Title: Viral delivery of an RNA-guided genome editor for transgene-free germline editing in *Arabidopsis*

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Abstract

Genome editing is transforming plant biology by enabling precise DNA modifications. However, delivery of editing systems into plants remains challenging, often requiring slow, genotype-specific methods such as tissue culture or transformation. Plant viruses, which naturally infect and spread to most tissues, present a promising delivery system for editing reagents. But most viruses have limited cargo capacities, restricting their ability to carry large CRISPR-Cas systems. Here, we engineered tobacco rattle virus (TRV) to carry the compact RNA-guided TnpB enzyme ISYmu1 and its guide RNA. This innovation allowed transgene-free editing of *Arabidopsis thaliana* in a single step, with edits inherited in the subsequent generation. By overcoming traditional reagent delivery barriers, this approach offers a novel platform for genome editing, which can greatly accelerate plant biotechnology and basic research.

Main text

Programmable RNA-guided endonucleases including CRISPR-Cas9 are driving advances in genome editing for both fundamental research and biotechnology. The ability to genetically

modify plant genomes has allowed for the creation of rationally designed phenotypes. However, efficient delivery of genome editing reagents to plants remains a major challenge. The most common strategy is to encode RNA-guided genome editors (e.g. CRISPR-Cas enzymes) within transgenes and use tissue culture and plant transformation approaches to make transgenic plants, after which genetic crosses are required to remove the transgenic material but retain the edits [1–3]. However, current plant transformation methods are limited to specific plant species and genotypes, often require considerable time, resources and technical expertise, and can cause unintended changes to the genome and epigenome [1].

One approach to circumvent these limitations is to use plant viral vectors to deliver genome editing reagents. For example, several viral vectors have been engineered to encode guide RNAs (gRNAs) for delivery to transgenic plants already expressing Cas9, resulting in somatic and germline editing and transmission of edits to the next generation [4–7]. Because plants have evolved mechanisms to restrict viral infection of meristems and germ cells, most viruses are rarely sexually transmitted [8]. However, transient invasion of meristem cells by viral RNAs encoding gRNAs can allow these cells to be edited, and for these edits to be seed transmissible [4–7]. While these approaches represent significant advances, they still require the use of transgenic plants to express the nuclease protein.

A strategy to avoid the need for transgenic plant materials has been the use of viral vectors with large cargo capacities, capable of expressing entire editing systems (e.g. Cas9 and the gRNA). This approach has been met with some success, however it still requires plant regeneration steps because these viruses do not cause germline editing and heritability of the edits [9–12]. On the other hand, encoding entire CRISPR systems in viruses that are capable of germline transmission has not been possible because of their limited cargo capacity [4–7,13].

To overcome this cargo size limit, we explored the potential of TnpB, a class of ultracompact RNA-guided endonucleases (~400 amino acids) [14–16], to be encoded in a plant RNA viral vector. As ancestors of Cas enzymes, TnpBs similarly utilize a programmable RNA guide,

83 called an omega RNA (ωRNA), to be directed to any target site and induce genome edits.
84 Previously, TnpBs ISDra2, ISYmu1 and ISAam1 were shown to be capable of targeted genome
85 editing in mammalian cells, and ISDra2 and ISYmu1 in monocot rice plant cells [14,17–19]. Here, we
86 tested the ISDra2, ISYmu1 and ISAam1 TnpBs for genome editing in the dicot plant, *Arabidopsis*.
87 Given the single cargo site in the TRV vector that is typically used, we sought to express both
88 the TnpB protein and its guide RNA within the same mRNA transcript under a single promoter,
89 similar to their natural expression arrangement [14–16].
90
91 To test the activities of TnpB and its gRNA encoded in a single transcript, we first
92 expressed these three TnpBs and assessed their RNA-guided plasmid interference activities in
93 bacteria. We co-expressed the TnpB and gRNA from the same promoter as a single transcript,
94 maintaining their natural sequences without codon optimization. We compared two
95 configurations of the 3'-guide region: one extended continuously without a terminator to mimic
96 the natural TnpB condition, and another capped by the hepatitis delta virus (HDV) ribozyme, as
97 previously used in bacteria [14] (Extended Data Fig. 1). Our results showed that without the HDV
98 ribozyme, only ISDra2 demonstrated plasmid interference activity whereas with the HDV
99 ribozyme, all three TnpBs exhibited robust activity at both 26°C and 37°C (Fig. 1a, Extended
100 Data Fig. 2). These findings revealed that single transcript expression cassettes with an HDV
101 ribozyme sequence at the 3' end are capable of cleaving plasmid DNA in bacteria.
102
103 To test the single expression cassette for targeted genome editing in *Arabidopsis*, we
104 used the *AtUBQ10* promoter to drive expression of the TnpB-ωRNA and a gRNA targeting the
105 *PHYTOENE DESATURASE3* (*AtPDS3*) gene region, followed by the HDV ribozyme and rbcS-E9
106 terminator (Fig. 1b). We tested twenty ISDra2 sites, ten ISYmu1 sites, and seven ISAam1 sites
107 for editing capabilities in *Arabidopsis* protoplast cells (Supplementary Table 1) [20]. ISDra2 and
108 ISYmu1 demonstrated active editing ranging from 0-4.8% and 0.1-4.2%, respectively, as
109 measured by next generation amplicon sequencing (amp-seq) (Extended Data Fig. 3a). ISAam1
110 was much less active, ranging from 0-0.3% editing efficiency (Extended Data Fig. 3a). On
111 average, we observed editing efficiencies of 1% for ISDra2, 2.1% for ISYmu1 and 0.1% for

112 ISAam1 (Fig. 1c). In line with previous reports, the DNA repair profiles consisted of deletion-
113 dominant repair outcomes for all three TnpBs (Extended Data Fig. 3b) [14,17,18]. These data
114 demonstrate that ISDra2, ISYmu1 and ISAam1 are all capable of targeted genome editing in
115 *Arabidopsis* plant cells using the single transcript expression design.
116
117 To evaluate TnpB-mediated editing in transgenic plants we selected ISYmu1, as it
118 demonstrated the highest average editing efficiency in *Arabidopsis* protoplast cells and was
119 shown to exhibit no off-target editing in rice [18]. Two gRNAs with the most active editing were
120 selected, each targeting a unique genomic context. gRNA2 targeted the coding region of
121 *AtPDS3* whereas gRNA12 targeted the promoter region directly upstream of the *AtPDS3* gene.
122 Transgenic plants were created via standard floral dip transformation utilizing the same
123 plasmids as for the protoplast experiments [21]. To test for sensitivity to temperature, transgenic
124 plants expressing ISYmu1 were either grown at room temperature or subjected to a heat shock
125 treatment. We tested editing in wild type (WT) plants, as well as in the *rna dependent rna*
126 *polymerase 6* (*rdr6*) mutant which is known to reduce transgene silencing [22]. Analysis using
127 amp-seq revealed an average editing efficiency of 1.6% and 2.5% for gRNA2 in WT and *rdr6*,
128 respectively (Fig. 1d). Analysis of gRNA12 revealed greater editing than gRNA2, averaging 44.9%
129 editing in WT and 75.5% in *rdr6* (Fig. 1e). Comparison of editing efficiency in the plants grown at
130 room temperature with those that received the heat shock treatment revealed a preference for
131 increased temperature for both target sites in the WT background, demonstrating 6.3-fold and
132 1.4-fold increases in editing for gRNA2 and gRNA12, respectively (Fig. 1d, e). In *rdr6* we
133 observed a 13-fold increase in editing for gRNA2, but little change in editing for gRNA12 (Fig.
134 1d, e). These data demonstrate that ISYmu1, encoded as a transgene, is capable of performing
135 efficient genome editing in *Arabidopsis* plants, and that heat treatment and the *rdr6* silencing
136 mutant can be used to increase editing efficiency.
137
138 Encouraged by the ISYmu1 activity in transgenic *Arabidopsis* plants, we next tested
139 ISYmu1 for TRV-mediated genome editing. TRV is a bipartite RNA virus composed of TRV1 and
140 TRV2 (Fig. 2a). Previous work has shown that the TRV2 RNA can be engineered by inserting a

