Efficient generation of null-segregant parthenocarpic tomato by CRISPR/Cas9 editing

Chihiro Abe Hara
Tokushima University
Risa Ueta
Tokushima University
Ryosuke Hashimoto
Tokushima University
Yuriko Osakabe
Tokushima University
Keishi Osakabe (kosakabe@tokushima-u.ac.jp)
Tokushima University https://orcid.org/0000-0002-6227-6913

Research article

Keywords: CRISPR/Cas9, null-segregant, parthenocarpy, SlIAA9, tomato

Posted Date: January 17th, 2020

DOI: https://doi.org/10.21203/rs.2.21137/v1

Abstract

Background Genome editing technique is a powerful tool in plant genetic engineering to accelerate the rapid breeding of crop plants. Currently, obtaining transgene-free mutant plants is an important issue. transgene-free mutant plants such as null-segregant, can be segregated out from selfing or back-crossed populations. However, such procedures are time consuming, and, therefore, the development of efficient systems to eliminate transgenes in mutant plants is required. Results CRISPR/Cas9 vectors targeting Sl IAA9 were introduced into the tomato cultivars, Ailsa Craig, Moneymaker, Super Roma and Rio Grande, via Agrobacterium transformation. Bi-allelic mutations were detected at ratios of 9–46% in CRISPR/Cas9-transgenic T0 shoots. Sequence analysis revealed that the bi-allelic mutations generated stop codons by frame-shift of SlIAA9 amino acid sequences, indicating that sliaa9 knockout mutants with parthenocarpic phenotypes were generated in the T0 generation with high efficiency in these cultivars. Subsequently, many null-segregant (transgene free) lines, as confirmed by PCR and Southern blot, were isolated rapidly in the T1 generation by self-pollination. Conclusions The method used in this study is effective in achieving rapid isolation of null-segregants with high efficiency, while adding important traits to various cultivars at the same time. Our developed system would further increase the advantages of using CRISPR/Cas9 for the production of novel crop varieties in the future.

Background

Plant genetic engineering is now a cornerstone of plant functional studies and creating key resources for crop improvement. Conventional crop breeding traditionally used various mutagens to generate large pools of genetic variation. These technologies introduce random mutations in plant genomes, requiring extensive screening for mutations of interest. Recent plant genetic engineering methodologies, referred to as New Plant Breeding Techniques (NPBTs) have contributed to the development of new traits in crop breeding without the time- and labor-intensive processes required in conventional breeding. One such new technology—genome editing—is now widely used as a powerful tool in plant genetic engineering to accelerate the rapid breeding of crop plants. One of the most convenient genome editing tools is the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) system, in which the Cas9 nuclease and a guide RNA (gRNA) form a complex, and induce a double-stranded DNA break (DSB) at the target site. In eukaryotes, DSBs are repaired by DNA repair pathways, error-free homologous recombination (HR) or error-prone non-homologous end joining (NHEJ), and NHEJ-mediated repair of a Cas9-generated DSB can be used to induce an error at the target locus to generate a null allele [1]. The CRISPR/Cas9 system has been developed for use in many plant species, such as Arabidopsis [2], rice [3], tobacco [4], maize [5], apple [6], grapevine [7], and potato [8].

Plant genome editing tools are introduced into plant cells mainly by *Agrobacterium*-mediated transformation. When transgenes, including genome editing tools, are integrated into mutant genomes, specific regulations, such as the Cartagena Protocol on Biosafety, must be adhered to, both in terms of public acceptance and to facilitate the commercialization of new crop varieties generated by genome editing [9]. Several methods for transgene-free plant genome editing, including RNP (ribonucleoprotein)

technology have recently been developed [10, 11, 12, 13]. Null-segregants, i.e., transgene-free mutant plants, can be segregated out from selfing or back-crossed populations. However, such procedures are time consuming, and, therefore, the development of efficient systems to eliminate transgenes in mutant plants is required.

Tomato (*Solanum lycopersicum*)—a model horticultural crop—is one of the most important vegetable and fruit crops worldwide. In wild-type tomato plants, fruiting can be induced by artificial pollination and application of exogenous auxin. Parthenocarpy, which allows fruit production without fertilization, is a hugely important agricultural trait, and has been produced by traditional breeding in limited species and cultivars. The fruiting of tomato is controlled by auxin signaling factors, tomato Auxin Responsive Factor 8 (SlARF8) and the auxin responsive transcription repressor INDOLE-3-ACETIC ACID 9 (SlIAA9) [14, 15]. It has been shown that downregulation of *SlIAA9* induces parthenocarpy [15, 16]. Mutants of *sliaa9* have been generated by ethyl methanesulfonate (EMS) treatment of several cultivars, such as Micro-Tom, Ailsa Craig, and M82 [17, 18]. We previously reported the efficient generation of *sliaa9* knockout mutants using the efficient CRISPR/Cas9 system [19]. To extend this technology to generate parthenocarpic tomatoes in the various genetic backgrounds of commercial cultivars, we used CRISPR/Cas9 to induce *SlIAA9* mutations in multiple cultivars (Ailsa Craig, Moneymaker, Super Roma, and Rio Grande) simultaneously. The newly generated mutants exhibited parthenocarpic phenotypes, and null-segregants from these *sliaa9* knockout lines were isolated systematically and rapidly in the T1 generation. The efficient generation of null-segregants of the mutant lines with the desired phenotype shown in this study is an important example of how useful traits can be generated rapidly in commercial cultivars by using NPBTs.

Results

Detection of *SlIAA9* mutations in commercial tomato cultivars

To generate parthenocarpic tomatoes in various commercial cultivars simultaneously, the CRISPR/Cas9 vectors pEgP237-2A-GFP or pEgPubi4_237-2A-GFP (Additional file 1: Figure S1), which targets exon2 of the *SlIAA9* gene (+217 to +236 from ATG, 5'-GAGCTCAGGCTCGGTCTACC-3') [19] were introduced into Ailsa Craig, Moneymaker, Super Roma, and Rio Grande, via *Agrobacterium* transformation. To detect CRISPR/Cas9-induced mutations in T0 regenerated shoots of commercial tomato cultivars, PCR-RFLP analysis using an *Acc*I restriction enzyme site located at the CRISPR/Cas9 target was performed. Many undigested bands were detected in the CRISPR/Cas9 transgenic lines (Fig. 1A), indicating that the CRISPR/Cas9 effectively induced mutations at the *SlIAA9* target site in the various commercial cultivars. The calculated mutation efficiencies in the regenerated T0 shoots were around 43–70% in Ailsa Craig, Moneymaker, and Super Roma, and <20% in Rio Grande (Fig. 1B). The 100% mutations determined as non-digested fragments in PCR-RFLP analysis (AC#52, MM#3, and RG#4 in Fig. 1A), which are presumed to be mutations introduced into almost all somatic cells, were isolated at a frequency of 23–46% in Ailsa Craig, Moneymaker, and Super Roma, and 9% in Rio Grande (Fig. 1B). The mutated sequences were then analyzed by Sanger sequencing of cloned PCR fragments from the target sites, amplified from shoots with 100% mutation from each of the various commercial cultivars (Fig. 1C, Additional file 1: Figure S2).

