Virus induced transgene- and tissue-culture free heritable genome editing in tomato

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Abstract

Genome editing has emerged as a powerful tool for crop breeding. However, most commonly used approaches rely on tissue culture and the use of transgenic materials, which are time-consuming and labor-intensive, and often exhibit strong genotype dependency. Here we utilized tobacco rattle virus (TRV)-mediated ISYmu1 genome editing, coupled with *in planta* shoot regeneration, to achieve somatic and heritable genome editing in different tomato cultivars. By targeting the *SlPDS* gene, we successfully generated virus- and transgene-free homozygous mutant progeny in one generation. We further verified the method by generating somatic edits in *SlDA1*, a gene with potential agronomic relevance. Given the wide host range of TRV and its compatibility with *in planta* shoot regeneration, our system could be broadly applicable for rapid, non-transgenic and heritable genome editing, thereby advancing functional genomics and crop improvement.

Introduction

The emergence of genome editing technology presents tremendous potential for accelerating crop improvement[1, 2]. However, despite the rapid adoption of genome editing in many major crops, two significant bottlenecks remain: efficient delivery of gene-editing reagents and the efficient generation of transgene-free plants. Conventional plant genome editing often relies on the use of tissue culture and plant transformation, which typically involves introducing the editing construct into plants through *Agrobacterium tumefaciens*-mediated transformation, selecting transgenic plants carrying the desired mutation, and subsequently removing the transgenic components by crossing[3, 4]. This method is time- and labor- intensive and is limited to specific crop species and genotypes, which hinders the application of genome editing in crop plants.

To overcome these barriers, several plant viruses have been engineered to deliver single-guide RNAs (gRNAs) directly to transgenic plants expressing SpCas9 for virus induced genome editing (VIGE) in both dicotyledonous and monocotyledonous crop species[5-8]. For example, some RNA viral vectors, such as *Tobacco Rattle Virus* (TRV) and *Barley Stripe Mosaic Virus* (BMSV) have been shown to deliver the gRNA to meristematic and germline cells to achieve heritable editing, in part with the help of RNA mobility sequences[9-12]. However, most plant viruses are excluded from meristematic tissues, limiting the access to the germline and thus reducing the potential to generated gene-edited progeny plants[13, 14]. Moreover, due to the limitated cargo capacity of most viruses, the application of these methods still requires the additional steps of using tissue-culture and transformation to obtain the overexpressing-Cas9 line as well as crosses to segregate away the transgenes after edits are obtained[15].

A recent strategy to circumvent these limitations was to engineer TRV to deliver the compact ISYmu1 TnpB nuclease and the gRNA[16], to enable transgene-free germline editing in *Arabidopsis*[17]. It remains unknown, however, whether this approach can be applied to additional species. Alternatively, large-capacity vectors[18], such as *Barley Yellow Striate Mosaic Virus* (BYSMV) have delivered both Cas9 and gRNAs to achieve

58 heritable editing in wheat[19]. However, this approach faces limitations due to its
59 complexity and dependence on insect transmission, making it difficult to adapt to other
60 plant species. Thus, the development of simple, rapid, and broadly applicable methods
61 for producing transgene-free, heritable genome-edited crops without the need for tissue
62 culture remains an urgent priority.

63 In this study, we established a strategy that utilized TRV-mediated delivery of the
64 compact ISYmu1 TnpB endonuclease and gRNA in tomato. Using this system, we first
65 targeted the *SlPDS* gene to successfully generate somatically edited shoots, and also
66 observed transmission of edited alleles to the next generation. We further extended the
67 approach to *SlDA1*, a gene of potential agronomic relevance, and achieved highly
68 efficient somatic editing. Together, these results demonstrate that our platform enabled
69 efficient somatic editing and stable transmission of targeted mutations in a crop, thereby
70 providing a streamlined one-step strategy for heritable and transgene-free genome
71 editing in tomato that bypass tissue-culture.

72

73 ## Results

74 **Viral Delivery of ISYmu1for somatic genome editing in tomato.**

75 Encouraged by our recent work demonstrating that TRV delivery of both ISYmu1 and
76 gRNA induced heritable transgene-free germline editing in *Arabidopsis*[17], we reasoned
77 that this strategy represents an ideal system for genome modification that can
78 completely bypass transgenics and tissue culture steps in crops like tomato. To this end,
79 we engineered a pTRV2-TnpB-gRNA vector fused with a truncated mutant FT
80 sequence of tomato (mSIFT) (Fig. 1A) , which has been shown to enhance mobility of
81 gRNA between cells[20]. We then generated a pTRV2-TnpB-gRNA^{SlPDS}-mSlFT vector
82 that expresses a gRNA targeting the tomato phytoene desaturase gene (*SlPDS*), which
83 was delivered to a red cherry cultivar via an *Agrobacterium tumefaciens* cotyledon
84 infiltration method (Fig. 1B, S1A). Three weeks post infiltration, DNA was isolated
85 from young leaves and used as a template for PCR, followed by NGS analysis of PCR
86 amplicons flanking the *SlPDS* target site. Of the 20 plants infiltrated we observed

