Generation of Parthenocarpy tomato on multiple commercial cultivars utilizing CRIPR/Cas9 system

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Abstract Tomato (*Solanum lycopersicum* L.) is one of the most important crops in the world for its fruit production. Many critical characteristics related to the quality and quantity of tomatoes have been developed using cutting-edge techniques. Recently, genetic techniques such as gene transformation and gene editing have emerged as powerful tools to promptly generate new plant varieties with superior traits. In this study, we successfully induced parthenocarpy traits in a population of elite tomato (ET) lines. The ET lines were screened for their adaptability to new genetic techniques to determine the lines that performed best by applying *ANT1* gene transformation and *IAA9* knock-out using the CRISPR/Cas9 system. ET5 and ET8 emerged as excellent materials for these techniques due to their high efficiency in applying those modern techniques. Phenotypes of knock-out *iaa9* were clearly shown in T-0 and T-1 plants, in which simple leaves and parthenocarpic fruits were observed. We generated T-DNA-free homozygous *iaa9* knock-out plants in the T-1 generation, indicating the high efficiency of CRISPR/Cas9 in developing new varieties with desired traits in the shortest time possible. Additionally, a simple artificial fertilization method was successfully applied to recover seed production from parthenocarpy plants, securing the varieties for use as breeding materials.

Keywords IAA9. Auxin signaling. CRISPR/Ca9. Elite tomato. Parthenocarpy. ANT1

Introduction

Tomato (*Solanum lycopersicum* L.) is an important fruit or vegetable crop due to its various applications in food production and health benefits. The quality and quantity of tomato are increased quickly in recent years (Grandillo et al. 1999). The breeding, including conventional breeding and modern biotechnology, have been involved in producing elite tomato cultivars which possess superior traits (Abewoy Fentik 2017; Sharma et al. 2019; Xia et al. 2021). New plant breeding technologies such as transgenesis and targeted mutagenesis have been adopted widely in recent years to quickly develop desired traits in tomatoes (Bai 2017; Vu et al. 2020; Tiwari et al. 2023). Gene editing systems, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9), base editors (BEs), and prime editors (PEs), have been applied in crops to target specific genes with high accuracy and produce transgene-free plants (Zhu & Zhu 2022).

Among these tools, CRISPR/Cas9 has shown significant potential in editing beneficial genes, promoting elite tomato production (Li et al. 2018; Pan et al. 2016; Reem & van Eck 2019; Santillán Martínez et al. 2020; Yang et al. 2022). This technology has been used to enhance biotic and abiotic stress resistance, improve fruit quality, and accelerate the domestication of tomatoes (Wang et al. 2019; Tiwari et al. 2023). However, the efficiency of gene editing tools depends on several factors, including the expression level of sgRNA and Cas proteins, the delivery efficiency of CRISPR/Cas reagents, the specificity of the gRNAs, and the transformation and regeneration systems (Ma et al. 2023).

Among the desired traits that were edited in tomatoes, parthenocarpy fruits are in high demand due to its better taste and higher industrial value (Gustafson 1942; Ueta et al. 2017; Xia et al. 2021). Modification of auxin signaling genes such as *SlIAA9*, *SlARF7*, and *SlARF5* resulted in parthenocarpy tomato, confirming the important role of this phytohormone in seed induction (Hu et al. 2018; Ueta et al. 2017). Knocking out the MADS-box gene *SlAGAMOUS-LIKE 6 (SlAGL6)* by CRISPR/Cas9 system resulted in facultative parthenocarpy and improved fruit yield under heat stress conditions (Klap et al. 2017). However, efficient modification of these parthenocarpy genes in commercial tomato cultivars is still limited. *SlAGL6* was successfully mutated in M82 cultivar to induce parthenocarpy (Klap et al. 2017), while seedless phenotype was observed only in Micro-Tom gene-editing *iaa9* (Saito et al. 2011), but not in M82 and Ailsa Crag backgrounds (Tran et al. 2021; Ueta et al. 2017). The *iaa9* parthenocarpic breeding lines were generated by crossing gene-edited Micro-Tom and inbred tomato lines. Although the mutant allele was adopted, only 35.8% of fruits in the T_4 population were seedless, and the fruit weight was significantly reduced (Tran et al. 2021). *Slagl6*/M82 (*sg*), on the other hand, produced around 80% of seedless fruits at R_1 population (Klap et al. 2017). These data raised the need to develop gene editing tools that are more efficient to direct introduce mutation to the parthenocarpy-related genes such as *SlIAA9* in the commercial tomato lines.

SlIAA9, a member of Aux/IAA family, is the key regulator of auxin signaling related to parthenocarpy induction (Wang et al. 2005). This negative regulator of auxin response pathway prevents ovary development prior to pollination via suppression of auxin production in flowers. The down-regulated mutants of *SlIAA9* released auxin signal in young flowers, thus activating the auxin-responsive genes which initiate fruits without fertilization. The *iia9* mutant plants are not shown a reduction of fruit yield as well as fruit weight and flesh consistency (Mazzucato et al. 2015), suggesting a wonderful option to produce parthenocarpy lines while maintaining other superior traits of background cultivars. Besides the parthenocarpy phenotype, *iaa9* mutants showed simple leave architecture, which is a convenient visible marker for breeding and selection on a large scale. However, the performance of the *iia9* mutants differs depending on the genetic backgrounds and the culturing conditions (Abe-Hara et al. 2021; Rahmat et al. 2023).

In recent years, many breeding programs resulted in a large number of tomato cultivars with improved quantity and quality traits (Cappetta et al. 2020). Productivity, sweetness, fruit shape, ripening time, and biotic and abiotic stress tolerance are of the focus of tomato breeders (Acquaah 2016). In a recent research project, a collection of elite tomato (ET) lines was developed in Vietnam through conventional breeding associated with genetic markers selection (unpublished data, preprint available at https://doi.org/10.1101/2022.11.28.518131). The F_8 populations of ET lines showed superior traits such as significantly high yield, early maturing, jointless pedicels, partial parthenocarpy, and TYLCV-resistant alleles harboring. These newly developed tomato lines not only contribute to the country's tomato production improvement but also provide an excellent material source for future breeding.