The results revealed that the mutant lines have only one or two types of mutation, which were found at similar levels in all sequenced clones (AC#52, MM#3, SR#12, RG#4) (Fig. 1C, Additional file 1: Figure S2), suggesting that they are bi-allelic mutations. Stop codons generated by frame shifts were found downstream of each target sequence (Fig. 1C, Additional file 1: Figure S2). These results confirm isolation of *sliaa9* knockout mutants of commercial tomato cultivars in the T0 generation following efficient CRISPR/Cas9 editing.

sliaa9-crispr T0 mutant phenotypes

The phenotypes of *sliaa9* T0 knockout mutants (*sliaa9-crispr*) in cultivars Super Roma, Moneymaker, and Rio Grande exhibited morphological changes in leaves, with similar tendency, i.e., the compound leaf shape was changed to a simple leaf with a longer petiole than that of wild-type (Fig. 2A)—a phenotype previously seen in the leaves and petioles of Ailsa Craig [19]. The growth rates of these mutants were similar to that of wild-type (Fig. 2A). Flower development was normal in all these mutants compared with wild-type plants (Fig. 2B); however, seedless fruits, or fruits with few seeds, were generated in the knockout mutants, suggesting a parthenocarpic phenotype (Fig. 2C, D). When mutations at the target locus were analyzed by PCR-RFLP analysis, 100% mutation was detected in fruits with parthenocarpic phenotypes (Additional file 1: Figure S3). We also counted fruit numbers in parthenocarpic phenotypes (Fig. 2E). In the wild-type, no fruits developed without pollination, whereas many seedless parthenocarpic fruits developed in the knockout mutants (Fig. 2E). There were no differences in fruit size between the mutants and wild-type (Additional file 1: Figure S4). These results showed that parthenocarpic phenotypes were efficiently introduced into the various commercial cultivars in the T0 generation by the CRISPR/Cas9 vectors.

Mutation analysis in next generation plants

PCR-RFLP analysis of the T1 generation isolated by self-pollination from the T0 knockout lines showed that some of *sliaa9-crispr* T1 plant lines in Ailsa Craig, Moneymaker, and Rio Grande cultivars showed 100% somatic mutation (Fig. 3A). Sanger sequencing using cloned PCR fragments at the target site in the T1 lines detected single mutations, suggesting that these lines were homozygotes, and that they were segregated from the parental T0 generation (Figs. 1C, 3A, Additional file 1: Figure S5). Altered leaf morphology and parthenocarpic fruits were also detected in these T1 lines (Fig. 3B, C). We next analyzed a candidate targets for off-target effects (Additional file 1: Figure S6) in mature leaves from T0 and T1 mutants by using amplicon sequence. The results showed that there were no off-target mutations (Additional file 1: Figure S6).

Isolation of null-segregants

Next, we examined null-segregants in the T1 generation. Since the latter were generated from T0 lines by self-pollination, the transgene, including the CRISPR/Cas9 cassette can be segregated out. To detect T-DNA insertion in the genomic DNA of *sliaa9-crispr* T1 progenies, the PCR primers shown in Additional file 1: Figure S1 were used to amplify the various T-DNA regions of the vector. Figure 4 shows results using the representative mutant lines, which indicate that no PCR bands from any T-DNA regions were detected in the several T1 lines tested (Fig. 4), suggesting that these lines are null-segregant candidates. Candidate lines for null-segregants were detected at a ratio of 33.3% (17/51 plants) in AC#33 T1 lines, 15.8% (3/19 plants) in AC#52 T1 lines, 25.0% (10/40 plants) in MM#2 T1 lines, and 28.6% (2/7 plants) in RG#4 T1 lines (data not shown). Subsequently, a search for T-DNA regions was also performed by PCR in the T2 generation (MM#2-13 and MM#2-14) (Additional file 1: Figure S7). T-DNA was not detected in MM#2-13, a T1 progeny of MM#2 (data not shown), and PCR analysis showed that the T2 progenies of MM#2-13 also did not possess any T-DNA (Additional file 1: Figure S7). On the contrary, individuals with both the presence and absence of T-DNA segregated in the next generation of MM#2-14 (Additional file 1: Figure S7), which did harbor T-DNA in its genome (data not shown).

We then performed Southern blot analysis to detect T-DNA insertion and copy number in various progenies, including null-segregants. Genomic DNA from T0 and T1 progenies were digested by the restriction enzyme HpaI (Additional file 1: Figure S1), and T-DNA was detected using probes for *gRNA* and *Cas9* (Fig. 5). Southern blot analysis suggested a single insertion of the transgene in several T0 and T1 lines. There were no hybridizing bands in several T1 progenies, in which PCR fragments corresponding to T-DNA were not detected (Figs. 4, 5), whereas hybridization bands for the *LHCB* gene used as a control were detected in all lines. These results indicate that many null-segregants lacking the CRISPR/Cas9 cassette can be efficiently isolated in various tomato cultivars in the T1 generation.

Discussion

In this study, parthenocarpic lines of various commercial tomato cultivars were generated by CRISPR/Cas9-mediated mutation of the *SlIAA9* gene. These lines are null-segregants, which were generated rapidly by self-pollination of T0 plants. Bi-allelic mutants of *SlIAA9* were generated in the T0 generation with high efficiency—a strong advantage in the rapid isolation of null-segregants in early generations. We have previously shown generation of a *sliaa9* mutant in Micro-Tom using the same CRISPR/Cas9 vector with similarly high mutation efficiency [19], suggesting that the optimized vector can be used universally among tomato cultivars, although slightly lower mutation efficiency was detected in Rio Grande among the four cultivars used here. This might be due to tissue culture difficulties with Rio Grande; further improvement of experimental procedures, including transformation steps, would increase mutant isolation efficiency in this cultivar.

Down-regulation of *SlIAA9* [15] and knockout mutants [16, 19] in Micro-Tom and Ailsa Craig have been shown to result in parthenocarpy and morphological changes in leaves. *sliaa9-crispr* mutants in various commercial cultivars also clearly show parthenocarpy, even in the T0 generation, which is useful in crops with agricultural applications. Our results suggest that the function of SlIAA9 in fruit development is

highly conserved among tomato varieties. Leaf morphological changes, however, showed slight differences among cultivars. The leaf morphology changes were more obvious in Moneymaker than in the other three cultivars; knockout plants in Moneymaker exhibited a longer petiole and shorter leaves than in the other cultivars. This might be caused by variations in SlIAA9 function among tomato cultivars. The leaf morphological changes in *sliaa9-crispr* mutants also affect tomato tree forms (Fig. 2). *SlIAA9* downregulation in Ailsa Craig leads to changes in flower morphology [15]; however, we found no apparent differences between *sliaa9-crispr* mutant and wild-type flowers in Ailsa Craig (Fig. 2C). Further study might be needed to identify differences in mutant flowers between knockdown and knockout of *SlIAA9* by CRISPR/Cas9; however, there is the possibility that expression levels of *SlIAA9* might affect flower phenotype.