141 cargo expression cassette downstream of the pea early browning virus promoter (pPEBV) (Fig.
142 2a) [23,24]. To test ISYmu1 for genome editing capabilities via TRV delivery to *Arabidopsis,* we
143 engineered two TRV2 Cargo Architectures. In TRV2 Architecture_A, the $tRNA^{Ileu}$ was directly
144 downstream of the TnpB and gRNA sequences (Fig. 2a). In TRV Architecture_B, we included an
145 HDV ribozyme sequence between the guide and $tRNA^{Ileu}$ sequences.(Fig. 2a). We included
146 $tRNA^{Ileu}$ in both designs as it was previously shown to promote systemic TRV movement and
147 transmission of edited alleles to the next generation [23,24].
148
149 First, we evaluated TRV-mediated editing potential with gRNA2 using both TRV2
150 Architecture_A and Architecture_B. gRNA2 was selected because it targets the *AtPDS3* coding
151 sequence, enabling easy phenotypic screening for editing due to white photobleaching of cells
152 containing biallelic mutations [23,24]. We delivered TRV vectors to both WT and the *ku70* genetic
153 mutant. *Ku70* plays a role in the nonhomologous end joining (NHEJ) double strand break (DSB)
154 repair pathway [25]. ISYmu1-mediated editing efficiency should be greater in the *ku70* genotype if
155 double stranded breaks generated by ISYmu1 are repaired through NHEJ. Each TRV2 plasmid
156 was co-delivered with the TRV1 plasmid to *Arabidopsis* plants using the agroflood method [24].
157 White speckles were observed on some of the leaves around three weeks post agroflooding,
158 suggesting that sectors of cells contained biallelic mutations in the target *AtPDS3* gene (Fig. 2b).
159 Amp-seq analysis revealed an average of 0.1% and 0% editing efficiency in leaf tissue of WT and
160 *ku70* plants agroflooded with TRV2 Architecture_A and grown under room temperature,
161 respectively (Fig. 2c). For the heat shock treated plants, we observed an average editing
162 efficiency of 0.4% in WT and 0.7% in *ku70* plants agroflooded with TRV2 Architecture_A (Fig.
163 2c). Using TRV2 Architecture_B we observed an average 0.6% and 2% editing in WT and *ku70*,
164 respectively, for the room temperature grown plants. For the plants that received TRV
165 Architecture_B and a heat shock, we observed an average editing efficiency of 3.3% in WT and
166 8.9% in *ku70* (Fig. 2c). These results show that Architecture_B, containing the HDV ribozyme,
167 generated higher editing than Architecture_A, and that the *ku70* mutant can enhance editing
168 efficiency.
169

170 Next, we tested gRNA12 utilizing the TRV2 Architecture_B, since this architecture
171 demonstrated the highest levels of editing for gRNA2. Using the same agroflood TRV delivery
172 method, we observed an average 8.51% and 4.27% editing efficiency in room temperature and
173 heat treatment growth conditions, respectively (Fig. 2d). Further, 6/57 plants displayed editing
174 greater than 40%, with four greater than 75%, when the room temperature treatment was
175 used (Fig. 2d). Again, analysis of the repair outcomes showed deletion dominant profiles for
176 ISYmu1 gRNA2 and gRNA12 (Fig. 2e, f).
177
178 To test for transmission of edited alleles to the next generation, we first screened the
179 progeny of a WT plant showing 54.54% somatic editing using the TRV2 Architecture_B design
180 with gRNA2 that underwent heat shock treatment. In total, 2318 seeds were sown on ½ MS
181 plates containing 3% sucrose. After ten days, 68 albino seedlings were observed, suggesting
182 biallelic mutations in the *PDS3* gene (Fig. 3a). To confirm *AtPDS3* was mutated we performed
183 Sanger sequencing on the two white seedlings shown in Fig. 3a, which revealed both plants to
184 be homozygous for a 4bp frame-shift deletion (Fig. 3b). To further characterize transmission of
185 edited alleles, amp-seq on 209 seedlings (41 albino and 168 green) showed that all of the albino
186 seedlings contained biallelic mutations, with the majority of mutations being the 4bp deletion
187 observed in Fig. 3b (Supplementary Table 2). Of the 168 green seedlings, eight were
188 heterozygous (4bp deletion/WT) (Supplementary Table 2).
189
190 Next, we characterized transmission of edited alleles from two individual lines, plant 54
191 (80.5% somatic editing) and plant 69 (77.1% somatic editing), that underwent agroflood using
192 gRNA12 TRV2 Architecture_B with the room temperature condition. Using Sanger sequencing,
193 we analyzed the genotypes of 148 and 75 progeny seedlings from plants 54 and 69,
194 respectively. Sanger sequencing analysis of the progeny from plant 54 revealed 27 (18%)
195 biallelic and 25 (17%) monoallelic edited plants (Supplementary Table 2, Extended Data Fig. 4a)
196 [26]. For plant 69 we observed higher transmission of edited alleles, totaling 32 (43%) biallelic and
197 15 (20%) monoallelic edited plants (Supplementary Table 2, Extended Data Fig. 4b) [26]. These

198 data demonstrate the heritability of edits generated via TRV-delivery of ISYmu1 at two distinct
199 target sites.
200
201 It has been demonstrated that TRV is not transmitted to the next generation following
202 agroflood inoculation of plants [23,24]. To confirm the TRV was not present in the progeny of a
203 TRV-infected plant, RT-PCR was performed on five albino plants harboring homozygous 4bp
204 deletions at *AtPDS3*. Consistent with the literature, TRV was not detected in any of the albino
205 plants (Extended Data Fig. 5) [23,24]. These data indicate that TRV-mediated biallelic edits using
206 ISYmu1 are heritable and virus-free.
207
208 To evaluate off-target editing we surveyed three individual albino plants harboring
209 biallelic mutations generated by ISYmu1 TRV2 Architecture_B gRNA2. Whole genome
210 sequencing was performed to generate an average 770x coverage, with greater than 99% of the
211 genome covered by mapped reads (Supplementary Table 3). In all three samples, we confirmed
212 the targeted mutations in the *AtPDS3* gene, as previously identified using amp-seq.
213 Additionally, we found a large number of variant differences compared to the Col-0 reference
214 genome both in the control and the edited plants (Supplementary Table 4), suggesting that
215 most of the variants detected are due to spontaneous mutations present in our lab strain of
216 *Arabidopsis*. To screen for variants potentially caused by ISYmu1 off-target editing, all variants
217 in the edited plants were filtered with variants already present in the control background.
218 Variants with coverage lower than 30-fold were also filtered out. The remaining variants were
219 checked manually for any false positive variant calling. In the three albino plants we sequenced,
220 only 5, 5, and 4 variants were detected, and these variants are all outside the predicted
221 potential off-target sites based on sequence similarity to the PDS3 gRNA2 sequence
222 (Supplementary Tables 4, 5) [27]. In line with ISYmu1 off-target analysis reported in rice and
223 human cells [17,18] , these data further demonstrate the high target site specificity of ISYmu1.
224
225 A long-term goal of plant scientists has been the development of fast and easy means of
226 editing plant genomes without the need for tissue culture and transgenesis. Here, we

227 developed an approach utilizing the ultracompact site-specific TnpB genome editor, ISYmu1,
228 together with tobacco rattle virus, for heritable plant genome editing. These results should
229 accelerate high throughput genome editing for both basic and applied research. We anticipate
230 this approach will be applicable to other novel TnpBs, various viral vectors and a number of
231 plant species for genome editing. Recent work has uncovered many TnpB systems from diverse
232 microbial sources, including enzymes with unique PAM sequence specificities [28], which can
233 increase the range of target DNA sequences that could be edited using this approach. The TRV
234 virus used in this study has a broad host range of over 400 species, including many solanaceous
235 plants such as tomato, ornamental plants, and other crops [29]. Additionally, plant viruses with
236 similar cargo capacities such as Potato Virus X and Barley Stripe Mosaic Virus are likely to be
237 amenable to this approach since it has been demonstrated they are capable of viral-mediated
238 heritable gene editing by delivering the gRNA to a Cas9-expressing transgenic plant [30]. In
239 addition to being an important tool for crop biotechnology, viral delivery of TnpBs could enable
240 high throughput CRISPR screens in model plant species such as *Arabidopsis*, further unlocking
241 their potential for genetic discovery.
242
243
244 **Materials and Methods:**

245 Plasmids used in this study

246 Plasmids used for bacterial assay were generated as follows. The single expression
247 cassette containing TnpB and ωRNA sequences were synthesized as geneblocks from IDT
248 (Integrated DNA Technologies), and were golden-gate cloned using BsmbI restriction enzyme
249 (cat E1602S) into a vector (chloramphenicol resistance) under a single tetracycline-inducible
250 promoter (TetR/pTet) to make the TnpB-ωRNA plasmid. Target sites with various PAM
251 sequences and target sites were golden-gate cloned with BBsI restriction enzyme(cat R3539S)
252 into a vector (ampicillin/carbenicillin resistance).