In order to evaluate the efficacy of gene editing tools in the newly developed ET lines and produce parthenocarpy tomatoes that retain superior traits and adapt well to the local conditions of Vietnam, we employed CRISPR/Cas9-mediated genome editing on the tissue culture of F8 plants to knock-out the *SlIAA9* gene. The gRNA targeted the first exon of the gene, resulting in truncated nonfunctional proteins. The transformation and gene editing efficiencies were evaluated in the T_0 plants. The phenotype of fruits and leaves were estimated in the T_0 and T_1 plants, revealing the variation in adopting genetic modification tools of different tomato lines. Finally, artificial fertilization was applied to recover seeds from the edited lines to prove that the seedless phenotypes were true parthenocarpy.

Materials and Methods

Plant materials and growth conditions

Ten Vietnamese varieties (ET1 – ET10) which were agronomically examined in the previous study (unpublished data, preprint available at https://doi.org/10.1101/2022.11.28.518131) were used as materials for transformation and gene editing. Hongkwang commercial variety was used to control transformation efficiency and gene editing experiments (Vu et al. 2019). Transgenic plants were

grown in soil pots at the greenhouse of Vietnam National University of Agriculture (VNUA) under optimized conditions.

Plasmids and transformation

pANT1ox plasmid (Vu et al. 2019) containing expression cassette of ANT1 driven by CAMV35S promoter was transformed into tested tomato lines to examine gene transformation efficiency.

In order to knock out the *IAA9* gene, the pEG-IAA9 vector was cloned following MoClo (Weber et al., 2011) and Golden Gate (Engler et al. 2014) protocols to introduce indel mutation to exon 1 of *IAA9*. The construct contains a Cas9 expression cassette driven by the CaMV 2x35S promoter and 5'UTR and guide RNA (Ueta et al. 2017) scaffold driven by the U6 promoter (Kamoun Lab, Addgene #46968).

Seven-day-old cotyledons were used to prepare explants for *Agrobacterium*-mediated transformation (Vu et al. 2019). pANT1ox and pEG-IAA9 vectors were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90) using the electroporation method. Ten and seven ET lines were used as materials for the transformation of pANT1ox and pEG-IAA9, respectively. Hongkwang cultivar was used as a control. Purple spots were counted on 21 days post-transformation (dpt). Transgenic shoots were grown in a growth chamber under long-day conditions (16 h light/8 h dark in 25 ºC) until roots were generated and transferred to soil pots. Transgenic plants were grown in the greenhouse until harvest. T-DNA insertion was determined by amplifying a 740-bp fragment within Kanamycin resistant gene located between the left border and right border of Ti plasmid using primer set (NptII-F5, 5'-TGGAGAGGCTATTCGGCTATG-3' and NptII-R5, 5'-CTCGTCAAGAAGGCGATAGAA-3'). Internal control, which is Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified by the primers GAPDH-F, 5'-GATTCGGAAGAATTGGCCG-3' and GAPDH-R, 5'-TCATCATACACACGGTGAC-3' to generate a 606 bp fragment.

Tomato tissue culture

Explants transformed by *A. tumefaciens* were transferred to co-cultivation media containing ABM-MS and 200 µM acetosyringone and were kept under dark condition (25 ºC) for two days. Samples were washed with distilled water containing 500 µM timentin, then transferred to selection media SEL4 (MS Gamborg B5 vitamins 4.4 g/L, MES 0.976 g/L, Maltose 30 g/L, IAA 0.05 mg/L, Zeatin ribose trans isomer 0.5 mg/L, putrescine 1 mM, pH 5.7) containing 300 µM timentin and 80 µM kanamycin. Transformed explants were grown at 28 ºC for 5 days under short-day conditions (8h light/16h dark) and moved to 25 ºC growth chamber under long-day conditions (16h light/8 h dark). Explant subculture to new media was performed every 14 days. The developed true shoots of explants were transferred to rooting media (MS salt containing B5 vitamins, sucrose 20 g/L, NAA 0.1 µM, IBA 0.3 µM, pH 5.8, timentin 300 µM). Transgenic plants were transferred to soil and grown in a greenhouse as described before (Vu et al. 2019).

Sanger sequencing and ICE analysis

Genomic DNA was collected from at least three different leaves of each plant. Edited alleles of IAA9 were identified by conventional PCR from extracted genomic DNA of T-0 and T-1 leaves using primer set (IAA9-F1, 5'-CCCAGGATTGTAGGAGTTATGACATAC-3' and IAA9-R1, 5'-GCCAACCAACAACCTGTGCCCTAA-3') flanking region covering cutting site in exon 1. PCR products were separated in agarose gel 1%, then the specific bands were purified by BioFACT HiGeneTM Gel & PCR purification system (Cat. No. GP104-100). Sanger sequencing was performed by SolGent Analysis Service (SolGent Co., Ltd., 43-10, Techno 5-ro, Yuseong-gu, Daejeon, 34014, Korea) with the sequencing primer (IAA9-sF1, 5'-GAGCTGTGCAGGATTATTAGAG-3'). Edited alleles were visualized on SnapGene software and decomposed using ICE analysis (https://ice.synthego.com).

With samples that produced multiple alleles which could not be decomposed by ICE, PCR products were cloned to pJET1.2/blunt vectors using the CloneJET PCR Cloning kit (Thermo Scientific, Cat. Nos. K1231). Cloned plasmids were transformed into *E. coli* competent cells using the heat shock method, then the transformed cells were grown in Carbenicillin 250mg/L media. Six colonies of each plate were analyzed by Sanger sequencing following plasmid extraction and PCR.

Artificial fertilization

Blooming flowers were shaken with an electric brush for 5–10 seconds. Artificial pollination was performed repeatedly every day in the morning (9:00-10:00 am) during the flowering period.