87 3(15%) plants with somatic editing, ranging from 0.8%~6.93% indels in systemically
88 infected leaves (Fig. S1B). We collected seeds from #13 plants showing 6.93% somatic
89 editing to screen for heritability of edited alleles, however, these somatic mutations
90 failed to be transmitted to the progeny seedlings. These data indicated that TRV can
91 deliver ISYMu1 and gRNA and induce somatic genome editing in tomato.
92

93 **Induction of Gene Edited *De Novo* Shoots via Viral Delivery of ISYMu1 and**
94 **gRNA^{SlPDS}**

95 Recent studies have demonstrated that heritable editing was achieved by inducing *de*
96 *novo* shoots from edited somatic cells through trimming TRV-infected plants, or
97 through TRV-mediated gRNA delivery to axillary meristems in tomato[21]. In addition,
98 emerging *in planta* regeneration approaches offer the potential for more efficient,
99 induction of *de novo* shoots[22, 23]. This inspired us to explore a heritable editing strategy
100 in which genome editing was first induced in somatic cells, followed by the
101 regeneration of *de novo* shoots from the edited cells. To do this we removed the upper
102 shoot and all the axillary shoots, and then injected *Agrobacterium* encoding TRV-TnpB-
103 gRNA^{SlPDS}-mSlFT into all wounding sites of the main stem and axillary regions (Fig.
104 1C, S2 A-C). Five weeks after injection, both callus-like tissue or new shoots arose
105 from the wounding sites, suggesting these *de novo* shoots arose from either direct or
106 indirect regeneration or both (S2 D, 2 E). A total of 49 plants were injected, from which
107 72 *de novo* shoots were initiated (Table S1). One of the *de novo* shoots (#3) showed
108 fully or partial white sectors on the plant similar to the known *slpds* mutant phenotype
109 (Fig. 1D-G), suggesting biallelic mutations in the *SlPDS* gene. To confirm this, we first
110 performed Sanger sequencing on three randomly selected green leaves of this shoot,
111 which all revealed 5-bp deletion as indicated by the presence of two sets of peaks in the
112 sequencing traces at the target site (Fig. 1I). Further amplicon-sequencing (amp-seq)
113 analysis showed that this plant exhibited 56.66% somatic editing efficiency in green
114 leaves (Fig. 1J), consisting of 48.51% of the 5-bp deletion and another 8.15% with a 4-
115 bp deletion. Interestingly, as the plant grew, the petals of all of the flowers were white

116 (Fig. 1H), but the fruit did not display a photobleaching phenotype. We also isolated
117 genomic DNA from different photobleached organs to measure the mutation frequency.
118 Amp-seq analysis showed that different tissues displayed different mutation patterns
119 and editing efficiencies (S3A, 3B), indicated that TRV-TnpB-gRNA^{SlPDS}#3 was
120 chimeric and that genome editing was continuing to occur late in the development of
121 this shoot.

122 For other new developed shoots without photobleaching phenotype (Fig. 1K-M),
123 we first performed Sanger sequencing as a preliminary screen. The results showed
124 mixed peaks in TRV-TnpB-sgRNA^{SlPDS}#7, #17, #43 and #70 lines out of 71 shoots
125 (S4A-F), indicating a high level of editing. To quantify this editing, tissue samples from
126 these lines were collected for amp-seq analysis, which revealed somatic mutation
127 frequencies ranging from 25.21% to 49.69% (Fig. 1N-P, S5). Notably, #7 and #17
128 shoots appeared to carry a single predominant deletion with a frequency of roughly 50%
129 (Fig. 1N, 1O), suggesting that these lines were heterozygous for a mutant allele that
130 likely arose very early in the development of the *de novo* shoot. These data demonstrate
131 TRV-TnpB-gRNA^{SlPDS}-mSlFT can induce a high level of somatic edits in *de novo*
132 shoots.

133

134 **Heritable Genome Editing via Viral Delivery of ISYmu1 and gRNA^{SlPDS}**

135 We next investigated whether targeted mutations could be transmitted to the next
136 generation. A total of 96 seeds from four lines (#3, #7, #17, #43) were sown on 1/2 MS
137 plates. After one week, albino seedings were observed in the progeny of shoots 3# and
138 #17, suggesting biallelic mutation in the *SlPDS* gene (Fig. 2A, 2B, 2E, 2G). To
139 characterize the genotypes of the seedlings we performed Sanger sequencing. Analysis
140 of seedlings from shoot #3 revealed 27 homozygous mutants (28%) with a 5-bp frame-
141 shift deletion, 39 heterozygous mutants (41%) and 30 wild type (31%), consistent with
142 a roughly 3:1 segregation ratio and confirming that the parent plant was likely
143 heterozygous for the 5-bp deletion (Fig. 2I). Similarly, the seedlings of shoot #17
144 showed 23 homozygotes for a 4-bp deletion (24%), 54 heterozygotes (51%) and 19