253 Plasmids were generated for protoplast and floral dip experiments in a two-step cloning
254 strategy. In step one, the ISDra2, ISYmu1, and ISAam1 protein coding sequences and their
255 ωRNAs were synthesized as geneblocks by IDT (Integrated DNA Technologies). Then, starting

256 with the pC1300_pUB10_pcoCASphi_E9t_MCS_version2 vector (Li et al. 2023), we used
257 NEBuilder HiFi DNA Assembly (catE2621) and PCR to assemble the TnpB-ωRNA geneblocks into
258 plant expression vectors with a toxic *ccdB* insert flanked by PaqCI sites immediately
259 downstream of the ωRNA scaffold and preceding an HDV ribozyme sequence. The HiFi
260 reactions were then transformed into One Shot *ccdB* Survival 2 T1R Competent Cells
261 (catA10460) to obtain the pMK003 (ISDra2), pMK025 (ISYmu1), and pMK024 (ISAam1)
262 intermediate vectors for facile guide sequence cloning (Supplementary Table 6). In step two,
263 guide sequences were synthesized as individual top and bottom strands with 4 base pair
264 overhangs from IDT, phosphorylated and annealed, and then used for golden-gate assembly
265 using the NEB PaqCI (catR0745) enzyme (Supplementary Table 7). When transformed into NEB
266 10-beta Competent E. coli (catC3019) vectors that still contained the *ccdB* gene would kill the
267 cells, leaving behind only the transformants possessing successfully assembled TnpB plant
268 expression vectors harboring a guide RNA sequence. A full list of all plasmids can be found in
269 Supplementary Table 6.

270 TRV vectors were created with the pDK3888 TRV2 plasmid as a base vector [24]. NEBuilder
271 HiFi DNA Assembly (catE2621) was used to clone the ISYmu1 gRNA2 Architecture_A, ISYmu1
272 gRNA2 Architecture_B, and ISYmu1 gRNA12 Architecture_B into the TRV2 cargo slot. First,
273 pDK3888 was digested using NEB ZraI (catR0659), NEB PmlI (catR0532), and NEB Quick CIP
274 (catM0525) overnight, and purified using Qiagen QiaQuick purification column (cat28104).
275 Next, three PCR reactions were performed to amplify the fragments needed for NEBuilder HiFi
276 DNA Assembly, followed by purification using Qiagen QiaQuick purification column (cat28104)
277 (Supplementary Table 8). Then, the digested and purified pDK3888 plasmid, and purified PCR
278 fragments were used to assemble the final TRV2 plasmid using NEBuilder HiFi DNA Assembly
279 according to the manufacturer's protocol. Finally, the NEBuilder HiFi DNA Assembly reaction
280 was transformed into NEB 10-beta Competent E.coli (catC3019). Correct plasmids were
281 confirmed using Primordium whole plasmid sequencing. Plasmids and their descriptions can be
282 found in Supplementary Table 6.

283

284 <u>Bacterial interference assay</u>

285 For the bacterial interference assay, we co-transfected 100 ng of the TnpB-ωRNA
286 plasmid and 100 ng of the target plasmid to 33 µl of NEB 10-beta electrocompetent E. coli cells
287 (cat C3020K). Specifically, the target plasmid contains a target site either flanking the canonical
288 PAM (TTGAT for ISYmu1 and ISDra2 and TTTAA for ISAam1) or flanking a non-canonical PAM
289 (GGGGG). The cells were recovered in 1 ml of NEB 10-Beta Stable/Outgrowth media(cat
290 B9035S) for 1 hour. Following recovery, a series of 5-fold dilutions of the recovery culture were
291 prepared. Each dilution (5 µl) was spot-plated onto LB-Agar plates containing double antibiotics
292 (34 µg/ml chloramphenicol, 100 µg/ml carbenicillin, and 2 nM anhydrotetracycline) and onto
293 control plates with a single antibiotic (34 µg/ml chloramphenicol and 2 nM
294 anhydrotetracycline). If no colonies were visible on the serial dilution plates, 400 µl of the 1 ml
295 recovery culture was plated entirely on the double antibiotic plate to enhance detection
296 sensitivity. Plates were left overnight at either 26°C or 37°C, and colony-forming units (CFU)
297 were counted on all plates the next morning. The normalized CFU were calculated by taking the
298 ratio of CFU on the double antibiotic plates to the CFU on the single antibiotic plates. The
299 normalized CFU in the canonical PAM conditions were compared to the one in the non-
300 canonical PAM conditions. Experiments were performed in triplicate.

301

302 Plant materials and growth conditions

303 For protoplast preparation, *Arabidopsis* Columbia ecotype (Col-0) seeds were suspended
304 in a 0.1% agarose solution and kept at 4°C in the dark for three days to stratify. Following
305 stratification, seeds were planted on Jiffy pucks and grown under a 12-h light/12-h dark
306 photoperiod, with low light condition for 3 to 4 weeks [20].

307 For the creation of transgenic plants, the *Arabidopsis* Col-0 ecotype was used. The *ku70*
308 (SALK_123114) genotype was provided from Feng Zhang lab at the University of Minnesota. The
309 *rdr6* genotype was created using CRISPR-Cas9, resulting in a 616 bp deletion in the gene body of
310 *rdr6*. Floral dip transformation was performed according to the protocol as previously outlined
311 using the Agl0 agrobacterium strain [21]. Transgenic T1 plants were screened using ½ MS plates
312 with 40 µg/mL hygromycin B under a 16-/8-h light/dark cycle at 23°C. After one week,

313 transgenic seedlings that passed selection were transferred to soil and moved to a greenhouse
314 (23°C) for the rest of their life cycle.
315 For agroflood experiments, sterilized seeds were sown on ½ MS agar plates and
316 stratified for five days. After five days, the seeds were moved to a growth room and grown
317 under a 16-/8-h light/dark cycle at 23**°**C for 8-10 days. The seedlings were then used for TRV
318 delivery.

319 A subset of transgenic T1 plants, and plants that underwent agroflood, were subjected
320 to a heat shock treatment modified from LeBlanc et al [31]**.** Seedlings that passed selection, or
321 underwent agroflood, were then transplanted to soil and grown in a greenhouse (23°C) for one
322 week. After one week, plants that did not receive a heat shock treatment continued to grow in
323 the greenhouse (23°C); however, plants that underwent heat shock treatment were exposed to
324 8 hours (9am - 5pm) of heat exposure at 37°C every day for 5 days, followed by 2 days of
325 recovery a greenhouse (23°C). This heat shock regime lasted for two weeks.

326

327 <u>Protoplast isolation and transfection</u>

328 Arabidopsis mesophyll protoplast isolation was performed as previously described [20].
329 Plasmid transfections into *Arabidopsis* protoplasts were performed using 20µg of plasmid,
330 similarly as [32]. The concentrations of plasmids were determined by nanodrop. Plasmids were
331 added to the bottom of each transfection tube, and the volume of the plasmids was
332 supplemented with water to reach 20 µL. 200 µL of protoplasts were added followed by 220 µL
333 of fresh and sterile polyethylene glycol (PEG)-CaCl2 solution. The samples were mixed by gently
334 tapping the tubes, and incubated at room temperature for 10 minutes. After 10 minutes, 880
335 µL of W5 solution was added and mixed with the protoplasts by inverting the tube two to three
336 times to stop the transfection. Next, protoplasts were harvested by centrifuging the tubes at
337 100 relative centrifugal force (RCF) for 2 min and resuspended in 1 mL of WI solution. The
338 protoplast cells were then plated in 6-well plates pre-coated with 5% calf serum. Protoplast
339 cells in the 6-well plates were incubated at 26°C for 48 hours. During the 48 hour incubation,
340 the protoplast cells were subjected to a 37°C heat shock treatment for 2 hours at 16 hours post

341 transfection. At 48 hours post transfection, protoplasts were harvested for genomic DNA
342 extraction.

343

344 TRV delivery to *Arabidopsis* seedlings

345 TRV delivery was performed as previously described [24]. TRV1 and TRV2 vectors were
346 first introduced into the GV3101 agrobacterium strain. The agrobacterium harboring TRV
347 vectors were then grown in 200 ml of lysogeny broth (LB) with antibiotics for 18 hours at 28°C.
348 Agrobacterium cultures were centrifuged for twenty minutes at 3500 RCF. The LB was
349 discarded and the agrobacterium cells were resuspended in 200 ml of sterile water. The
350 resuspended agrobacterium was centrifuged for 10 minutes at 3500 RPM. The supernatant was
351 discarded and the pellet was resuspended in sterile agro-infiltration buffer containing 10 mM
352 MgCl2, 10 mM 2-(N-morpholino) ethanesulfonic acid, and 250 µM acetosyringone to OD600 =
353 1.5. The agrobacterium cells were then incubated at 23°C for three hours with slow shaking.
354 After three hours, the agrobacterium harboring TRV1 and TRV2 were mixed in a 1:1 ratio. 15 ml
355 of the 1:1 ratio of TRV was delivered to seedlings at 8-10 days old. After four days of agroflood
356 co-culture, seedlings were transplanted to soil.

357

358 Screening the progeny of TRV-infected plants for edits

359 Seeds were harvested from the TRV-infected plants about 12 weeks after TRV delivery.
360 The seeds were sown on ½ MS plates supplemented with 3% sucrose and stored at 4°C in the
361 dark for five days to stratify. After five days, the seeds were moved to a growth room and
362 grown under a 16-/8-h light/dark cycle at 23°C for 10-12 days. Amp-seq or Sanger sequencing
363 was then performed on a subset of the progeny plants using primers listed in Supplementary
364 Table 9.

