. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat. Biotechnol. 2011;29:24–6.

25. Goecks J, Nekrutenko A, Taylor J. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol. 2010;11:R86.

26. Blankenberg D, Kuster G Von, Coraor N, Ananda G, Lazarus R, Mangan M, et al. Galaxy: A web-based genome analysis tool for experimentalists. Curr. Protoc. Mol. Biol. 2010;1–21.

27. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, et al. Galaxy: A platform for interactive large-scale genome analysis. Genome Res. 2005;15:1451–5.