Results

Transformation efficiency of F_8 lines

In order to examine the ability to adopt foreign genes, the transformation of pANT1ox was performed in all ten ET lines with Hongkwang as a control. Overexpression of ANT1 by pANT1ox construct (Fig. 1A) causes purple spots on tomato callus, in which each spot is correlated to one transformed cell (Fig. 1B). These purple cells can generate purple shoots and plants with purple leaves and fruits (Fig. 1B). After 21 days of transformation, purple spots were counted, and the results of three experiments were shown in Fig. 1C and Supplemental Table 1. Hongkwang (HK) cultivar has been widely used as a model commercial tomato in previous studies (Tran et al. 2021; Vu et al. 2019) due to its high acceptance rate for foreign genes. As a result of this characteristic, it has been a good material for genetic technique application.

Out of 10 lines tested, ET1 and ET5 exhibited transformation efficiencies comparable to the HK. While HK had the highest average number of purple spots (p.p.) (16.94 p.p./explant on average), ET1 and ET5 also showed an increased number of purple spots, which were 16.19 and 16.88 per explant on average, respectively. ET8 (14.32 p.p./explant) and ET9 (12.35 p.p./explant) displayed a slightly lower transformation efficiency than HK, but it was relatively high compared

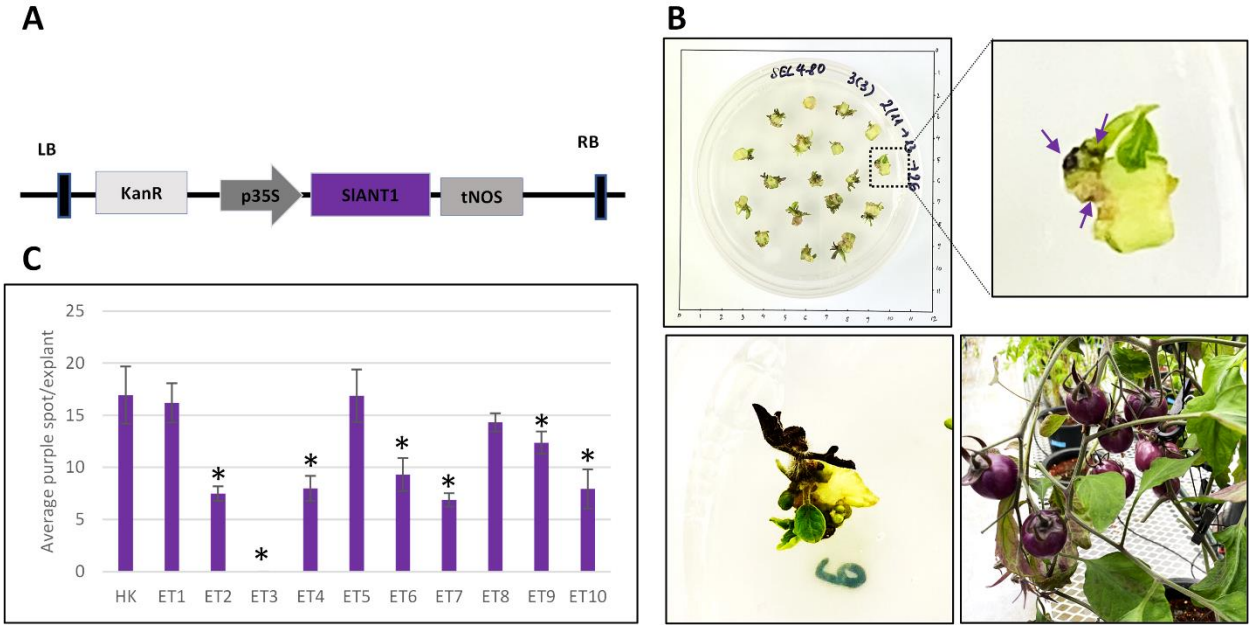

Fig. 1 Transformation efficiency. 10 selected F8 lines were used as materials for transformation of SlANT1 overexpressing construct. 7-day-old cotyledons were cut from seedlings and were transformed by SlANT1 constructs, then grown on Kanamycin selection media. At least 30 explants of each line were used in one experiment. Three biological repeats were conducted. A: pANT1 construct. KanR: Kanamycin resistance gene, P35S: CAMV35S long-promoter. B: ANT1 expression in tomato cotyledon (representative pictures). Upper panel: ANT1 expression at 21 days after transformation (DAT). Purple arrows indicated purple spots. Lower panel: purple shoots (left) and fruits (right). C: Transformation efficiency. x-axis showed an average number of purple spots per explant; y-axis indicated tomato F_8 lines tested in this experiment.

with the remaining lines. Remarkably, ET1 and ET8 were the progeny of the same parental lines, so their high transformation efficiency might be a heritable trait. However, this correlation was not observed between ET9 and ET4, which were generated from the same parents, suggesting segregation of the trait.

Notably, ET3 did not show any purple spot in all three replicates, indicating that pANT1-ox did not work in this line (Fig. 1C and Supplemental Table 1)**.** While the seedling of other lines exhibited purple shoots, ET3 had green shoots. Additionally, segregation lines of ET2, ET4, ET5, and ET9, which also had green shoots, were tested, but none of them showed any purple spots (Supplemental Table 2). These findings suggest that the ANT1 gene was not expressed in the green shoot plants, and the suppression of the ANT1 expression pathway resulted in the inhibition of anthocyanin production in both shoots and calluses. Based on the transformation efficiency and agronomical performance, 7 out of 10 ET lines were selected for gene editing experiments. Between ET1 and ET8, which were generated from the same parents' combination, ET8 was chosen for being superior in productivity. ET3 and ET10 were eliminated due to low or undermined transformation efficiency (Fig. 1C) and a high rate of contamination (data not shown).