365

366 Next generation amplicon sequencing

367 DNA was extracted from protoplast samples with Qiagen DNeasy plant mini kit (Qiagen
368 69106). Tissue was collected from transgenic plants by sampling and pooling leaf tissue from
369 three random leaves on a single plant three weeks after being transplanted to soil. For the
370 plants that underwent agroflood, leaf tissue was sampled by collecting and pooling tissue from
371 three random (however, if white sectors visible they were sampled) leaves on a single plant
372 distal to the TRV delivery site three weeks after being transplanted to soil. Once tissue samples
373 were collected, they were frozen at -80C overnight. The samples were then ground and DNA
374 was extracted using the invitrogen Platinum Direct PCR Universal Master Mix (refA44647500)
375 according to the manufacturer's instructions. For the progenies of plants that underwent
376 agroflood, a single leaf tissue was sampled and DNA was extracted using invitrogen Platinum
377 Direct PCR Universal Master Mix (REF A44647500) according to the manufacturer's instructions.
378 The DNA was then used for Next Generation Amplicon Sequencing.

379 As similarly performed by Li et al [32], editing efficiency was characterized using single-
380 end Next Generation Sequencing on the Illumina NovaSeqX platform. Libraries were prepared
381 via a 2-step PCR amplification method. In the first round of amplification, each target site was
382 amplified using primers flanking the target site (Supplementary Table 9). After 25 cycles of
383 amplification, the reactions were cleaned using a 1.0X Ampure XP bead purification (Beckman
384 Coulter A63881). Next, each sample went through 12 additional cycles of amplification using
385 Illumina indexing primers. The samples were cleaned using a 0.7X Ampure XP purification.
386 Samples were checked for purity on a 2% agarose gel, quantified using Nanodrop, normalized,
387 and pooled.

388

389 <u>Next generation amplicon sequencing analysis</u>

390 Amplicon sequencing analysis was performed similarly as Li et al [32]. Only single-end
391 reads were used for analysis. Reads were adapter trimmed using Trim Galore default settings.
392 Remaining reads were mapped to the target genome region using the BWA aligner (v0.7.17,
393 BWA-MEM algorithm). Sorted and indexed bam files were used as input files for further analysis
394 by the CrispRvariants R package (v1.14.0). Each mutation pattern with corresponding read

395 counts was exported by the CrispRvariants R package. After assessing all control samples, a
396 criterion to classify reads as edited was established: only reads with a >= 3 bp deletion or
397 insertion (indel) of the same pattern (indels of same size starting at the same location) with
398 >=10 read counts from a sample were counted as edited reads. SNVs were also filtered out.
399

400 Off-target analysis

401 Off-target analysis was performed as previously described in Li et al[32]. DNA from single
402 Arabidopsis seedlings was extracted with the Qiagen DNeasy plant mini kit and sheared to 300
403 bp size with a Covaris. Library preparation was performed with Tecan Ovation Ultralow V2 DNA-
404 seq kit. For variant calling, WGS reads were aligned to the TARI10 reference genome using BWA
405 mem (v0.7.17)[33] with default parameters. GATK (4.2.0.0)[34] MarkDuplicatesSpark was used to
406 remove PCR duplicate reads. Then GATK HaplotypeCaller was used to call raw variants. Raw SNPs
407 were filtered with QD < 2.0, FS > 60.0, MQ < 40.0, and SOR > 4.0. Raw InDels were filtered with
408 QD < 2.0, FS > 200.0, and SOR > 10.0 and used for base quality score recalibration. The
409 recalibrated bam was further applied to GATK and Strelka (v2.9.2) SNPs/InDel calling. Only
410 SNPs/InDels called by both GATK and Strelka were used for further filtering. The intersection of
411 SNPs/InDel called by GATK with Strelka (v2.9.2)[35] is obtained by BedTools (v2.26.0) [36]. SNPs/InDel
412 filtered with wild type background were conducted by BedTools (v2.26.0). Variants with coverage
413 lower than 30 depth were filtered.

414

415 RT-PCR

416 Total RNA from TRV-infected progeny plants was extracted using Zymo Research Direct-
417 zol RNA MiniPrep kit (catR2052). Total RNA was converted to cDNA using the invitrogen
418 SuperScript IV VILO Master Mix (cat11766050). The RT-PCR control was performed using
419 primers targeting the *AtIPP2* gene (Supplementary Table 10). PCR was performed to check for
420 the presence/absence of the TRV vector using SP9238 and SP9239 (Supplementary Table 10) [24].
421 PCR was performed with New England Biolabs Q5 High-Fidelity 2X Master Mix (catM0492L)
422 according to the manufacturer's instructions, using 2 µl of cDNA in a 25 µl reaction. PCR

423 conditions included a 98°C initial denaturation step for 30 seconds, 35x(98°C, 10 sec; 55°C, 20
424 sec; 72°C, 10 sec), 72°C for 2 min. 10 µl of PCR amplicons were analyzed by 2% agarose gel
425 electrophoresis.
426
427
428 **Data availability:**
429 All the amp-seq data generated in this study will be accessible at NCBI Sequence Read Archive
430 under BioProject PRJNA1124592. Whole genome sequencing data is accessible at BioProject
431 PRJNA1146711
432
433
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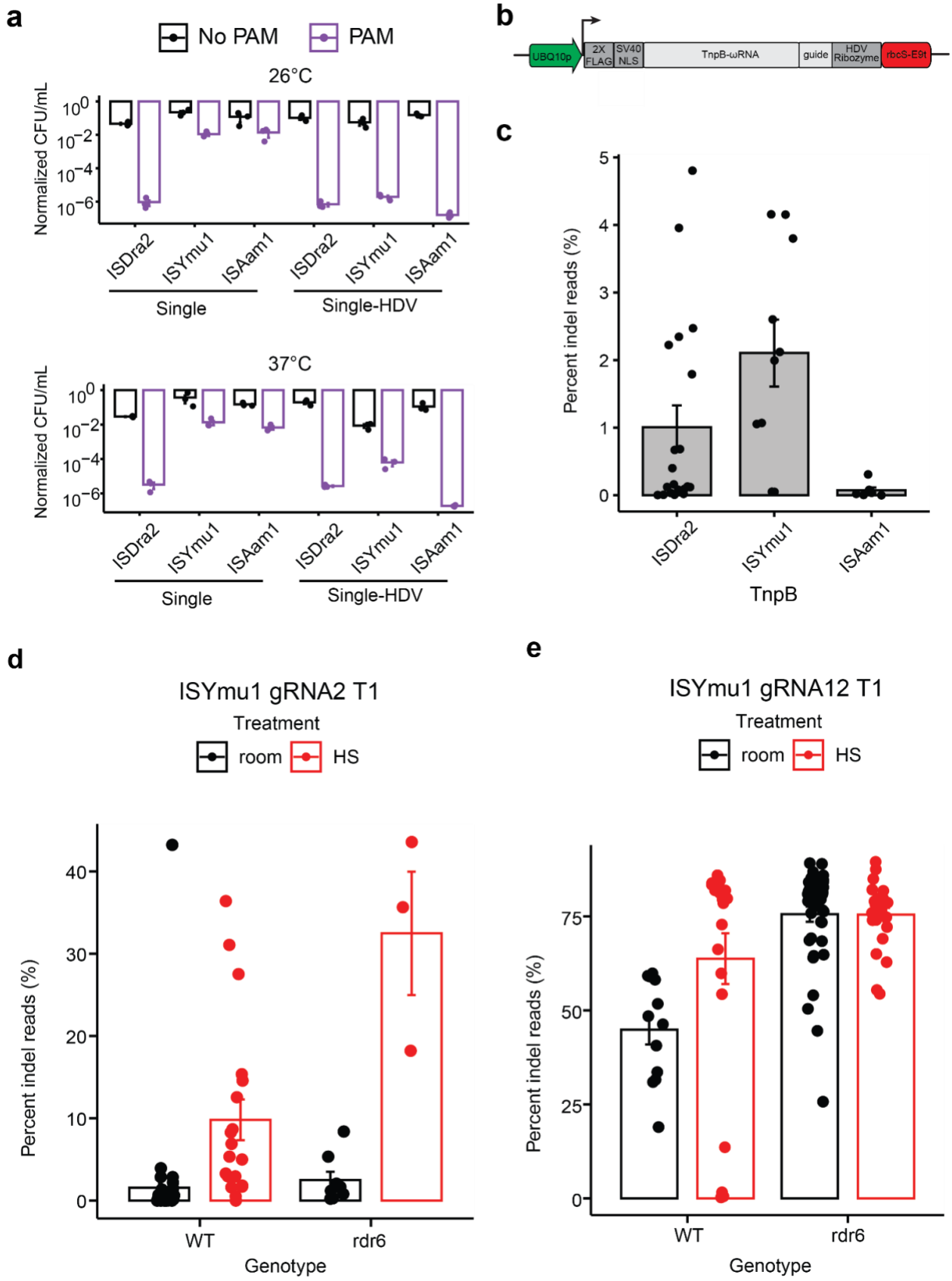
445 suggestions.
446
447
448 **Author Contributions:**
449 TW, MK, HS, ZL, BAA, JB, JAD and SEJ designed the research; TW, HS, ZZ, JAD, and SEJ
450 interpreted the data; TW and SEJ wrote the manuscript; TW, MK, HS, ZL, JA, MS, KV, GW, SC,
451 CA, NS, AS, and DS performed experiments.