Agrobacterium-mediated transformation leads to T-DNA insertion into the genome, resulting in generation of a mutant line; however, null-segregant mutant lines generated by CRISPR/Cas9 can be developed through segregation of the T-DNA by self-pollination [20, 21, 22]. In this study, we isolated many null-segregants of *sliaa9-crispr* mutants in T1 progenies. Using conventional PCR-based analysis to test for various regions of the T-DNA in the progeny, no trace of T-DNA was found in the null-segregants. However, it is difficult to eliminate the possibility that short regions of T-DNA have been inserted in the mutant genome that may escape detection by PCR-based analysis. In such cases, alternative technologies such as next generation sequencing might be required. The mutant lines we analyzed for off-target effects (Additional file 1: Figure S6) did not harbor any such small varied sequences that may have occurred upon T-DNA insertion (data not shown). In our PCR analysis, one T1 progeny (AC#33-3) showed an alternate PCR pattern, in which regions 1–8 were amplified, whereas region 9 was not detected, suggesting that the left border (LB)-flanking region of T-DNA was missing, as is sometimes reported in Ti-plasmid-mediated transformation. Finally, Southern blot analysis confirmed the segregation of T-DNA in the null-segregants.

Conclusions

Generally, much time and labor are needed to isolate null-segregant mutants of homozygotes by back-crossing with wild-type. The method used in this study is effective in achieving rapid isolation of null-segregants with high efficiency, while adding important traits to various cultivars at the same time. While classical breeding requires a long time and much labor to produce new traits, the generation of new crop varieties that are otherwise difficult to cross-breed can be achieved using genome editing. Recent studies report transgene free systems as well as novel RNP methods and the optimization of transformation and tissue culture systems [10, 11, 13, 23, 24, 25, 26], which will further increase the advantages of using CRISPR/Cas9 for the production of novel crop varieties in the future.

Methods

Plant materials and growth conditions

Four varieties of *Solanum lycopersicum L.*, *i.e.,* cv. Ailsa Craig (AC), Moneymaker (MM), Super Roma (SR), and Rio Grande (RG), were purchased from Thompson & Morgan (UK: abbreviations used in vector and plant line names). Tomato calli and shoots were grown in a growth chamber under conditions of 21–25◦C with 16 h light at 4000– 8000 lx/8 h dark. Tomato plants were grown in a growth chamber or greenhouse under conditions of 21–25◦C with 16 h light at 7000–10,000 lx/8 h dark.

gRNA and CRISPR/Cas9 vectors

The selected *SlIAA9* target sequence in genomic DNA [Solyc04g076850, 5'-GAGCTCAGGCTCGGTCTACC-3'—named gRNA2 in our previous study [19]—was completely identical in all four tomato cultivars. The CRISPR/Cas9 expression vectors pEgP237-2A-GFP or pEgPubi4_237-2A-GFP [19] (Additional file 1: Figure S1A) were used, which comprise a gRNA under control of the Arabidopsis *U6 snRNA-26* (*AtU6-26*) promoter, and an Arabidopsis codon-optimized spCas9 (AtCas9) fused to GFP via a 2A peptide. *Cas9* expression is driven by the 2 × *CaMV35S* promoter with the omega translational enhancer in pEgP237-2A-GFP, and a parsley *ubi4-2* promoter was used in pEgPubi4_237-2A-GFP. Cas9 contains the 3 × NLS on its C-terminal. In both vectors, *NPT II* was used as a selection marker in plants.

Transformation

The CRISPR/Cas9 expression vector pEgP237-2A-GFP or pEgPubi4_237-2A-GFP (Ueta et al., 2017) was introduced into *Agrobacterium tumefaciens* (*GV2260*) and used to transform tomato leaf disks as described previously [19, 27]. Briefly, leaf discs prepared from cotyledons were soaked in *Agrobacterium* infection medium [3% sucrose, 1 × MS (pH 5.7), 0.0003% 2-mercaptoethanol, 100 μM acetosyringone, *Agrobacterium* (OD_{600} = 0.01)] for transformation and then cultured on co-culture medium [3% sucrose, 1 × MS (pH 5.7), 40 μM acetosyringone] for several days in the dark. The leaf discs were then transferred to callus induction medium (CIM) 1 [3% sucrose, 1 x MS, 100 mg/L kanamycin, 1.5 mg/L trans-zeatin, and 25 mg/L meropenem] and cultured for 4 weeks for cultivars Ailsa Craig, Moneymaker, and Super Roma. Leaf discs from Rio Grande were incubated in CIM2 [3% sucrose, 1 x MS, 100 mg/L kanamycin, 1 mg/L BA, 0.1 mg/L NAA, and 25 mg/L meropenem] for 2 weeks after co-culture, then transferred to CIM1 for 2 weeks. After the appearance of calli, shoot induction medium [3% sucrose, 1 x MS, 100 mg/L kanamycin, 1.0 mg/L trans-zeatin, and 25 mg/L meropenem] was used for further incubation. Transgenic shoots were transferred to root induction medium [1.5% sucrose, 0.5 × MS, 50 mg/L kanamycin, and 25 mg/L meropenem] for 2-4 weeks, and then to soil pots.

Mutation Analyses in CRISPR/Cas9 Target Sites

Genomic DNA was isolated from tomato leaves and fruits using an SDS-based DNA extraction method. Fragments, including the target sequence, were amplified PCR using PrimeSTAR GXL DNA Polymerase (TaKaRa, Japan). The recognition sequence of *Acc*I is included at the predicted DSB position on the *SlIAA9* target sequence. In PCR-RFLP, PCR fragments were digested with *Acc*I (NEB, Japan) and analyzed by agarose-gel electrophoresis. For Sanger sequencing analysis, PCR fragments purified from agarose-gel were cloned by the Seamless ligation cloning extract (SLiCE) method [28] into cloning vector pNEB193 (NEB, Japan). All primers used for PCR are listed in Additional file 1: Table S1.

PCR for detection of null-segregant plants

Nine regions of the T-DNA in the CRISPR/Cas9 vector were selected and amplified using PrimeSTAR GXL polymerase (TaKaRa, Japan). Between 20 and 50 plants in the T1 generation and 8 plants from T2 generations were randomly selected for PCR-based detection of T-DNA regions. All primers used for PCR are listed in Additional file 1: Table S1. The amplicon sizes are also indicated in Additional file 1: Table S1. Technical replicates were performed three times for each line.