Design of gene editing construct to knock-out IAA9 gene in the ET lines

gRNAs for knocking out IAA9 gene were tested in Micro-Tom and Ailsa Crag varieties by Ueta et al. (2017). Although all the gRNAs were effective, the editing efficiency was significantly lower in Ailsa Crag compared to Micro-Tom. Furthermore, the parthenocarpy fruits were not reported in the AC background. To choose the most appropriate gRNA, the sequence of IAA9 gene in the ten ET lines was compared with that of HK and Micro-Tom to examine the sequence variability.

gRNA3 (20 bp) position showed 100% sequence match in the exon 1 of all the ET lines (Supp. Fig. 1), so we chose this gRNA for knocking-out *IAA9* gene in the tested lines. The *AtU6-9gRNA-7xT* fragment was cloned into level-1 plasmid (*SlIAA9-gRNA3*) (Supp. Fig. 2A). Enzyme digestion by *BpiI* of three clones shows the expected 4352 bp and 203 bp bands, and Sanger sequencing confirmed the correct level-1 sequences (Supp. Fig. 2B-C). The level-2 construct *pGE-IAA9* was designed and cloned using Golden Gate system. Expression cassette of Kanamycin resistance gene *NptII* under the regulation of NOS promoter and OCS terminator with the omega enhancer sequence was placed in position 1 of *pGE-IAA9* (Fig. 2A). SpCas9 driven by 2x35S CaMV promoter and NOS terminator and *AtU6-9gRNA-7xT* scaffold were placed in position 2 and position 3 of the level-2 plasmid, respectively. Enzyme digestion using *NheI* and *PmlI* showed expected bands of 5333 bp, 3977 bp, and 2758 bp. Additionally, Sanger sequencing revealed that the construct was cloned successfully with correct junctions (Supp. Fig. 3).

IAA9 gene editing efficiency of tested lines

pEG-IAA9 construct (Fig.2A) was expected to introduce indel mutations into exon 1 of the *SlIAA9* gene, thereby knock-out or knock-down this gene in the studying ET lines. The highest number of true shoots (63) and T0 plants (59) were generated from 120 transformed explants of ET8 **(**Fig.2B**).** Among them, 40 plants carried T-DNA insertion of the construct, which was 33.33%, and 19 plants, equal to 15.83% exhibited the simple leaves phenotype. Despite only 27 true shoots (27%) generated from ET5, we obtained 25 T0 plants, with 13% exhibiting the simple leaves phenotype, making ET5 the second most promising candidate for IAA9 gene editing. The percentage of simple-leaf plants generated from ET2, ET4, and ET9 was relatively low at 3%, 2.5%, and 1.67%, respectively. ET6 and ET7 produced a small number of true shoots (10% and 11.25%), and no plants with the simple leaf phenotype were observed.

The leaf and fruit phenotypes of T_0 plants were affected by the ratio of knock-out alleles. For example, plant ET5-#2, which showed normal leaves, had 15% knock-out alleles, whereas

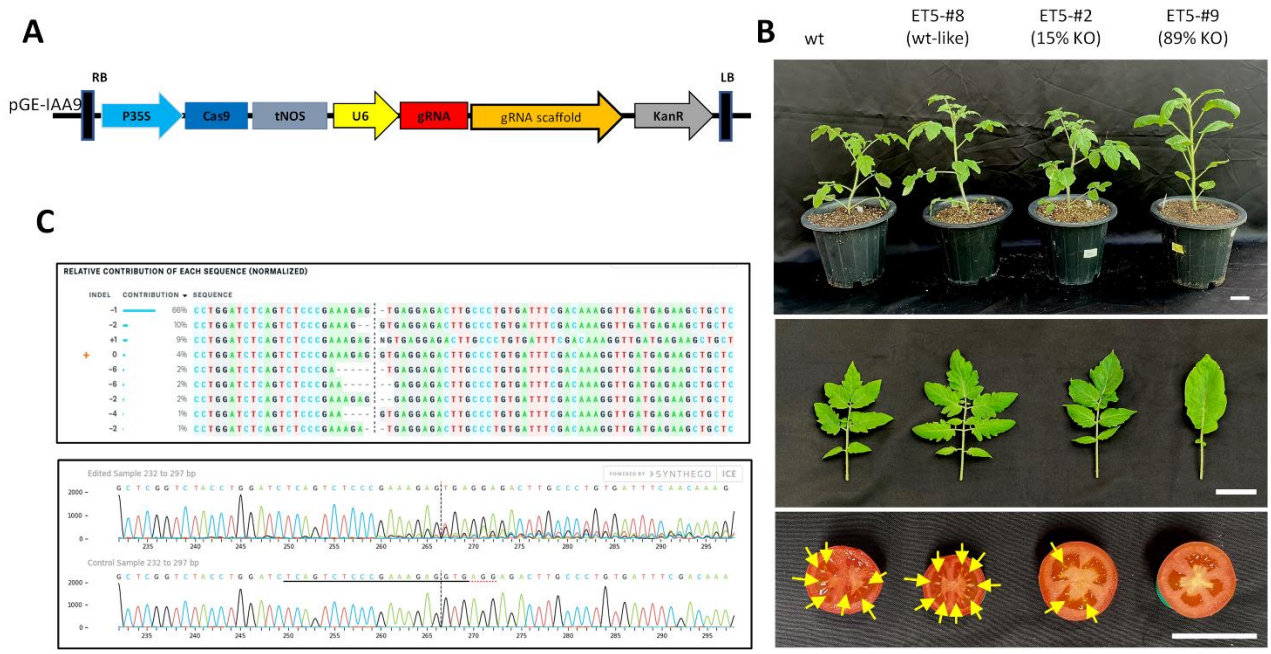

Fig 2. Gene editing on elite tomato lines. Cotyledons of 7-day-old plants were transformed by Agrobacterium carrying Cas9-gRNA^{IAA9} plasmids, then grown and selected by antibiotic-containing shoot induction media. A: Map of IAA9 editing construct. Cas9 protein was expressed under the CAMV35S promoter, while gRNA was expressed under U6 promoter. KanR: Kanamycin-resistant gene. B: IAA9 gene edited T_0 plants (representative pictures). Shoot was transferred into root induction media for 3 weeks before moving to the soil. Pictures of T0 plants (upper panel) and leaves (middle panel) were captured 4 weeks after moving to the soil. Yellow arrows in the lower panel indicate seeds in fruits of T_0 individuals. Bar = 5cm. C: Allele contribution in plant ET5-#9. *IAA9* sequences were analyzed by ICE-Synthego online software (https://ice.synthego.com). Sequences of mutated alleles were compared with wild-type (marked with "+" symbol) and proportions of each allele were shown. The black vertical dotted line indicates the cut site.