Competing interests:

T.W. ,M.K, J.A, Z.L, H.S, B.A.A., J.A.D. and S.E.J. have filed a patent covering aspects of this work.

Figures and Tables:


a
No PAM
PAM
26°C
Normalized CFU/mL
ISDra2
ISYmu1
ISAam1
Single
Single-HDV
37°C
b
UBQ10p
2X FLAG
SV40 NLS
TnpB-ωRNA
guide
HDV Ribozyme
rbcS-E9t
c
Percent indel reads (%)
TnpB
d
ISYmu1 gRNA2 T1
Treatment
room
HS
WT
rdr6
Genotype
e
ISYmu1 gRNA12 T1

459

460 **Figure 1: Expression of TnpB and guide RNA in a single transcript for plant genome editing. (a)**
461 Barplots of interference assay testing the single transcript expression TnpB vectors for cleavage
462 in E. coli. The barplot on the top displays data from experiments performed at 26°C and the
463 barplot below depicts data from experiments performed at 37°C. Black bars indicate absence of
464 a PAM on the target plasmid and purple bars indicate the presence of a PAM on the target
465 plasmid. The Y-axis is a log10 scale of the normalized colony forming units (CFU)/milliliter (mL).
466 The X-axis displays the three TnpBs tested using the single expression transcript design without
467 a HDV ribozyme or the single expression design with a HDV ribozyme. The standard error of the
468 mean (SEM) was calculated for each experiment. **(b)** Schematic of the single expression
469 transcript TnpB-ωRNA plasmid design used for plant genome editing. The green arrow
470 symbolizes the At*UBQ10* promoter; the dark gray boxes indicate the 2X-FLAG, SV40 NLS and
471 HDV ribozyme sequences; the light gray boxes indicate the TnpB-ωRNA and guide sequences;
472 the red box symbolizes the rbcS-E9 terminator; the black arrow indicate the orientation of the
473 TnpB-ωRNA expression cassette. **(c)** Barplot displaying the average editing frequency for
474 protoplast experiments using ISDra2, ISYmu1 and ISAam1. Each dot represents the average
475 editing efficiency of a gRNA from Extended Data Fig. 3a. The Y-axis indicates the editing
476 efficiencies (percent indel reads (%)). The X-axis displays the ISDra2, ISYmu1 and ISAam1 TnpBs
477 tested. The standard error of the mean (SEM) was calculated for each experiment. **(d-e)** ISYmu1
478 somatic editing in T1 transgenic plants. Panels d and e display a barplot for ISYmu1 gRNA2 and
479 ISYmu1 gRNA12, respectively. Each dot indicates a single T1 transgenic plant. The room and HS
480 treatments stand for room temperature and heat shock plant growth conditions, respectively.
481 The genotypes are plotted along the X-axis and the editing efficiencies (percent indel reads (%))
482 are plotted on the Y-axis. The standard error of the mean (SEM) was calculated for each
483 experiment. WT is an abbreviation for wild type, and *rdr6* is an abbreviation for a mutation in
484 the *RNA-DEPENDENT RNA POLYMERASE 6* gene.

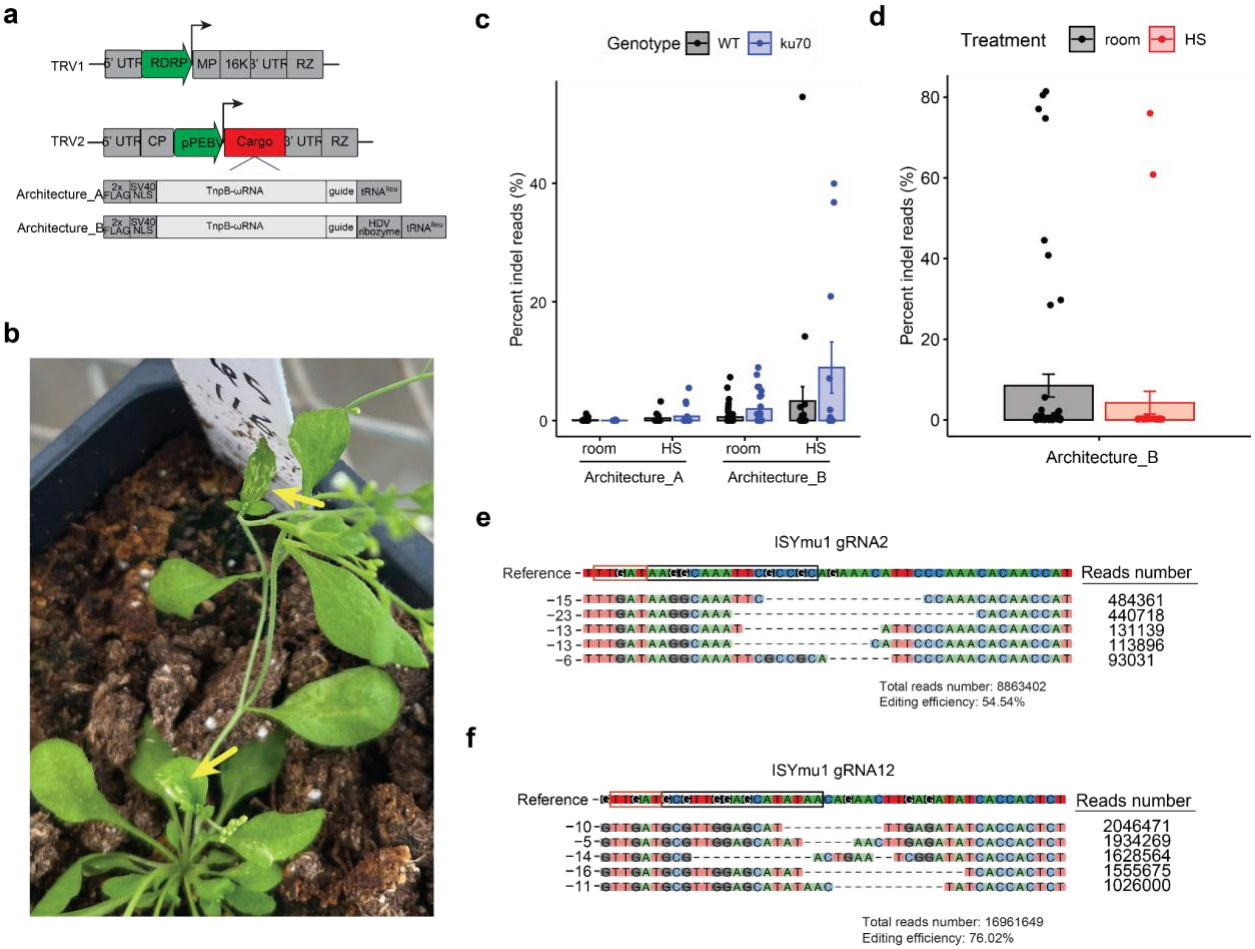


Figure 2: Somatic editing of *Arabidopsis* using TRV to deliver ISYmu1 TnpB and guide RNA. (a) Schematic of the TRV1 and TRV2 plasmids. Green arrows indicate the RNA dependent RNA polymerase (RDRP) and pea early browning virus (pPEBV) promoters for TRV1 and TRV2, respectively; the gray boxes in TRV1 and TRV2 indicate the native TRV components; the red Cargo box in TRV2 indicates the location of either Architecture_A or Architecture_B; below TRV2 are schematics of the components, Architecture_A or Architecture_B, cloned into the TRV2 Cargo slot. **(b)** Representative picture of a plant displaying white sectors about three weeks after TRV delivery. The yellow arrows indicate leaves containing white sectors. **(c)** Barplot displaying the somatic editing efficiencies for ISYmu1 gRNA2 in wild type (WT) and *ku70* genetic backgrounds. WT samples are black and *ku70* samples are blue. The TRV2 Cargo Architectures are plotted along the X-axis with either room or HS treatment. The room and HS along the X-axis stand for room temperature and heat shock plant growth conditions, respectively. The Y-axis indicates the editing efficiencies (percent indel reads (%)). Each dot