Southern blot analysis

Total DNA was extracted from leaves of WT and *sliaa9-crispr* mutants by a CTAB-based DNA isolation method. Briefly, tomato leaves were crushed after freezing with liquid nitrogen, and incubated in 2% CTAB solution. The lysates were extracted with chloroform: isoamyl alcohol (CI) (24:1), and then 1% CTAB solution was added to the water layer. Total DNAs were obtained by CsCl-EtOH precipitation.

Total DNA was digested with *Hpa*I, which has a single restriction recognition site in the vector (Additional file 1: Figure S1), fractionated on 1% agarose gels, and transferred to nylon membrane (Zeta-Probe, Bio-Rad, USA) by capillary transfer. After UV cross-linking (UVP CL-1000 crosslinker, Analytik Jena, Upland, CA, USA), the membranes were hybridized with DIG-labelled probes for *gRNA*, *Cas9*, and *LHCB* (Solyc02g070970) genes. Probes were amplified using pEgP237-2A-GFP (*gRNA* and *Cas9*) or tomato genomic DNA (*LHCB*) using the primers listed in Additional file 1: Table S1, and labeled with DIG by using a DIG High Prime DNA Labeling and Detection Kit (Roche, Basel, Switzerland). Probe labeling, membrane hybridization, and detection were carried out according to the manufacturer's instructions. Hybridization signals were detected and imaged with PXi (SYNGENE, Bangalore, India).

Off-target analysis by deep sequencing using MiSeq

Deep sequencing was performed using MiSeq Reagent Kit v2 Nano (Illumina, Japan). Genomic DNAs, including the region of the CRISPR/Cas9 target sites and off-target sequences, were amplified by PCR.

One off-target candidate sequence (SL2.50 ch6 :26946923-26946946) was selected by Cas-OT to examine the mutation. PCR products were first separated by electrophoresis, then purified from the agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega, Japan) and used as templates for a second round of PCR. Second PCR primers were subjected to TruSeq (Illumina, Japan). All primers used for PCR are listed in Additional file 1: Table S1. MiSeq data was analyzed using CLC Genomics Workbench software version 7.5.1 (CLC bio, Japan), mapped on the off-target candidate using Integrative Genomics Viewer (IGV) (Broad Institute).

Abbreviations

BA: Benzyl adenine

CaMV35S: Cauliflower mosaic virus 35S RNA

CIM: Callus induction medium

CRISPR: Clustered regularly interspaced short palindromic repeats

CTAB: Cetyl trimethylammonium bromide

DSB: Double-stranded DNA break

EMS: Ethyl methanesulfonate

gRNA: Guide RNA

HR: Homologous recombination

LB: Left border

NAA: 1-Naphthaleneacetic acid

NHEJ: Non-homologous end joining

NPBTs: New plant breeding techniques

RNP: Ribonucleoprotein

PAM: Protospacer adjacent motif

PCR-RFLP: Polymerase chain reaction mediated restriction fragment length polymorphism

SLiCE: Seamless ligation cloning extract

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

YO is the guest editor of this journal. The authors declare that they have no competing interests.

Funding

KO was funded by JSPS KAKENHI Grant Number JP19H02932, and YO was founded by Program on Open Innovation Platform with Enterprises, Research Institute and Academia (OPERA). The funders had no role in the experimental design, data collection and analysis or preparation of the manuscript.

Authors' contributions

CH performed most of experiments, analyzed the data, and wrote the manuscript. RU and RH designed gRNA and transformation. YO supervised the research and wrote the manuscript. KO designed, led, and coordinated the overall study. All authors have read and approved the manuscript

Acknowledgments

We thank N. Nakashima, M. Fukuhara, and Dr. N. Kira for their technical assistance. We also thank Dr. K. Yamada, Dr. N. Wada, and Dr. T. Miyaji for their critical discussion of this manuscript.