ET5-#9 had 89% knock-out alleles and displayed simple leaf phenotypes (Fig. 2C). Mildly affected phenotype plants such as ET5-#2 fruits had few seeds, which was less than ten seeds per plant. Severe knock-out plants such as ET5-#9 showed parthenocarpy fruits. The T0 generation genotype and phenotype are summarized in Table 1 and Table 2. No T-DNA-free plants exhibiting the simple leaves phenotype were observed in the T_0 population.

Up to ten plants of each ET line were subjected to sequencing analysis. Sequence analysis through Sanger sequencing and pJET cloning followed by ICE analysis revealed that multiple alleles were generated in each edited T_0 plant (Fig. 2D, Supp. Fig. 4). Most frame-shift mutations led to truncated proteins (Supplemental Table 3). The phenotypes of the plant were determined by the dominant alleles in the pool. In the case of ET5-#9, 89% of alleles were knocked-out; and

Table 1 Assessment of transformants obtained from the transformation of pEG-IAA9 construct on tested tomato lines

Lines	Explants	True shoots	T0 plants	T-DNA insertion plants	Simple leaf plants	Seedless
ET2	100	18 (18%)	13 (13%)	10 (10%)	3 (3%)	3 (3%)
ET4	120	47 (39.17%)	13 (10.83%)	9 (7.5%)	2 (1.67%)	1 (0.83%)
ET5	100	27 (27%)	25 (25%)	21 (21%)	9 (9%)	13 (13%)
ET6	100	10 (10%)	1 (1%)	1 (1%)	0	0
ET7	80	9 (11.25%)	1 (1.25%)	1 (1.25%)	0	0
ET8	120	63 (52.5%)	59 (49.17%)	40 (33.33%)	15 (12.5%)	7 (5.83%)
ET9	120	14 (11.67%)	7 (5.83%)	6 (5%)	2 (1.67%)	1 (0.83%)

True shoots were transferred into root induction media to generate T0 plants. T-DNA insertion was confirmed by PCR using Kanamycine resistant gene (*NptII*) primers. Leaf shape was observed on mature T0 plants.

Table 2 Genotyping and phenotyping of IAA9 mutant T-0 plants generated by CRISPR/Cas9 system

No.	Plant #	T-DNA insertion	Reading score	Mutation rate	KO score	Simple leaf	Seed phenotype
1	ET2-#1	X	1	0	0	N	N
2	ET2-#2	O	0.97	0.52	0.52	N	X, F
3	ET2-#3	O	0.94	0.94	0.47	N	X
4	ET2-#4	O	0.94	0.94	0.48	O	F
5	ET2-#5	O	0.98	0.27	0.27	N	F
6	ET2-#6	O	0.97	0.2	0	N	N
7	ET2-#7	O	1	0	0	N	F
8	ET2-#8	O	0.9	0.62	0.62	O	X
9	ET2-#9	O	1	0	0	N	F
10	ET2-#10	O	-	0.33*	0.16*	N	F
11	ET4-#1	O	1	0	0	N	N
12	ET4-#2	X	1	0	0	N	N
13	ET4-#3	X	1	0	0	N	N
14	ET4-#4	X	1	0	0	N	N
15	ET4-#5	O	1	0	0	N	N
16	ET4-#6	O	1	0	0	N	N
17	ET4-#7	O	0.97	0.49	0.49	O	N
18	ET4-#8	O	1	0	0	N	N
19	ET4-#9	O	1	0	0	N	N
20	ET4-#10	O	0.96	0.67	0.67	O	X
21	ET5-#1	O	-	1*	1*	O	X
22	ET5-#2	O	0.86	0.17	0.17	N	F
23	ET5-#3	X	1	0	0	N	F

24	ET5-#4	O	-	0.83*	0.83*	N	X
25	ET5-#5	O	-	1*	0.83*	O	X
26	ET5-#6	O	0.96	0.68	0.08	N	F
27	ET5-#7	O	0.97	0.93	0.93	O	X
28	ET5-#8	O	1	0	0	N	X
29	ET5-#9	O	0.97	0.93	0.89	N, O	X
30	ET5-#10	X	-	0*	0*	N	N
31	ET6-#1	O	0.98	0.2	0.14	N	N
32	ET7-#1	O	0.91	0.91	0.61	N	X
33	ET8-#1	O	0.97	0.93	0.93	O	N
34	ET8-#2	O	1	0	0	N	N
35	ET8-#3	X	1	0	0	N	N
36	ET8-#4	O	0.99	0.19	0.19	N	N
37	ET8-#5	O	0.98	0.94	0.94	N, O	X
38	ET8-#6	O	0.96	0.51	0.51	N	F
39	ET8-#7	O	0.96	0.95	0.85	O	X, N
40	ET8-#8	X	1	0	0	N	N
41	ET8-#9	O	0.97	0.85	0.8	O	F
42	ET8-#10	O	0.97	0.8	0.71	O	X
43	ET9-#1	O	0.99	0.07	0.07	N	F
44	ET9-#2	O	0.95	0.56	0.43	N	N
45	ET9-#3	X	1	0	0	N	N
46	ET9-#4	O	1	0	0	N	N
47	ET9-#5	O	1	0	0	N	N
48	ET9-#6	O	-	1*	1*	O	X
49	ET9-#7	O	0.95	0.95	0.95	O	N

KO: knock-out; X: no T-DNA insertion/seedless; O: has T-DNA insertion/simple leave phenotype; N: normal leaf shape/ normal seed number; F: few seeds (<10 seeds/fruit). Asterisks (*) represent data obtained by PJET cloning followed by Sanger sequencing and ICE analysis.