145 wild type (20%) (Fig. 2I), and shoot #7 produced 27% homozygotes (9-bp deletion)
146 and 51% heterozygotes. The progeny of shoot #47 on the other hand showed a lower
147 transmission of mutations, with only 2% homozygotes and 24% heterozygotes for a 3-
148 bp deletion. It is not surprising that we did not observe albino plants in the progeny of
149 #7 and #43 plants (Fig. 2B, 2D, 2F, 2H), since these lines carried 9-bp or 3-bp deletions,
150 respectively, which would be in frame deletions. Together these data indicate that TRV
151 deliver of ISYMu1 and gRNA fused to mSlFT is capable of inducing heritable editing
152 in tomato.

153 Previous studies have shown that TRV is not transmitted to the next generation in
154 plants[17, 24]. To confirm that the TRV was absent in the progeny of a TRV-infected plants,
155 reverse transcription PCR was performed on 5 albino progenies of both #3 and #17
156 parent shoots (Fig. 2J). As expected, TRV and TnpB mRNA signals were not present in
157 any of the albino offspring seedings, confirming that the TRV-TnpB-gRNA-mSlFT
158 system can be used to generate edited virus-free progeny.

159 **Efficient Somatic Editing of *SlDA1* in *De Novo* Shoots Using TRV To Express**
160 **ISYMu1 and gRNA**

161 Many prior demonstrations of novel genome-editing approaches have relied on
162 targeting genes with an easy phenotypic readout, such as *PDS* [25-27]. To demonstrate that
163 our system can support editing of other genes, we targeted the *DA1* gene of tomato,
164 whose homologous genes play an important role in the regulation of seed and organ
165 size in other plant species[28-31], but which remains uncharacterized in tomato. To this
166 end, we first conducted a Blast search using the *Arabidopsis* DA1 protein sequence as
167 a query against the tomato genome database and, combined with phylogenetic analysis,
168 identified that *Solyc04g079840* is the likely tomato *DA1* homolog (Fig. 3A), hereafter
169 referred to as *SlDA1*. We then generated a TRV–TnpB-gRNA^{SlDA1}-mSlFT construct and
170 utilized the same method (Fig. S2) to infect a different tomato cultivar, M82, so that we
171 could simultaneously test whether our system functions effectively in different tomato
172 cultivars (Fig. 3B). Approximately 5 weeks after injection, 41 *de novo* shoots were
173 initiated from 27 injected M82 plants (Table S2). Three random leaves from each of the

174 41 *de novo* shoots were collected for genomic DNA extraction and Sanger sequencing
175 analysis. We observed mixed sequencing peaks near the *SlDA1* target site in four of the
176 TRV–TnpB-gRNA^{SlDA1}-mSlFT infected shoots, #3, #23, #32 and #38 (S6 A-F),
177 suggesting the presence of mutations. Further amp-seq analysis of these four shoots
178 detected indel frequencies from 63.36% to 99.7% in the *SlDA1* gene (Fig. 3C, S7).
179 Notably, shoots #3 and #23 contained more than 99% edited reads consisting of roughly
180 50% of one type of indel and 50% of another type of indel, suggesting that these two
181 shoots are biallelic mutants with two different mutations arose on the two homologous
182 chromosomes very early during shoot development. These results show that the TRV–
183 TnpB-gRNA-mSlFT system can efficiently induce somatic genome editing in *de novo*
184 shoots targeting *SlDA1*.

185 **Discussion**

186 To advance modern crop genome editing it is important to develop rapid and convenient
187 strategies to generate transgene-free and heritable genome edited crop plants without
188 relying on tissue culture. In a number of recent studies, tissue-culture-free heritable
189 gene editing was achieved by delivering the gRNA to Cas9-expressing tomato plants
190 using TRV[20, 21, 32]. However, this approach still requires the generation of Cas9-
191 expressing plants via tissue culture and plant transformation, as well as self- or back-
192 crossing to remove the transgenic components after the edits are obtained. In this study,
193 we delivered TRV vectors expressing TnpB and its gRNA into wounding sites, which
194 efficiently induced targeted mutations at the *SlPDS* and *SlDA1* in *de novo* shoots.
195 Further, we demonstrated that the *SlPDS* mutations were stably transmitted to the
196 progeny. Thus, our method simultaneously overcomes two major obstacles for tomato
197 genome editing, dependence on tissue culture and the requirement of transgenic
198 components. We targeted both the *SlPDS* gene, a commonly used marker gene with an
199 easily screenable phenotype, as well as the *SlDA1* gene not yet studied in tomato, and
200 utilized two tomato ecotypes in the study. This suggests that our system will be useful
201 for gene function analysis and crop breeding, and that it may be effective in a wide
202 range of tomato genotypes.