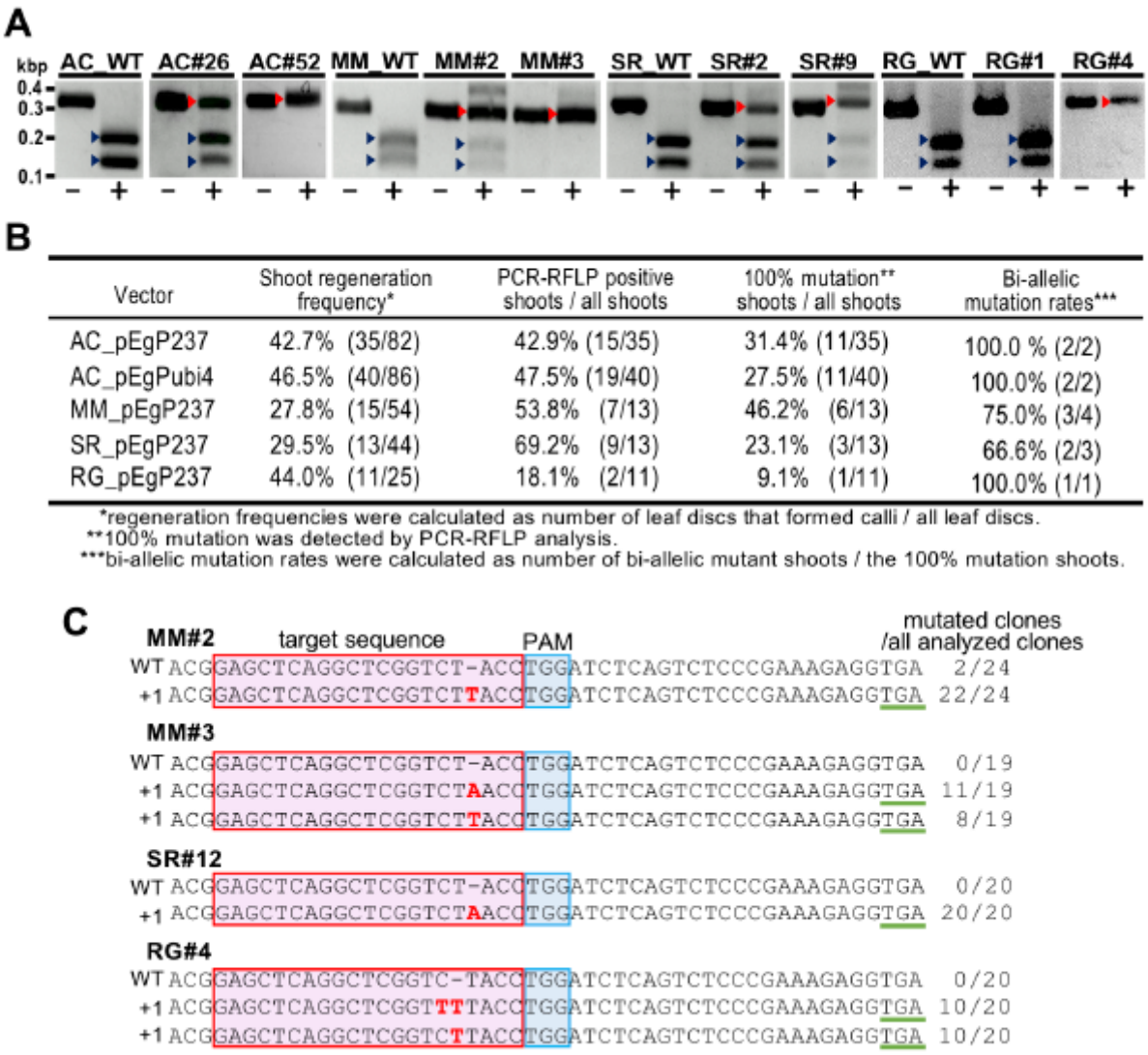
References

1. Osakabe Y, Osakabe K. Genome editing with engineered nucleases in plants. Plant Cell Physiol. 2015;56:389-400. doi: 10.1093/pcp/pcu170.
2. Osakabe Y, Watanabe T, Sugano SS, Ueta R, Ishihara R, Shinozaki K, et al. Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. Sci. Rep. 2016;6:26685. doi: 10.1038/srep26685.
3. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol. 2013;31:686-688. doi: 10.1038/nbt.2650. No abstract available.

4. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat Biotechnol. 2013;31:691-693.
5. Chen R, Xu Q, Liu Y, Zhang J, Ren D, Wang G, et al. Generation of Transgene-Free Maize Male Sterile Lines Using the CRISPR/Cas9 System. Front Plant Sci. 2018;9:1180. doi: 10.3389/fpls.2018.01180.
6. Nishitani C, Hirai N, Komori S, Wada M, Okada K, Osakabe K, et al. Efficient Genome Editing in Apple Using a CRISPR/Cas9 system. Sci Rep. 2016;6:31481. doi: 10.1038/srep31481.
7. Ren C, Liu X, Zhang Z, Wang Y, Duan W, Li S, et al. CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (*Vitis vinifera L.*). Sci. Rep. 2016;6:32289. doi: 10.1038/srep32289.
8. Wang S, Zhang S, Wang W, Xiong X, Meng F, Cui X. Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. Plant Cell Rep. 2015;34:1473–6. doi: 10.1007/s00299-015-1816-7.
9. Voytas DF, Gao C. Precision genome engineering and agriculture: opportunities and regulatory challenges. PloS Biol. 2014;12:e100187. doi: 10.1371/journal.pbio.1001877.
10. Hamada H, Liu Y, Nagira Y, Miki R, Taoka N, Imai R. Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat. Rep. 2018;8:14422. doi: 10.1038/s41598-018-32714-6.
11. Osakabe Y, Liang Z, Ren C, Nishitani C, Osakabe K, Wada M, et al. CRISPR–Cas9-mediated genome editing in apple and grapevine. Nat. Protocol. 2018;13:2844-2863. doi: 10.1038/s41596-018-0067-9.
12. Hua K, Zhang J, Botella JR, Ma C, Kong F, Liu B, et al. Perspectives on the application of genome-editing technologies in crop breeding. Mol Plant. 2019;12:1047-1059. doi: 10.1016/j.molp.2019.06.009.
13. Toda E, Koiso N, Takebayashi A, Ichikawa M, Kiba T, Osakabe K, et al. An efficient DNA- and selectable-marker-free genome-editing system using zygotes in rice. Nat Plants. 2019;5:363-368. doi: 10.1038/s41477-019-0386-z.
14. Goetz M, Vivian-Smith A, Johnson SD, Koltunow AM. AUXIN RESPONSE FACTOR8 is a negative regulator of fruit initiation in Arabidopsis. Plant Cell. 2006;18:1873-86.
15. Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, et al. The Tomato Aux/IAA Transcription Factor IAA9 Is Involved in Fruit Development and Leaf Morphogenesis. Plant Cell. 2005;17:2676-92.
16. Gorguet B, van Heusden AW, Lindhout P. Parthenocarpic fruit development in tomato. Plant Biol. 2005;7:131–9.
17. Zhang J, Chen R, Xiao J, Qian C, Wang T, Li H, et al. A single-base deletion mutation in *SlIAA9* gene causes tomato (*Solanum lycopersicum*) entire mutant. J Plant Res. 2007;120:671-8.
18. Saito T, Ariizumi T, Okabe Y, Asamizu E, Hiwasa-Tanase K, Fukuda N, et al. TOMATOMA: a novel tomato mutant database distributing Micro-Tom mutant collections. Plant cell physiol. 2011;52:283-96. doi: 10.1093/pcp/pcr004.
19. Ueta R, Abe C, Watanabe T, Sugano SS, Ishihara R, Ezura H, et al. Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. Sci. Rep. 2017;7:507. doi: 10.1038/s41598-017-00501-4.

20. Gao X, Chen J, Dai X, Zhang D, Zhao Y. An Effective Strategy for Reliably Isolating Heritable and Cas9-Free Arabidopsis Mutants Generated by CRISPR/ Cas9-Mediated Genome Editing. Plant Physiol. 2016;171:1794-1800. doi: 10.1104/pp.16.00663.
21. Tsutsui H, Higashiyama T. pKAMA-ITACHI Vectors for Highly Efficient CRISPR/Cas9-Mediated Gene Knockout in Arabidopsis thaliana. Plant Cell Physiol. 2017;58:46-56. doi: 10.1093/pcp/pcw191.
22. He Y, Zhu M, Wang L, Wu J, Wang Q, Wang R, et al. Programmed Self-Elimination of the CRISPR/Cas9 Construct Greatly Accelerates the Isolation of Edited and Transgene-Free Rice Plants. Mol Plant. 2018;11:1210-1213. doi: 10.1016/j.molp.2018.05.005.
23. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS, et al. DNA-Free Genetically Edited Grapevine and Apple Protoplast Using CRISPR/Cas9 Ribonucleoproteins. Front Plant Sci. 2016;7:1904. doi: 10.3389/fpls.2016.01904.
24. Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, et al. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol. 2015;33:1162-4. doi: 10.1038/nbt.3389.
25. Kim H, Kim ST, Ryu J, Kang BC, Kim JS, Kim SG. CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat Commun. 2017;8:14406. doi: 10.1038/ncomms14406.
26. Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, et al. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Commun. 2017;8:14261. doi: 10.1038/ncomms14261.
27. Hashimoto R, Ueta R, Abe C, Osakabe Y, Osakabe K. Efficient Multiplex Genome Editing Induces Precise, and Self-Ligated Type Mutations in Tomato Plants. Front Plant Sci. 2018;9: 916. doi: 10.3389/fpls.2018.00916.
28. Motohashi K. A simple and efficient seamless DNA cloning method using SLiCE from Escherichia coli laboratory strains and its application to SLiP site-directed mutagenesis. BMC Biotechnol. 2015;15:47. doi: 10.1186/s12896-015-0162-8.