among them, 66% were one-nucleotide deletion alleles. This mixture of alleles resulted in simple leaves and seedless fruits phenotype. At T-1 generation, homozygous plants of alleles such as -1, -2, -4, -5, -6, -7, +1 bp were generated. Heterozygous and chimeric genotypes were also observed and summarized in Table 3**.** In one case, a rather long deletion of 16 nucleotides at the cutting site of *IAA9* was inherited in ET2-#5-5. Additionally, ET8#10-1 showed a significantly long deletion allele of -218 bp, together with a -1 bp mutant allele, and both plants expressed simple leaves phenotype. Besides, all the homozygous knock-out T1 plants showed a simple leaf phenotype (Fig.3A, Table 3). Furthermore, there are heterozygous and chimeric plants that contain up to four edited alleles of *IAA9*. Indeed, four edited T-1 plants were T-DNA-free, including a homozygous plant with two nucleotide deletion alleles (ET2-#2-5). These results indicate that the edited alleles were stably inherited in subsequent generations.

Table 3. Genotyping and phenotyping of *iaa9*-T1 generation

Plant#	IAA9 sequence	Editing pattern	Allele contribution	KO rate (%)	Genotype	Leaf shape	T-DNA insertion	Seed phenotype
wt	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	100%	homo	N	X	N
ET2-#2-1	TCTCAGTCTCCCGAAAGAGGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	+1	100%	100%	homo	S	O	X
ET2-#2-2	TCTCAGTCTCCCGAAAGAGGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	+1	100%	100%	homo	S	O	NF
ET2-#2-3	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	100%	100%	homo	S	O	X
ET2-#2-4	TCTCAGTCTCCCGAAAGAGGNTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	+1	48%	97%	chimeric	S	O	X
	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	45%					
	TCTCAGTCTCCCGAAAGA - - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	4%					
ET2-#2-5	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	100%	100%	homo	S	X	NF
ET2-#2-6	TCTCAGTCTCCCGAAAGAGGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	+1	100%	100%	homo	S	O	X
ET2-#2-7	TCTCAGTCTCCCGAAAGAGGNTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	+1	48%	97%	chimeric	S	O	X
	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	45%					
	TCTCAGTCTCCCGAAAGA - - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	4%					
ET5-#9-1	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	O	N
ET5-#9-2	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	O	N
ET5-#9-3	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	X	N
ET5-#9-4	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	O	N
ET5-#9-5	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	O	N
ET5-#9-6	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	X	N
ET5-#9-7	TCTCAGTCTCCCGAAAGAGGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	0	100%	0%	homo	N	O	N
ET7-#1-1	TCTCAGTCTCCCGA - - - - GGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	100%	100%	homo	S	O	N
ET7-#1-2	TCTCAGTCTCCCGA - - - - GGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	100%	100%	homo	S	O	F
ET7-#1-3	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	100%	0%	homo	N	X	N
ET7-#1-4	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	100%	0%	homo	N	O	N
ET7-#1-5	TCTCAGTCTCCCGA - - - - GGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	100%	100%	homo	S	O	X
ET7-#1-6	TCTCAGTCTCCCGA - - - - GGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	100%	100%	homo	S	O	X

ET7-#1-7	TCTCAGTCTCCCGAA - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	47%	47%	chimeric	N	O	N
	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	44%					
	TCTCAGTCTCCCGA - - - - - - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	5%					
ET7-#1-8	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	100%	0%	homo	N	X	N
ET7-#1-9	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	100%	0%	homo	N	X	N
ET7-#1-10	TCTCAGTCTCCCGAA - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	46%	46%	chimeric	N	O	N
	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	43%					
	TCTCAGTCTCCCGA - - - - - - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	7%					
ET7-#1-11	TCTCAGTCTCCCGA - - - - GGTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-4	100%	100%	homo	S	O	N
ET8-#7-1	TCTCAGTCTCCCGAAAGAG -TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-1	48%	97%	chimeric	S	O	R
	TCTCAGTCTCCCGA - - - - - - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-7	41%					
	TCTCAGTCTCCCGAAAGA - - - - - - - AGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-7	6%					
ET8-#7-2	TCTCAGTCTCCCGAAAGAG - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-1	100%	100%	homo	S	O	X
ET8-#7-3	TCTCAGTCTCCCGAAAGAG - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-1	100%	100%	homo	S	O	X
ET8-#7-4	TCTCAGTCTCCCGAAAGAG - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-1	100%	100%	homo	S	O	X
ET8-#7-5	TCTCAGTCTCCCGAAAGAG - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-1	48%	95%	chimeric	S	O	R
	TCTCAGTCTCCCGA - - - - - - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-7	40%					
	TCTCAGTCTCCCGAAAGA - - - - - - - AGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-7	7%					
ET8-#7-6	TCTCAGTCTCCCGAAAGAG - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-1	47%	95%	chimeric	S	O	R
	TCTCAGTCTCCCGA - - - - - - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-7	38%					
	TCTCAGTCTCCCGAAAGA - - - - - - - AGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-7	10%					
ET8-#9-1	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	46%	45%	chimeric	N	O	N
	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	37%					
	TCTCAGTCTCCCGAAAGAG - -GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	8%					
	TCTCAGTCTCCCGA - - - - - - TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	2%					
ET8-#9-2	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	44%	45%	chimeric	N	O	N
	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	37%					
	TCTCAGTCTCCCGAAAGAG - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	8%					

	TCTCAGTCTCCCGAA - - - - - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	3%					
ET8-#9-3	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	45%	46%	chimeric	S	X	F
	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	40%					
	TCTCAGTCTCCCGAAAGAG - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	6%					
	TCTCAGTCTCCCGAA - - - - - - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	1%					
ET8-#9-4	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	100%	100%	homo	S	O	N
ET8-#9-7	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	45%	45%	chimeric	N	O	N
	TCTCAGTCTCCCGAAAG - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	38%					
	TCTCAGTCTCCCGAAAGAG - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-2	7%					
	TCTCAGTCTCCCGAA - - - - - - - GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	2%					
ET8-#9-8	TCTCAGTCTCCCG - - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	100%	0%	homo	N	O	N
ET8-#9-9	TCTCAGTCTCCCG - - - - - - GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGAT	-6	100%	0%	homo	N	X	N

KO: knock-out; X: no T-DNA insertion/seedless; O: has T-DNA insertion/simple leave phenotype; N: normal leaf shape/ normal seed number; F: few seeds (<10 seeds/fruit).