203 The TRV vector in this study possesses a broad host range of more than 400 plant
204 species[33], suggesting that the approach described here should work in many additional
205 crops. In addition, ISYmu1 is compact in size and can likely therefore be incorporated
206 into other viral vectors with cargo capacities comparable to TRV, such as *Potato virus*
207 *X* (PVX) and *Pea early browning virus* (PEBV)[7, 34], further expanding the range of
208 crops that could benefit from this approach. Given that *in planta* viral infection
209 strategies have already been demonstrated with many different viruses and plant species,
210 the potential number of systems that could utilize ISYmu1 is large. Our approach should
211 be especially useful in recalcitrant species that are difficult to genetically transform. In
212 the future, further improving the editing efficiency and target sequence range of
213 ISYMu1 and related TnpB enzymes will be essential to fully realize the potential of this
214 strategy.

215 In summary, by integrating the advantages of a compact RNA-guided nuclease, viral
216 infection, and would-induced shoot regeneration, this work demonstrates a method that
217 could advance functional genomics and precision breeding in tomato and other crops.

218

219 Methods and Materials

220 Plant material and growth conditions

221 In this study, two tomato genotypes were used for viral infection, a red cherry type
222 tomato (Amazon.com) and M82 (USDA). Seeds were soaked in water at 55°C for 20
223 minutes, then planted in germination trays and placed in a long-day growth chamber
224 (16-h light and 8-h dark; day 25°C/night 21°C; light intensity: 150 μmol/m^2/s; 50%
225 humidity) for about 3 weeks.

226 A different procedure was followed for growing plants used for seed harvesting. Two
227 weeks after germination, small seedlings were transferred from germination trays into
228 large pots. They were grown in a greenhouse with long-day conditions (16-h light and
229 8-h dark; day 24°C/night 21°C; light intensity:150 μmol/m^2/s; 50% humidity) for about
230 3 months.

231

232 **Plasmids construction**

233 To generate the TRV2-TnpB-wRNA-mSlFT (pYL001) plasmid, the TnpB-wRNA-
234 *ccdb*-HR-mSlFT sequence with 20 bp overlapping as a gene block was synthesized
235 from Integrated DNA Technologies (IDT). The pMK435 vector was digested by SacI
236 (NEB, R3156S) and PmlI (NEB, R0532S) for 2 hours and the largest fragment was
237 purified using QIAQuick Gel Cleanup kit (Qiagen, 28704). Next, the gene block and
238 digested purified pMK435 plasmid fragment were used to make the pYL001 plasmid
239 using NEBuilder HiFi DNA Assembly (NEB, E2621) according to the manufacturer's
240 protocol. Finally, the reaction was transformed into the ccdB Survival2 T1R Competent
241 Cells (Invitrogen, A10460).

242 The pYL001 plasmid was used as a base vector to construct the TRV2 vector with
243 *SlPDS* and *SlDA1* gRNA using the Anneal and Golden Gate strategy. First, two target
244 site oligos were synthesized from Integrated DNA Technologies (IDT) and diluted to
245 100 μm, and then phosphorylated and annealed. Next, the annealed double-stranded
246 DNA and the pYL001 plasmid were used in a Golden Gate reaction using PaqCI® (NEB,
247 R0745) and T4 DNA Ligase (NEB, M0202), followed by the Golden Gate Assembly
248 Protocol. Finally, the reaction was transformed into the 10-beta competent *E. coli* (NEB,
249 C3019). All the plasmids were confirmed using Primordium whole-plasmid sequencing.
250 The target site primers are listed in Table S2

251

252 **Viral infection and *de novo* shoot induction**

253 TRV1 and TRV2 related plasmids were transformed into *Agrobacterium* strain
254 GV3101 electrocompetent cells (Golabio, CC-207) and grown on LB plates with Kan
255 (50 μg/ml) and RIF (30 μg/ml) for 48 hours at 28°C. A single colony was selected and
256 moved to 2 ml LB liquid media with antibiotics and shaken overnight at 28°C. After
257 confirmation by bacterial PCR, 1 ml of Agrobacterium liquid was transferred into 100
258 ml of LB liquid media containing KAN (50 μg/ml), RIF (30 μg/ml), 10mM MES and
259 25uM acetosyringone and grown overnight at 28°C incubator. The next day, the
260 Agrobacterium culture was centrifuged for 15 min at 4000 rpm. The supernatant was

261 discarded and the pellet was resuspended in infection buffer containing 10 mM MgCl2,
262 10 mM 2-(*N*-morpholino) ethanesulfonic acid and 250 μM acetosyringone to an OD_{600}
263 of 0.6. The *Agrobacterium* cells were then incubated at room temperature for 3 hours
264 in darkness. After approximately 3 hours, the TRV1 and TRV2 Agrobacterium were
265 mixed in a 1:1 ratio and prepared for injection into the tomato plants.