Figures



Vector	Shoot regeneration frequency*	PCR-RFLP positive shoots / all shoots	100% mutation** shoots / all shoots	Bi-allelic mutation rates***
AC_pEgP237	42.7% (35/82)	42.9% (15/35)	31.4% (11/35)	100.0 % (2/2)
AC_pEgPubi4	46.5% (40/86)	47.5% (19/40)	27.5% (11/40)	100.0% (2/2)
MM_pEgP237	27.8% (15/54)	53.8% (7/13)	46.2% (6/13)	75.0% (3/4)
SR_pEgP237	29.5% (13/44)	69.2% (9/13)	23.1% (3/13)	66.6% (2/3)
RG_pEgP237	44.0% (11/25)	18.1% (2/11)	9.1% (1/11)	100.0% (1/1)

*regeneration frequencies were calculated as number of leaf discs that formed calli / all leaf discs.
**100% mutation was detected by PCR-RFLP analysis.
***bi-allelic mutation rates were calculated as number of bi-allelic mutant shoots / the 100% mutation shoots.

Figure 1

Generation of SlIAA9 mutants in T0 generation. (A) PCR-RFLP analysis of SlIAA9 in CRISPR/Cas9 transgenic plants. Target sites in the SlIAA9 gene were amplified by PCR using total DNA isolated from shoots in sliaa9-crispr T0 mutant lines. PCR products were digested with the restriction enzyme AccI, the recognition site of which is located at the CRISPR/Cas9 target site. −; untreated, +; treated with AccI, red arrowheads; mutated bands, blue arrowheads; wild-type fragments digested with AccI. (B) Mutation efficiency in regenerated shoots. (C) The mutation sequence junctions in the SlIAA9 gene were analyzed by Sanger sequencing. Wild-type sequences (WT) are on top in each alignment. The numbers to the left of the sequences indicate the shift in the mutated nucleotide sequence. The numbers of clones analyzed are shown to the right of the sequences. # numbers; individual T0 lines, red box; target sequence, blue box; PAM sequence, green underline; a newly generated stop codon following CRISPR/Cas9 editing, red characters; mutated sequences. Tomato cultivars: AC; Ailsa Craig, MM; Moneymaker, SR; Super Roma, RG; Rio Grande, WT; wild-type.

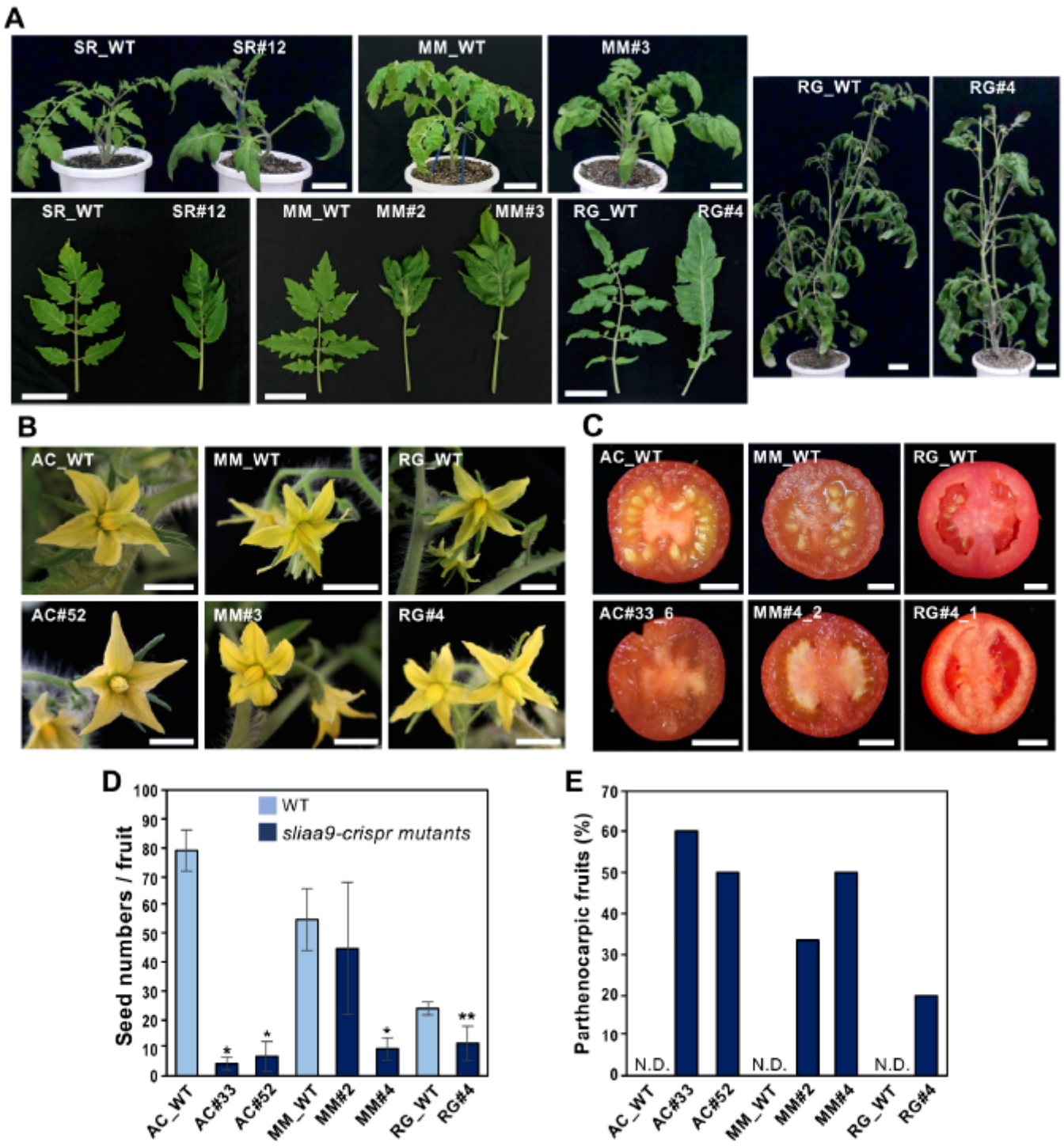


Figure 2

Phenotypes of sliaa9-crispr knockout mutants. (A) sliaa9-crispr T0 mutants of Super Roma (SR), Moneymaker (MM), Rio Grande (RG) and wild-type (WT) exhibit changes in leaf morphology. Names of individual lines are indicated. Bars = 5 cm. Flowers (B) and seedless fruit formation (C) of the sliaa9-crispr T0 mutants of Ailsa Craig (left), Moneymaker (middle), Rio Grande (Right). Bars = 1 cm. (D) Seed formation rates in sliaa9-crispr T0 mutants of Ailsa Craig, Moneymaker, and Rio Grande. Average seed numbers were calculated in mutant fruits (N = 3–12) without pollination and in wild-type (WT) fruits (N

=5–28) with pollination. Error bars indicate SE (standard error). *; P < 0.01. **; P < 0.05. (E) Frequency of seedless mature fruits developed without pollination (parthenocarpic fruits) in sliaa9-crispr mutants. N.D. = not detected.