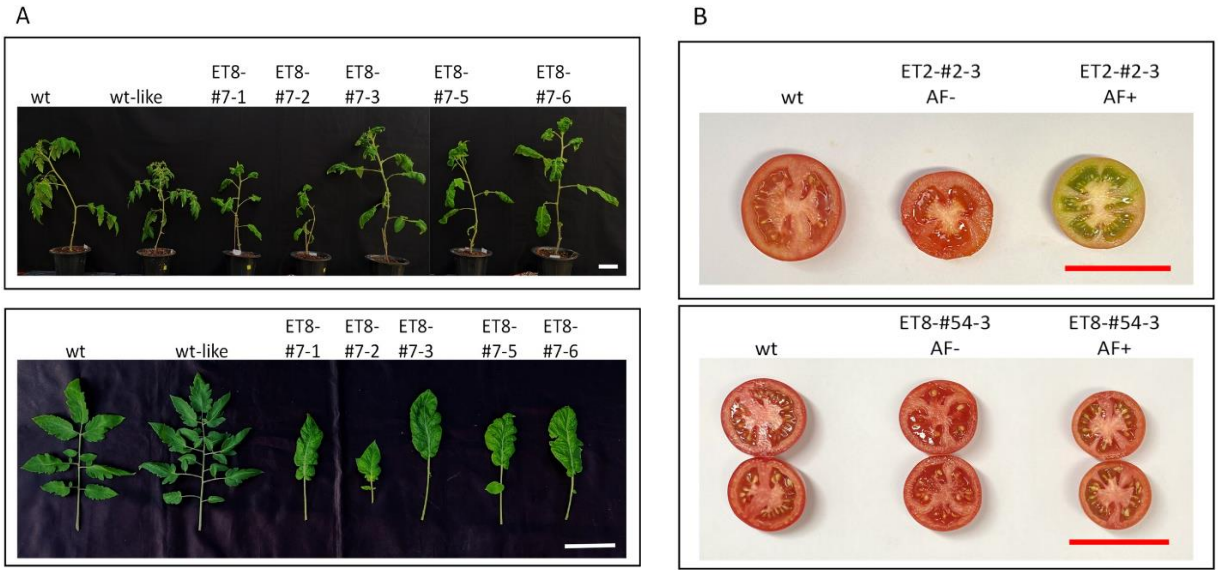

Fig 3. Phenotype of IAA9-knockout T1 generation. Seeds were obtained from T0 plants and were selected based on leaf phenotypes and T-DNA insertion. A. Plant and leaf shape of representative IAA9 editing T1 lines generated from ET8-#7 (T0). B. Seed phenotypes of T1 fruits. wt: wild-type; AF-: fruits without artificial fertilization; AF+: Fruit with artificial fertilization. White bars = 10cm; red bars = 5cm

Artificial fertilization helps recover seeds from parthenocarpy lines

Artificial fertilization was conducted by shortly shaking young to blooming flowers using an electric toothbrush. In the absence of artificial pollination, the original fruits of T1 knock-out exhibited either no seed (ET2-#2-3) or a low number of seeds (ET8-#54-3). However, the artificially fertilized ones showed as many seeds as the wild types (Fig. 3B). This phenomenon was observed in various lines from different backgrounds, indicating that malfunctioning of *IAA9* promoted fruiting without fertilization, while pollen viability remained unaffected. The parthenocarpy phenotype, therefore, is greatly influenced by culturing conditions. This observation can serve as a useful method to obtain seeds for maintaining true parthenocarpy lines without the need for tissue culture or complicated propagation methods.

Discussion

Anthocyanin markers work selectively between the ET sublines

Anthocyanin production has been widely used as a visual selection marker in plant transformation as well as in CRISPR technology (Kortstee et al. 2011; Ruan et al. 2021). Overproduction of the *ANT1* gene generated high anthocyanin pigment in transformants indicating the approximate level of transformation in plant tissues (Vu et al. 2019). However, the result in Fig. 1C and Supplemental Table 1 suggest that the marker did not work in ET3, whose young shoot

color was green instead of purple. A rough test experiment revealed that all the green shoot sub-lines of other ET lines failed to produce anthocyanin pigments despite the pANT1-ox transformation effort (Supplemental Table 2). It is suggested that in green-shoot tomato lines, there is an ANT1-inhibition mechanism that prevents this gene from producing purple spots. One or more of the structural genes involved in ANT1-dependent anthocyanin biosynthetic pathways might malfunction, including early enzymes such as CHS, CHI, and F3H, or late enzymes such as DFR and 3-GT (Mathews et al. 2003; Schreiber et al. 2012). These data proved that not all visual markers could be used to assist genome editing and it is important to understand the genetic background of the materials.

Transformation and editing efficiency vary among tomato lines

The editing efficiency of *IAA9* differs between Micro-Tom and the commercial line Ailsa Craig (Ueta et al. 2017). The use of pEgP237-gRNA2(20b)-2A-GFP resulted in a 100% mutation rate in Micro-Tom shoots but only 42.9% in Ailsa Craig shoots. Unlike Micro-Tom, Ailsa Crag edited plants were not reported to produce parthenocarpy fruits (Tran et al. 2021; Ueta et al. 2017). Table 1 and Table 2 reveal a high degree of variation in both transformation and editing efficiency among the tested population of ET lines. While some of these lines share parental origins, they do not exhibit the same level of foreign construct adoption potential. Notably, the transformation efficiency using pANT1-ox plasmid and pEG-IAA9 are closely correlated in elite lines such as ET5 and ET8 (Table 1 and Supplemental Table 1). ET5 presented 16.88 purple spots per explant on average, and 21% of pEG-IAA9 transformed plants possessed T-DNA insertion. These numbers in ET8 were 14.32 and 33.33%, respectively. These two lines are the best F8 ET lines to be used as materials for genetic modification technologies due to their well-response to foreign gene transformation. Between these two lines, ET5 showed higher editing efficiency, presented by the amount of simple leaf and seedless plants in the T-0 population (Table 1). However, the high productivity and survival rate of ET8 benefit this line to maintain and transfer the edited alleles to the next generations (Table 3). For the generation of commercial genome editing tomato, ET8 is the best recommended option which provides beneficial traits such as high yield, high transformation efficiency, and low fruit cracking ratio (unpublished data, preprint available at https://doi.org/10.1101/2022.11.28.518131).