266 Three-week-old tomato seedlings with about 4 true leaves were pruned, removing
267 the main shoot and all the axillary shoots. This produced wounding sites on the stem
268 and axillary sites, only leaving two true leaves and two cotyledons. TRV1 and TRV2
269 *Agrobacterium* mix was injected near the wounding site using 1 ml syringes. Successful
270 injection was confirmed by observing the *Agrobacterium* buffer seeping from the
271 wounding site. Plants were then placed in dark and high-humidity (80%) growth
272 chamber at 25°C for 2 days, and then grown at 20°C for 2 days. Next, plants were grown
273 in a greenhouse at 16/8-hour day/night cycle at 24/21°C. We removed any new shoots
274 that appeared at the wounding sites in the first month to promote the *de novo* shoot
275 initiation. One month later, all the new shoots were genotyped.

276

277 **Edited shoots screening and mutation frequency analysis**

278 Approximately 35 DPI, 3 random green leaves from each shoot were harvested for
279 extracting genomic DNA by using Qiagen DNeasy Plant Mini Kit (Qiagen, 69106)
280 according to manufacturer instructions. Target sites were amplified using Q5 DNA
281 polymerase (NEB, M0491s) and PCR products were directly sent for Sanger
282 sequencing. If edited was observed, plants were transferred to larger pots. Otherwise,
283 the unedited shoots were cut at their base to promote new shoot initiation.

284 Deep next-generation amplicon sequencing was performed for analysis of the
285 mutation frequency of edited plants. DNA libraries were preparted by using a 2-step
286 PCR amplification method. For the first round of PCR, target regions were amplified
287 with primers close to the target site using Phusion™ High-Fidelity DNA Polymerases
288 (Thermo Fisher, F530S) and the reaction was performed under the following cycling
289 conditions: 98 °C for 30s, 98 °C for 30 s, 55°C for 20 s, 72 °C for 30s, 25 cycles (Step2);

290 72 °C for 5 min. Then, the PCR products were purified by using 1.0× Ampure XP bead
291 purification (Beckman Coulter, A63881). Purified PCR products were then used as the
292 template for the second round PCR by using Illumina indexing primers under the
293 following cycling conditions: 98 °C for 30s, 98 °C for 30s, 60°C for 20s, 72 °C for 30s,
294 12 cycles (Step2); 72 °C for 5 min. Subsequently, the second-round PCR products were
295 purified and quantified. Finally, equal amounts of the second-round PCR products were
296 mixed and single-end next-generation sequencing on the Illumina NovaSeqX platform
297 was performed.
298

299 **Next-generation amplicon sequencing analysis**

300 Amplicon sequencing analysis was conducted as previously described[35]. Single-end
301 reads were processed by adapter trimming with Trim Galore using default parameters,
302 and the resulting reads were aligned to the target genomic region with BWA (v0.7.17)
303 employing the BWA-MEM algorithm. The resulting BAM files were sorted and
304 indexed, and subsequently analyzed with the CrispRvariants R package (v1.14.0). For
305 each sample, distinct mutation patterns and their associated read counts were extracted
306 using CrispRvariants. Based on the assessment of control samples, a stringent
307 classification criterion was applied; only reads containing indels of ≥1 bp (insertions or
308 deletions of identical size starting at the same position) with a minimum of 10
309 supporting reads per sample were considered edited. Single-nucleotide variants were
310 excluded from the analysis.
311

312 **Progeny genotyping**

313 Seeds were harvested from the editing parent plants and dried in a 37°C incubator for
314 3 days. Seeds were sterilized using a 55°C water incubator for 10mins, 75% ethanol for
315 1 min, and washed three times with sterile water, 3% NaClO for 7 mins. Seeds were
316 then put in 1/2 MS plates and incubated in darkness at 23°C for 3 days, followed by
317 growth with long-day conditions (16-h light and 8-h dark; day 25°C/night 20°C; light
318 intensity:150 μmol/m^2/s; 50% humidity) for about 5 days. Once the cotyledons were

319 fully expanded, half of a cotyledon was collected for DNA extraction using the
320 Invitrogen Platinum Direct PCR Universal Master Mix (A44647500) following the
321 manufacturer's protocol. The extracted DNA was subsequently used as a template for
322 PCR, and the PCR products were subjected to Sanger sequencing. Quantification of
323 indel frequencies was performed with the ICE CRISPR Analysis Tool.

324 Genotypes were classified according to indel frequencies as follows: Shoots or
325 seedlings with a total indel frequency of 0–10% were considered wild type (WT);
326 samples with a single unique sequence modification at 35–65%, the nonmodified
327 sequence frequency between 35% and 65%, and the total frequency of single unique
328 sequence modification and nonmodified sequence between 85%~100% was designated
329 as heterozygous (Het); samples with a single unique sequence modification at 85–100%
330 were classified as homozygous (Ho); and samples with two distinct sequence
331 modifications each at 35–65% and the total modification frequency at 85–100% were
332 designated as biallelic for two different alleles.