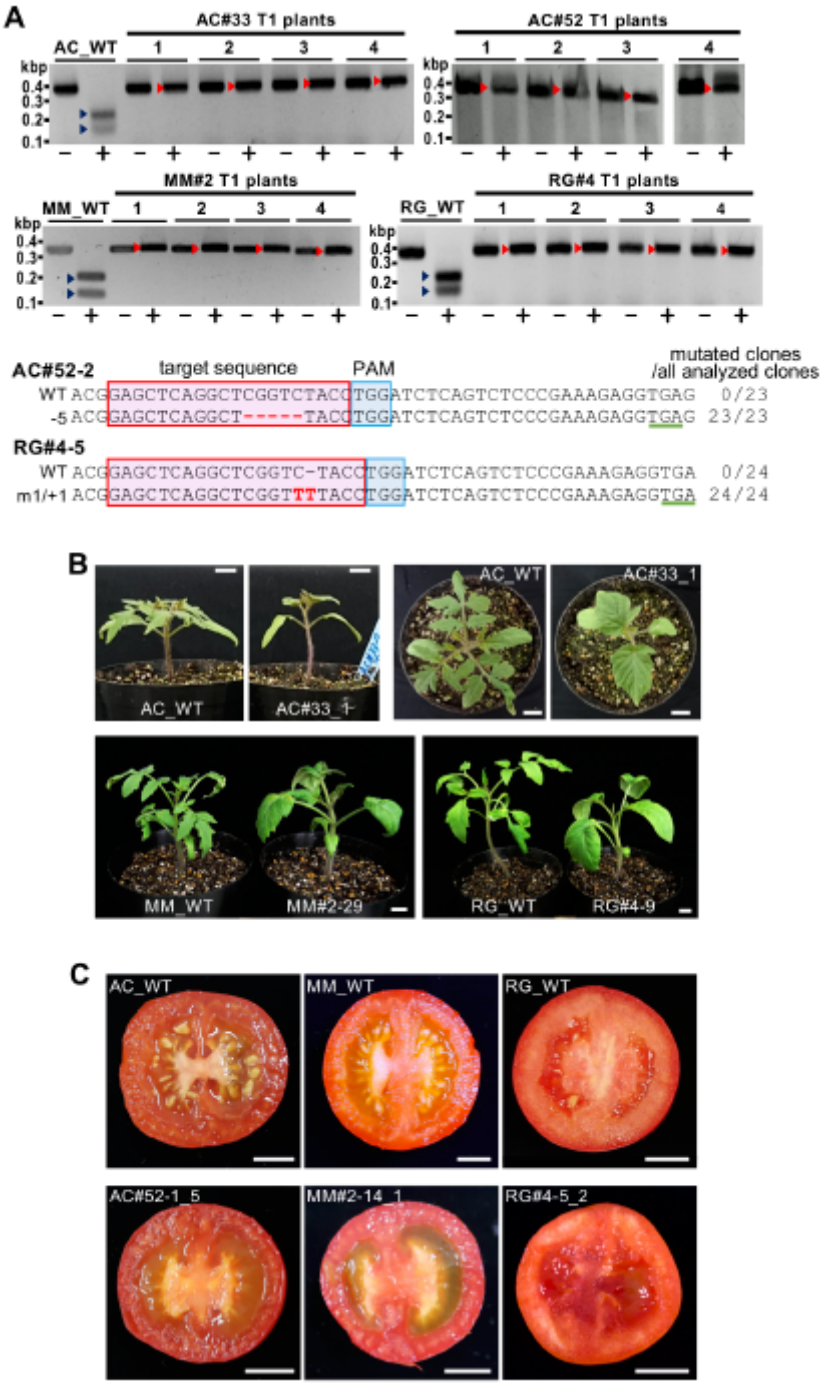


Figure 3

Isolation of the sliaa9-crispr knockout mutants. (A) PCR-RFLP analysis of mutations in sliaa9-crispr T1 plants of cultivars Ailsa Craig, Moneymaker, and Rio Grande. PCR products were digested (+) or not (−) with restriction enzyme AccI. Red arrowheads; mutated bands, blue arrowheads; wild-type fragments digested with AccI. Sanger sequencing analysis of mutations in sliaa9-crispr T1 plants of lines AC#52-2

and RG#4-5. Top line, Wild-type sequence (WT); bottom line, mutant sequence. Numbers of clones analyzed are noted to the right of the sequences. (B) Leaf morphology in young sliaa9-crispr T1 plants (3 weeks after sowing). Bars = 1 cm. (C) Phenotypes of sliaa9-crispr T1 fruits. Bars = 1 cm.