499 represents an individual plant that underwent agroflood TRV delivery. The standard error of the
500 mean (SEM) was calculated for each experiment. **(d)** Barplot displaying the somatic editing
501 efficiencies for ISYmu1 gRNA12 in wild type (WT). Room temperature treatment (room)
502 samples are black and heat shock (HS) samples are red. The TRV2 Cargo Architecture_B is
503 plotted along the X-axis. The Y-axis indicates the editing efficiencies (percent indel reads (%)).
504 Each dot represents an individual plant that underwent agroflood TRV delivery. The standard
505 error of the mean (SEM) was calculated for each experiment. **(e-f)** Panel e displays the DNA
506 indel repair profile for an individual WT plant that underwent delivery of TRV Cargo
507 Architecture_B with ISYmu1 gRNA2 under the heat shock treatment. Panel f shows the DNA
508 indel repair profile for an individual WT plant that underwent delivery of TRV Cargo
509 Architecture_B with ISYmu1 gRNA12 under the heat shock treatment. The top five most
510 common indel types are listed on the left. The read counts for each indel are listed on the right.
511 The PAM is identified by the red box, and the target site is outlined by the black box, in the
512 Reference sequence. The total read number and editing efficiency are listed below each indel
513 profile.

a

b

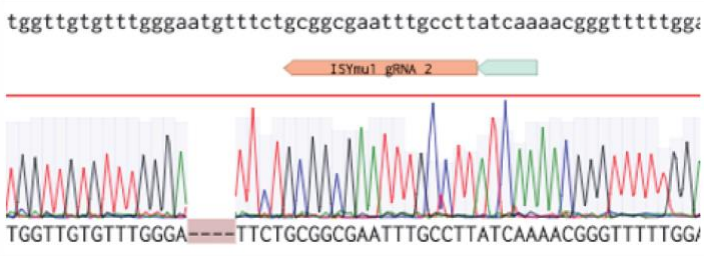
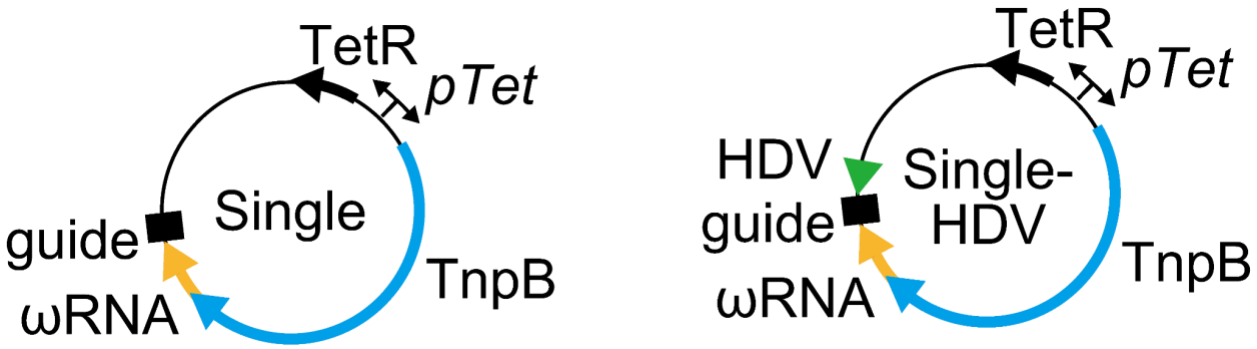
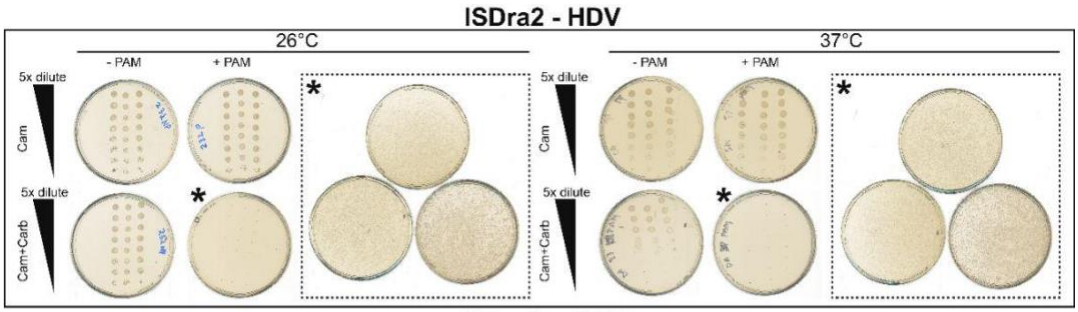
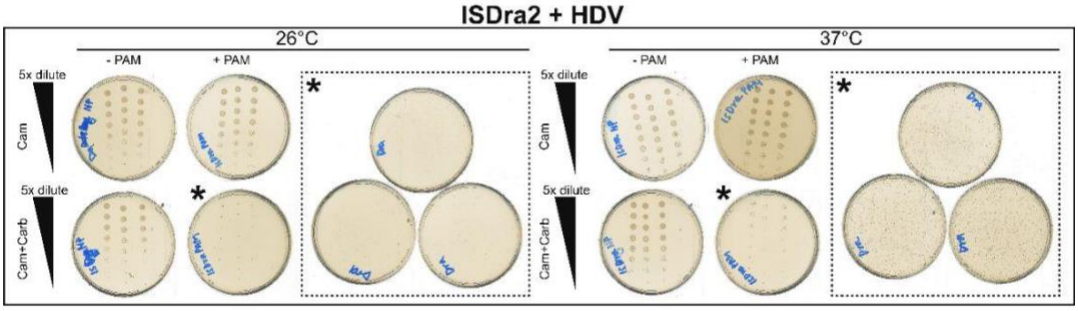


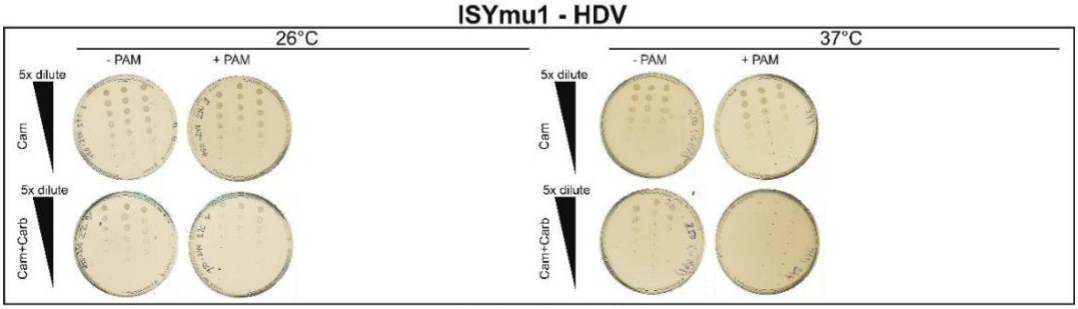
Figure 3: Inheritance of edited alleles. (a) Representative image of albino and green progeny seedlings from a WT plant showing 54.54% somatic editing using the TRV2 Architecture_B design with gRNA2 that underwent heat shock treatment. **(b)** Sanger sequencing trace file screenshot from one of the albino plants in Fig 3a. The sequence at the top is the wild type reverse complement; below that are the ISYmu1 gRNA2 target and PAM (gray box); the ab1 trace file displays a homozygous 4bp deletion.

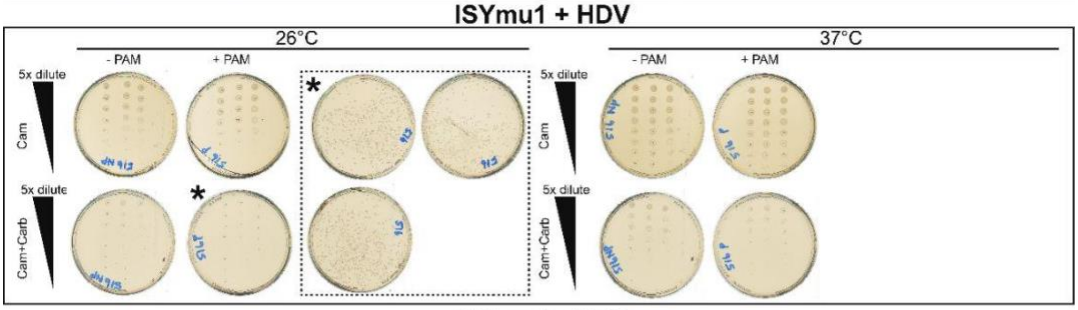


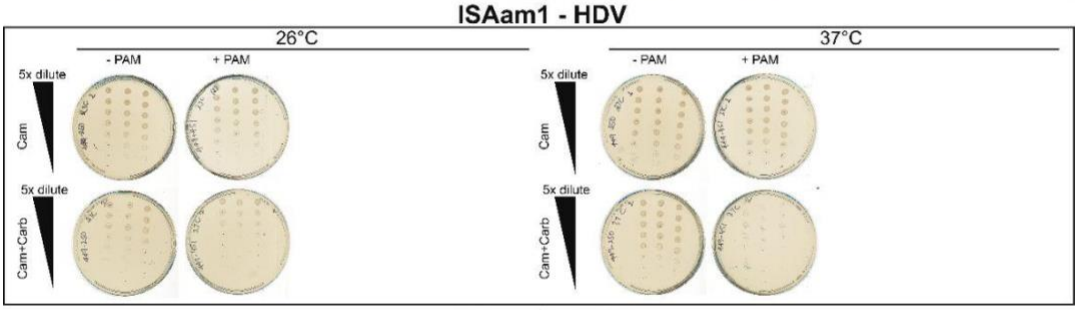
Extended Data Fig. 1: Schematic of interference assay plasmids containing either the TnpB-ωRNA Single or TnpB-ωRNA Single-HDV design. The blue arrow indicates the TnpB sequence; the yellow arrow indicates the ωRNA sequence; the black rectangle indicates the guide sequence; the green arrow indicates the HDV ribozyme sequence. The plasmids contain the tetracycline resistance gene (TetR). A tetracycline promoter (pTet) was used to drive expression of the TnpB-ωRNA single or Single-HDV sequences, and the tetracycline resistance gene.