333

334 **RT-PCR**

335 Total RNA from progeny seedlings was extracted using Zymo Research Direct-zol
336 RNA MiniPrep kit (R2052). Total RNA was reverse transcribed to synthesize first-
337 strand cDNA using the Invitrogen SuperScript IV VILO Master Mix (11766050). The
338 synthesized cDNA was diluted and then used for PCR with specific primers to detect
339 the TRV virus and TnpB expression by using New England Biolabs Q5 High-Fidelity
340 2× Master Mix (M0492L) according to manufacturer instructions. The *SlActin* gene
341 was used as the internal control. PCR products were fractionated by 2% agarose gel
342 electrophoresis.

343

344 Author contributions: Y.L. and S.E.J. designed research. Y.L., J.L., J.P., S.Y.C.C., E.R.,
345 J.A. and S.H.F. performed experiments; Y.L., T.W. and M.K. analyzed data; Y.L. and
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347

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349

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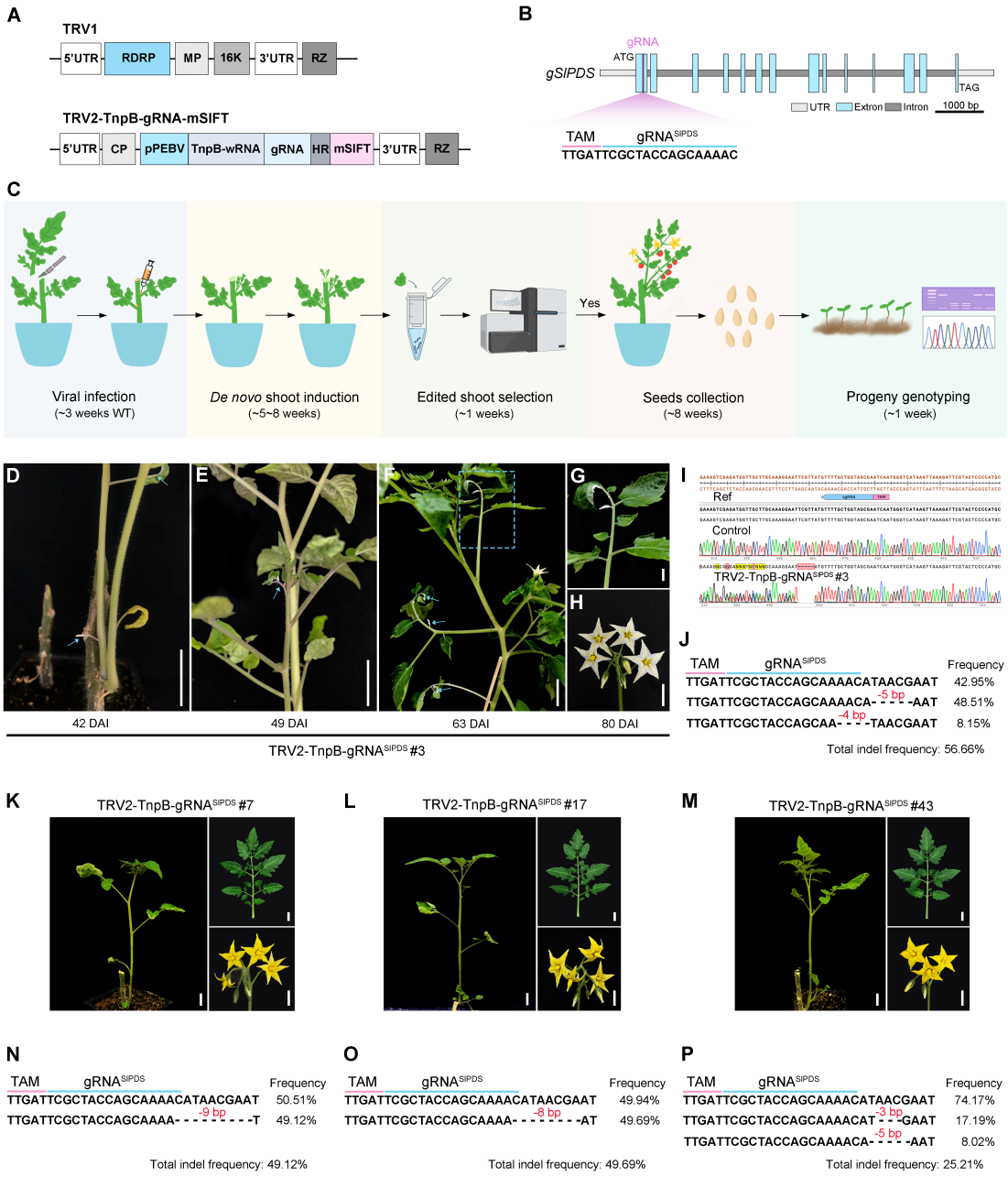
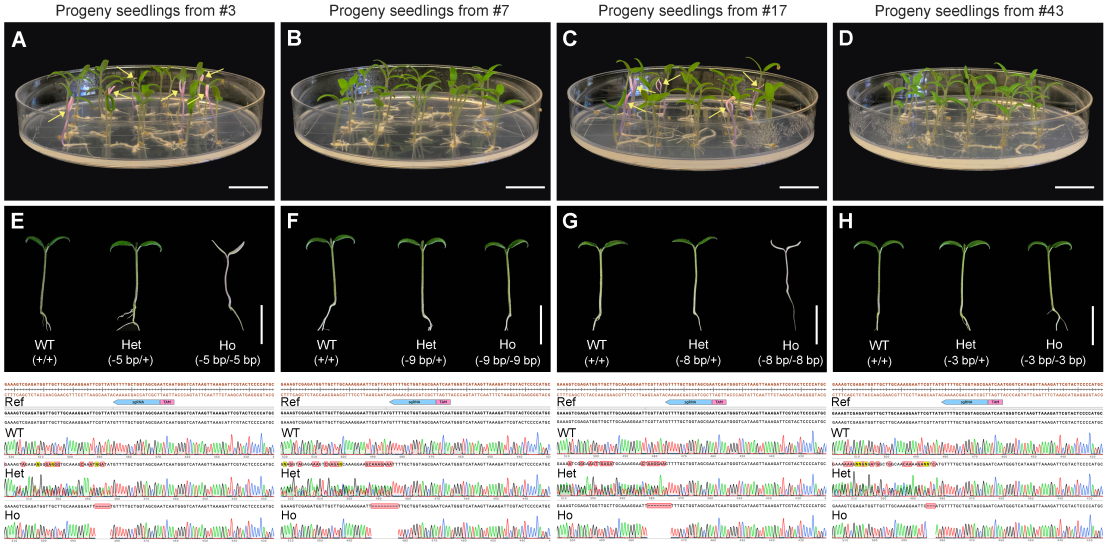