Seed number of parthenocarpy lines is culture-dependent

The number of seeds in IAA9-edited tomatoes exhibited variability not only between different editing events but also within each T-1 subline that has a similar genetic background. The phenotypes observed regarding seed number in T-0 and T-1 plants included the standard seed forming, a few seeds (less than ten seeds in each fruit), and zero seeds, which are referred to as seedless. Additionally, even within a single plant, each fruit could produce a different number of seeds. However, applying artificial fertilization to parthenocarpy plants significantly recovers seeds in T-1 plants (Fig. 3B). This result revealed that culturing conditions such as physical

impacts on blooming flowers would easily lead to a change in the seed number of the fruits. Parthenocarpy generated by modifying auxin biosynthesis and regulation pathways is known to induce fruit forming without fertilization (Wang et al. 2005; Wang et al. 2009), so the fruit forming would be initiated earlier than normal. It is predicted that natural physical impacts on young and blooming flowers, such as wind and insects, can restore seed formation in parthenocarpy fruits. This characteristic could be exploited to develop an efficient method for maintaining parthenocarpy lines and to consult the optimal conditions to produce a high proportion of seedless tomatoes.

Author contribution C.C.N. and J.-Y.K. conceived the idea and designed the experiments. C.C.N. designed experiments, performed experiments, analyzed data, and wrote the manuscript; C.C.N., T.V.V., and N.N.T. performed the experiments; R.M.S. contributed to the interpretation and draft of the output; R.M.S., T.V.V., J.-Y.K. and T.D.K revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material The data used in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable

Conflict of interest The authors declare that they have no conflicts of interest.

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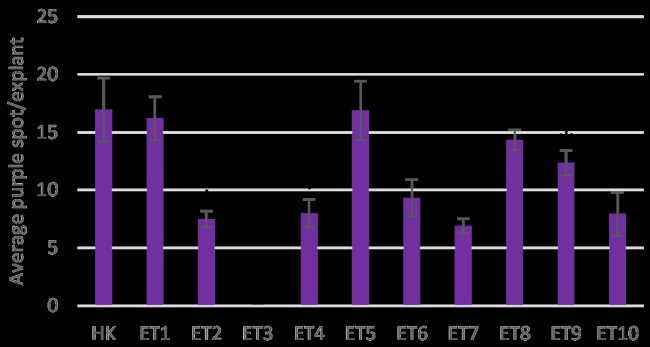
KanR
p35S
SlANT1
tNOS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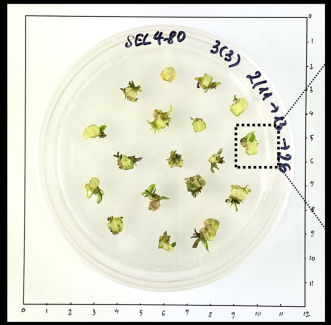
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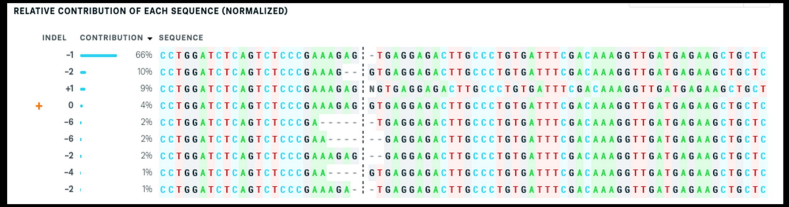
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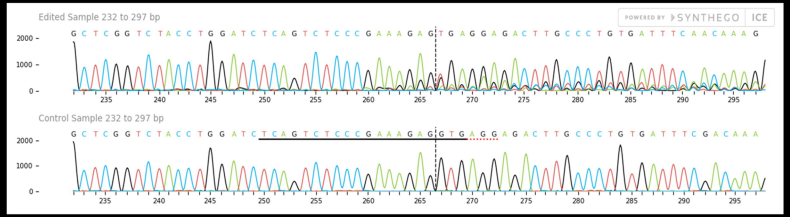
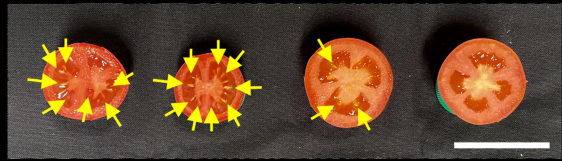
+ 0 4% CCTGGATCTCAGTCTCCCGAAAGAG GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGATGAGAAGCTGCTC
-6 2% CCTGGATCTCAGTCTCCCGA----- -TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGATGAGAAGCTGCTC
-6 2% CCTGGATCTCAGTCTCCCGAA---- --GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGATGAGAAGCTGCTC
-2 2% CCTGGATCTCAGTCTCCCGAAAGAG --GAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGATGAGAAGCTGCTC
-4 1% CCTGGATCTCAGTCTCCCGAA---- GTGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGATGAGAAGCTGCTC
-2 1% CCTGGATCTCAGTCTCCCGAAAGA- -TGAGGAGACTTGCCCTGTGATTTCGACAAAGGTTGATGAGAAGCTGCTC


Edited Sample 232 to 297 bp
POWERED BY SYNTHEGO ICE
Control Sample 232 to 297 bp