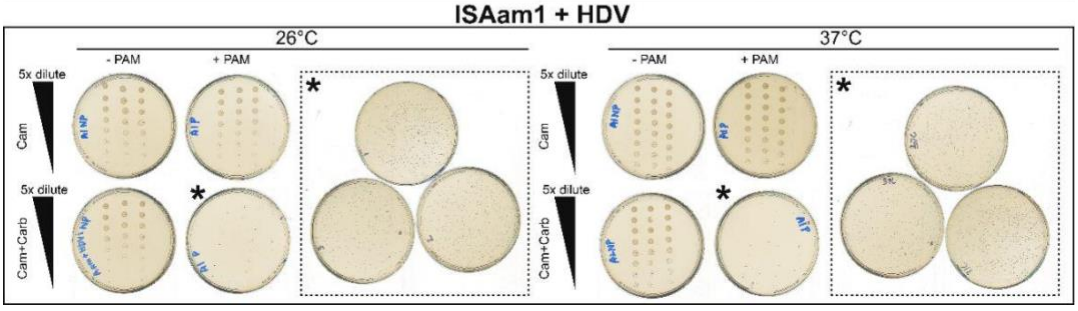

ISDra2 - HDV
26°C
37°C
- PAM
+ PAM
5x dilute
Cam
Cam+Carb
*


ISDra2 + HDV
26°C
37°C
- PAM
+ PAM
5x dilute
Cam
Cam+Carb
*

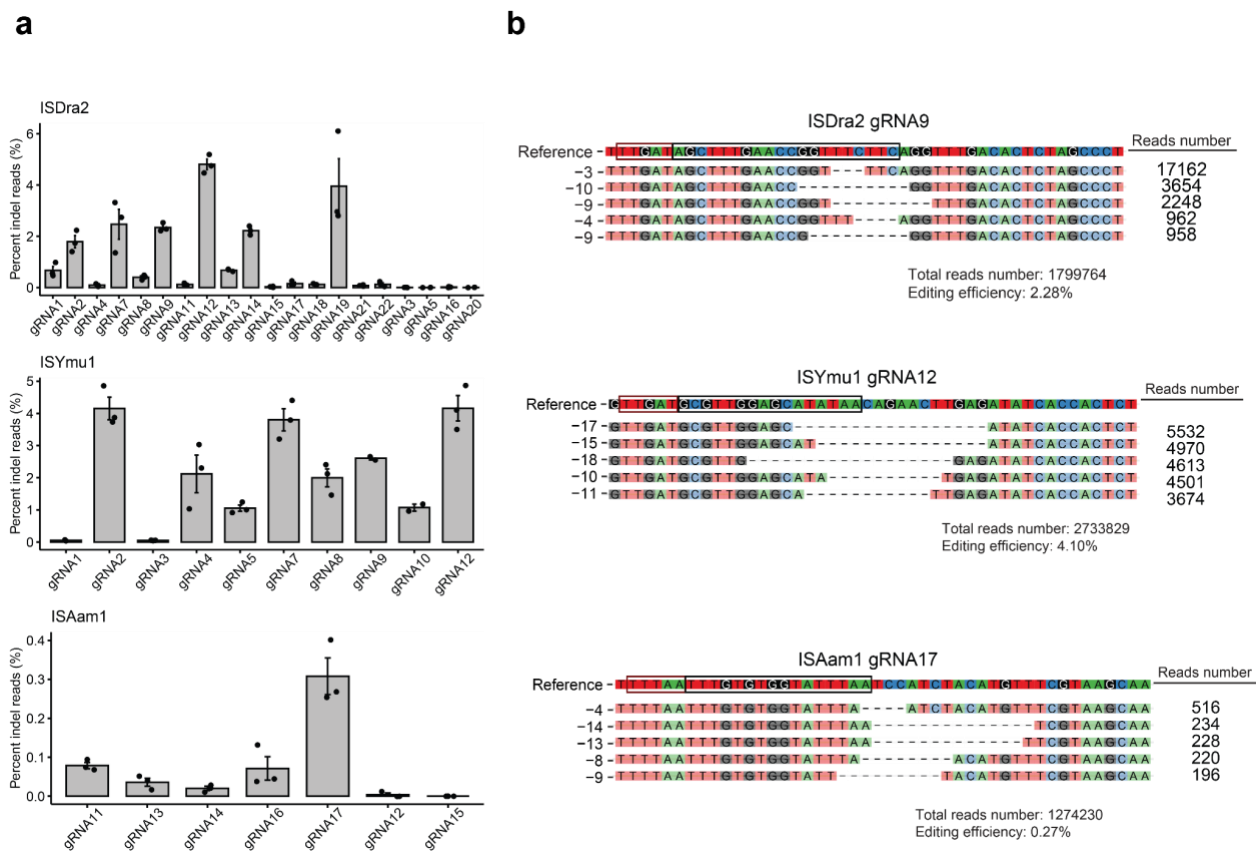

ISYmu1 - HDV
26°C
37°C
- PAM
+ PAM
5x dilute
Cam
Cam+Carb


ISYmu1 + HDV
26°C
37°C
- PAM
+ PAM
5x dilute
Cam
Cam+Carb
*

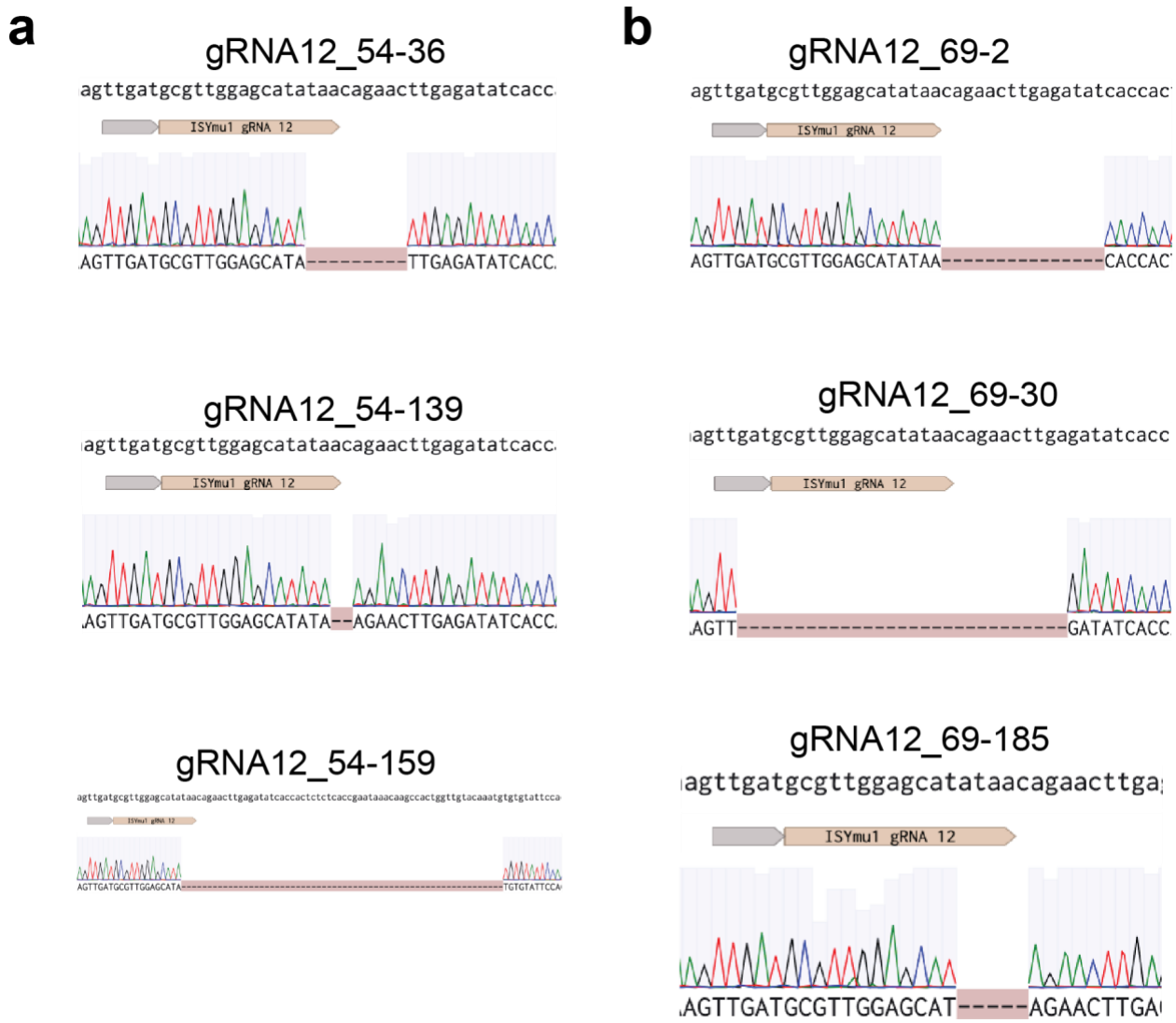

ISAam1 - HDV
26°C
37°C
- PAM
+ PAM
5x dilute
Cam
Cam+Carb


ISAam1 + HDV
26°C
37°C
- PAM
+ PAM
5x dilute
Cam
Cam+Carb
*

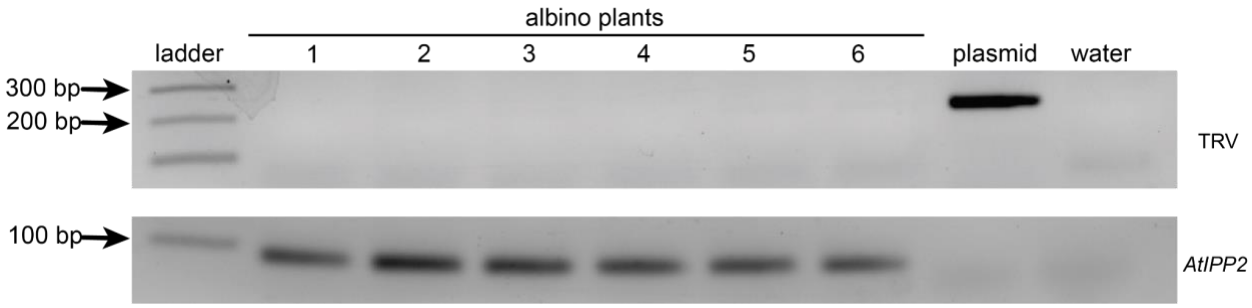
529 **Extended Data Fig. 2: Plate images of bacterial plasmid interference assay.** Five-fold serial
530 dilutions (5 µL) from the 1 mL recovery culture post transformation were plated on both single
531 antibiotic LB-Agar plates (Cam, upper row) and double antibiotic LB-Agar plates (Cam+Carb,
532 lower row). Plates without visible colonies (indicated by an asterisk) had 400 µL of the original 1
533 mL recovery culture plated on the double antibiotic plates (dash insets). Experiments were
534 performed in triplicates.



Extended Data Fig. 3: Editing efficiency and repair profiles for ISDra2, ISYmu1 and ISAam1 in protoplast experiment. (a) The name of each TnpB tested is at the upper left of each barplot. The gRNAs are plotted along the X-axis and the editing efficiency (percent indel reads (%)) is plotted on the Y-axis. Each dot indicates a single transfection. The standard error of the mean (SEM) was calculated for each target site. **(b)** DNA repair indel profiles for individual transfection samples. The top five most common indel types are listed on the left. The read counts for each indel are listed on the right. The PAM is identified by the red box, and the target site is outlined by the black box, in the Reference sequence. The total read number and editing efficiency are listed below each indel profile. The name of each TnpB and gRNA is displayed above the reference sequence of each indel repair profile.



Extended Data Fig. 4: Sanger sequencing screenshots of the progeny plant genotypes for ISYmu1 gRNA12. (a-b) Panels a and b correspond to the progeny from plants 54 and 69, respectively. The sequence at the top is the wild type genomic sequence; below that are the ISYmu1 gRNA12 target and PAM (gray box); the ab1 trace file displays the mutation.



Extended Data Fig. 5: RT-PCR gel showing absence of the TRV in the progeny of a TRV-infected plant. RT-PCR was performed using total RNA extracted from albino plants homozygous for a 4bp deletion at the *AtPDS3* gene. Gel electrophoresis image for RT-PCR performed using primers targeting TRV (upper panel) and *AtIPP2* gene (bottom panel). The lanes are indicated (from left to right) as ladder, six individual albino plants, plasmid control, and a water control. Black arrows indicate the amplicon size in base pairs (bp).