Fig. 1. Virus-induced genome editing in *de novo shoots* of tomato using TRV-TnpB-gRNA^{SlPDS} -mSlFT. (**A**) Schematic diagram of the TRV1 and TRV2 plasmids. The tomato mutated *FT* mobility element (mSlFT) was fused to the 3' end of the gRNA in TRV2. RdRP, RNA dependent RNA polymerase; MP, movement protein; CP, coat protein; sgP, *Pea Early Browning Virus* subgenomic promoter; HR, HDV ribozyme, mSlFT; mutated tomato *FT* mobility element. (**B**) Schematic representation of *SlPDS* genomic structure. The light blue box represents exons, light grey represents the UTR sequence, and dark grey represents introns. The TAM sequence and target sequence are

highlighted by the pink and blue lines, respectively. (**C**) The procedure for TRV-TnpB-gRNA-mSlFT mediated genome editing system in tomato plants. Edited *de novo* shoots were screened and validated by Sanger and NGS amplicon sequencing. (**D-H**) Different growth stages of TRV-TnpB-gRNA^{SlPDS}#3 plant displaying albino sectors in leaves and flowers. White leaflets in leaves are indicated by blue arrows, and the flowers exhibited white petals (**H**). **G** is a close-up view of **F**; DAI: Days After Injection. (Scale bars, 1 cm). (**I**) Sanger sequencing of the *SlPDS* gene target site in TRV-Control (top) and TRV-TnpB-gRNA^{SlPDS}#3 (bottom) plants. (**J**) Indel frequency analysis of the *SlPDS* gene in the TRV-TnpB-gRNA^{SlPDS}#3 shoot. Genomic DNA was extracted from green leaf tissue and subjected to amplicon sequencing using next-generation sequencing (NGS). The TAM sequence and target sequence are highlighted by the pink and blue lines, respectively. Deleted residues are marked by black dotted lines. The different indel types are listed on the left. The percentage of reads corresponding to each indel type is shown on the right. The indel efficiency was calculated by the total indel reads number divided by total reads number. (**K-M**) Phenotype of the TRV-TnpB-gRNA^{SlPDS}#7 (**K**), #17 (**L**) and #43 (**M**) shoots, respectively., showing no obvious albino sectors. Images include the whole plant (left), a leaf (top right) and flowers (bottom right). (Scale bars, 1 cm). (**N-P**) Indel frequency analysis of the *SlPDS* gene from the TRV-TnpB-gRNA^{SlPDS}#7 (**N**), #17 (**O**) and #43 (**P**) shoots. The reads of sequences with frequencies >5% are shown as well as the total indel frequency.