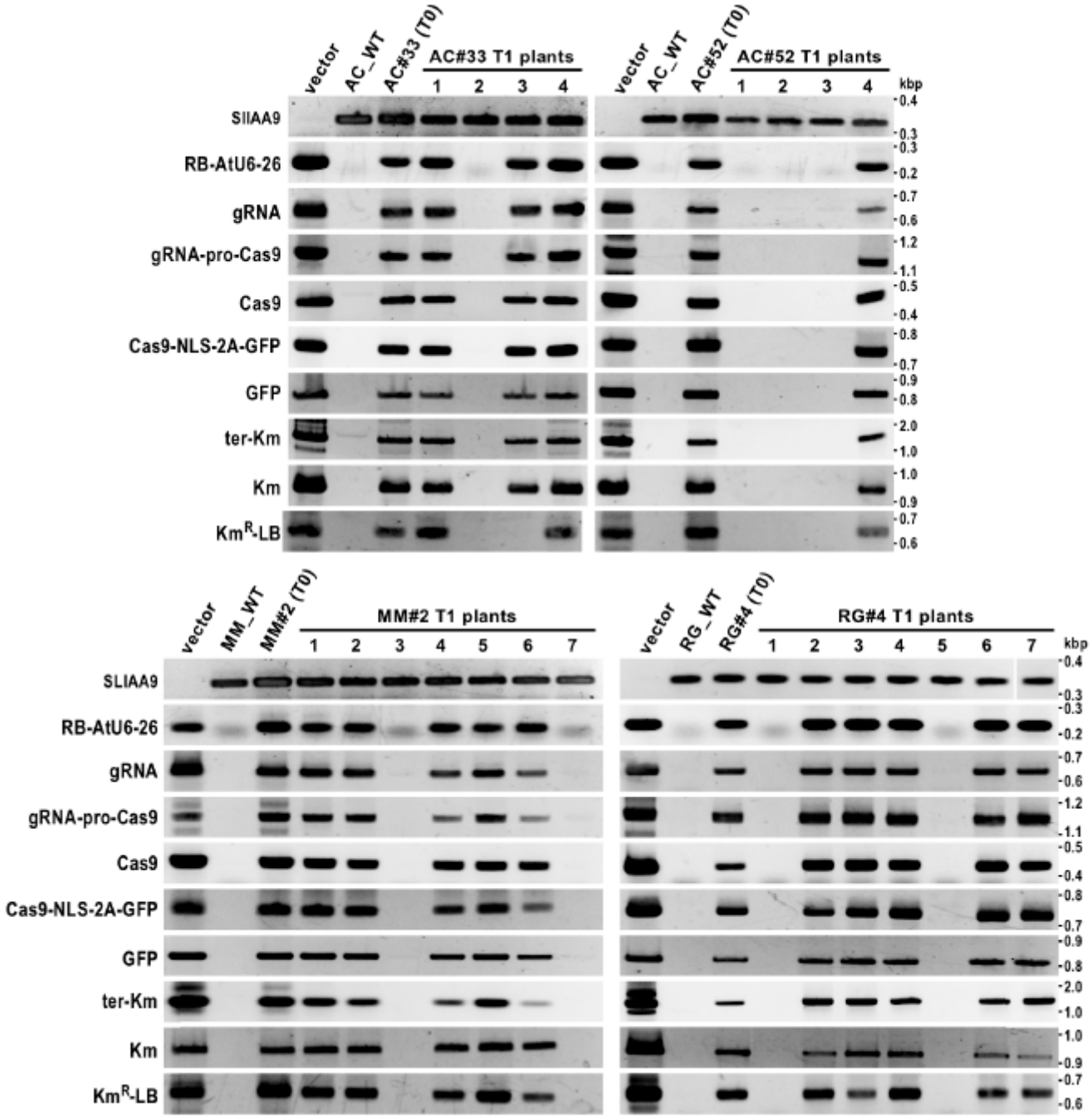


Figure 4

PCR-based T-DNA detection in the T1 generation of the sliaa9-crispr mutants. Detection of T-DNA insertion in sliaa9-crispr T1 plants was performed by PCR using various primer sets designed to cover the whole T-DNA region as indicated by each gene name (Additional file 1: Figure S1). The SlIAA9 gene was

used as an endogenous control. MM; Moneymaker. AC; Ailsa Craig, # numbers; individual T0 lines, numbers; individual T1 lines.

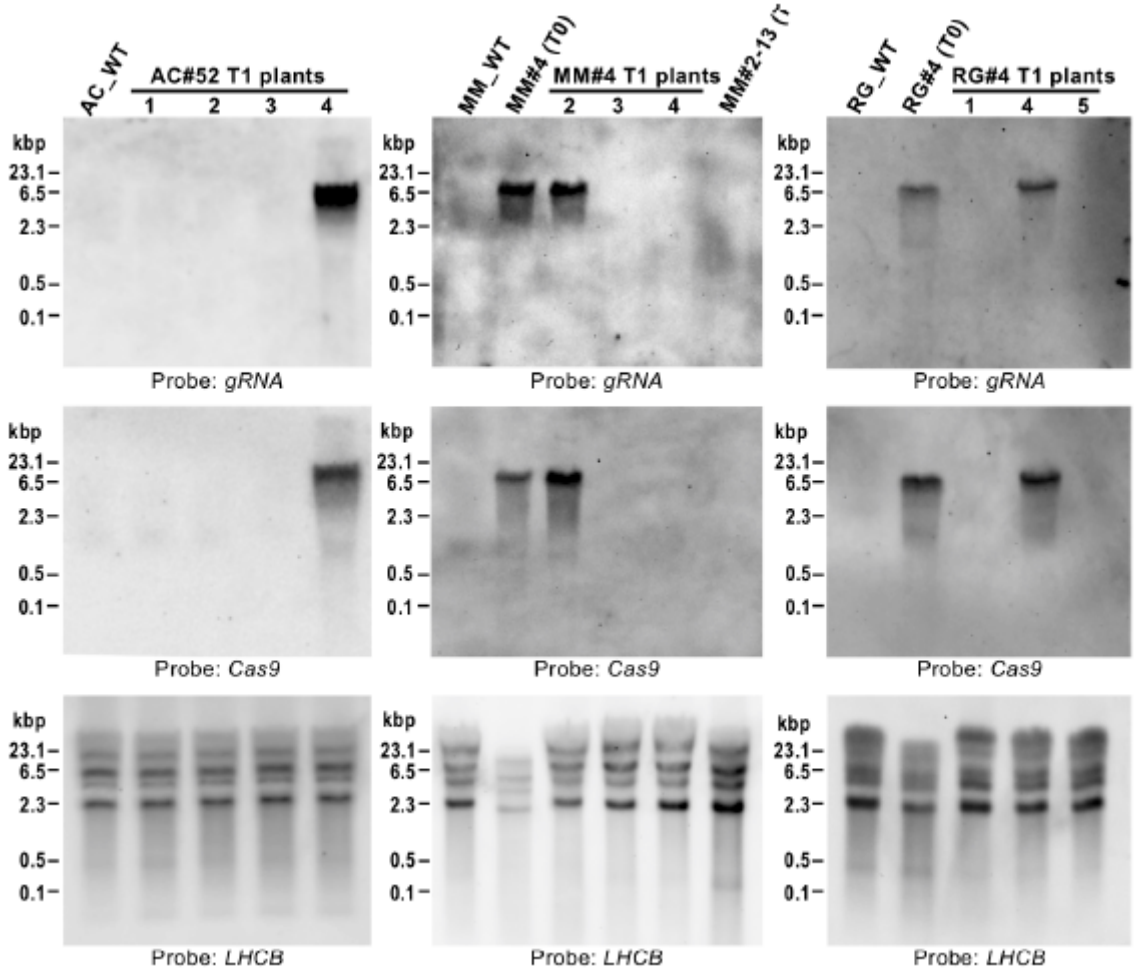


Figure 5

Isolation of null-segregants in the T1 generation of the sliaa9-crispr mutants. Southern blot analysis was performed to isolate null-segregants of sliaa9-crispr mutants. DIG-labeled gRNA and Cas9 probes were used to detect T-DNA insertion in HpaI-digested genomic DNA of sliaa9-crispr mutants. The LHCB gene was used as an endogenous control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementarymaterialsHaraetal.pdf