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Parent plants		Progeny seedlings		
Line	Mutation(%)	Planted number	Heterozygous(%)	Homozygous(%)
3	56.7%	96	39 (41%)	27 (28%)
7	49.1%	96	54 (56%)	23 (24%)
17	49.7%	96	49 (51%)	26 (27%)
43	25.2%	96	25 (26%)	2 (2%)

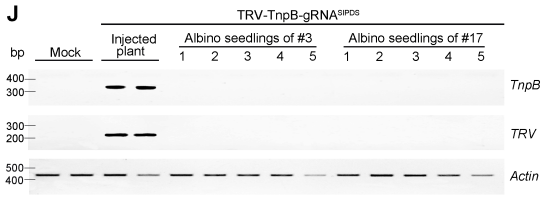

Fig. 2. Transgene-free and heritable genome editing in TRV-TnpB-gRNA^{SlPDS}-mSlFT infected tomato plants. (**A-D**) Phenotype of 1-week old progeny seedlings from TRV-TnpB-gRNA^{SlPDS}-mSlFT#3 (**A**), #7 (**B**), #17 (**C**) and #43 (**D**) parent plants. The albino seedlings are marked with yellow arrows. (Scale bars, 2 cm). (**E-H**) Phenotyping and genotyping analysis of progeny seedings from TRV-TnpB-gRNA^{SlPDS}-mSlFT#3 (**E**), #7 (**F**), #17 (**G**) and #43 (**H**) parent plants. Representative image of 1-week old progeny seedlings (top panel). Sanger sequencing trace file of PCR product from progeny seedlings (bottom panel). Ref: Reference sequence; WT: Wild Type; Het: Heterozygous; Ho: Homozygous. (Scale bars: 1 cm). (**I**) Table summarizing the percentage of progeny seedlings with heterozygous and homozygous mutations from the four parent plants shown in panels A-H. (**J**) RT-PCR performed using total RNA from 2 control plants, 2 TRV injected plants (tissue shortly after injection), and 10 albino progeny seedlings to detect *TnpB* (upper panel) or TRV mRNA (middle panel) expression. The *SlActin* gene served as an endogenous control (bottom panel). The experiment was repeated three times with similar results.

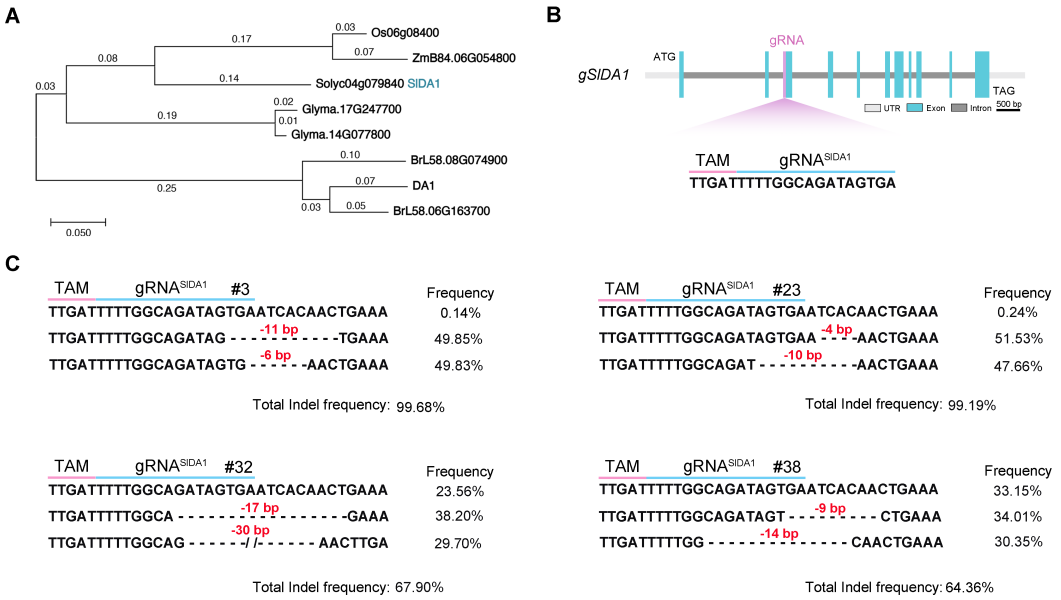

Fig.3. Somatic editing in tomato using TRV to deliver TnpB and guide RNA targeting *SlDA1*. (**A**) Phylogenetic analysis of tomato DA1 (SlDA1) and its homologs from other species. The SlDA1 is marked with cyan color. (**B**) Schematic diagram of genomic structure. The cyan box represents exons, light grey represents the UTR sequence and dark grey represents introns, purple box represents the gRNA site. The TAM and target sequences are highlighted by the pink and blue lines, respectively. (**C**) Indel frequency analysis of the *SlDA1* gene from the TRV-TnpB-gRNA^{SlDA1} #3, #23, #32 and #38 shoots. The mutant sequences with frequencies >5% are shown as well as the total indel frequency.