558

TnpB	Guide	gRNA sequence	Genomic location
ISDra2	gRNA1	ttacgaattgatgaccatat	4:8195186-8195206
ISDra2	gRNA2	aaggcaaattcgccgcagaa	4:8194730-8194749
ISDra2	gRNA3	tcacattaagcctagaaact	4:8193921-8193940
ISDra2	gRNA4	cccaagttctccaaataaat	4:8193476-8193495
ISDra2	gRNA5	tacccatcctaaagtatggg	4:8192944-8192963
ISDra2	gRNA7	aaattcaacatctttctcta	4:8190835-8190854
ISDra2	gRNA8	tggtctcactttccgaatta	4:8194167-8194186
ISDra2	gRNA9	agctttgaaccggtttcttc	4:8192305-8192324
ISDra2	gRNA11	cgaaaactgaagaacacata	4:8191494-8191513
ISDra2	gRNA12	gcgttggagcatataacaga	4:8195556-8195575
ISDra2	gRNA13	taaagagaggaaattgcagg	4:8194513-8194532
ISDra2	gRNA14	ggtagagctgataagatata	4:8193625-8193644
ISDra2	gRNA15	aaaattggattaatgtgcac	4:8193203-8193222
ISDra2	gRNA16	tactattaaatgtcaaaatc	4:8193077-8193096
ISDra2	gRNA17	caatacaaataaatacatgc	4:8193117-8193136
ISDra2	gRNA18	caattcaagctaattataga	4:8193095-8193114
ISDra2	gRNA19	gagcttaacttggtagagta	4:8192577-8192596
ISDra2	gRNA20	ttgtcagctttcttatggat	4:8192528-8192547
ISDra2	gRNA21	gttgattaacttgtactacc	4:8192131-8192150
ISDra2	gRNA22	taacttgtactacctcatcc	4:8192125-8192144
ISYmu1	gRNA1	ttacgaattgatgacc	4:8195187-8195202

ISYmu1	gRNA2	aaggcaaattcgccgc	4:8194730-8194745
ISYmu1	gRNA3	tcacattaagcctaga	4:8193921-8193936
ISYmu1	gRNA4	cccaagttctccaaat	4:8193476-8193491
ISYmu1	gRNA5	tacccatcctaaagta	4:8192944-8192959
ISYmu1	gRNA7	aaattcaacatctttc	4:8190839-8190854
ISYmu1	gRNA8	tggtctcactttccga	4:8194171-8194186
ISYmu1	gRNA9	agctttgaaccggttt	4:8192309-8192324
ISYmu1	gRNA10	taacttgtactacctc	4:8192129-8192144
ISYmu1	gRNA12	gcgttggagcatataa	4:8195560-8195575
ISAam1	gRNA11	caattcatctggtatc	4:8191755-8191770
ISAam1	gRNA12	ccaagaacaagcctta	4:8191347-8191362
ISAam1	gRNA13	gttttgtcctcttctc	4:8190412-8190427
ISAam1	gRNA14	caatttacctatctta	4:8193797-8193812
ISAam1	gRNA15	cacataattgaaaaga	4:8194919-8194934
ISAam1	gRNA16	aatttgttacacaact	4:8195755-8195770
ISAam1	gRNA17	tttgtgtggtatttaa	4:8195158-8195173

Supplementary Table 1: ISDra2, ISYmu1 and ISAam1 target sites. The table consists of four columns. The TnpB and Guide columns indicate the TnpB gRNA used. The gRNA sequence column lists the gRNA sequence used for targeted genome editing. The Genomic location column provides the location of each gRNA target site in the *Arabidopsis* genome.

563

Plant ID	Phenotype	Genotype (allele1 / allele2)	Sequencing type
gRNA2_HS_116-1	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-2	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-3	WT	WT / WT	amp-seq
gRNA2_HS_116-4	WT	WT / WT	amp-seq
gRNA2_HS_116-5	WT	4bp del / WT	amp-seq
gRNA2_HS_116-6	WT	WT / WT	amp-seq
gRNA2_HS_116-9	WT	WT / WT	amp-seq
gRNA2_HS_116-10	WT	WT / WT	amp-seq
gRNA2_HS_116-12	WT	WT / WT	amp-seq
gRNA2_HS_116-13	WT	WT / WT	amp-seq
gRNA2_HS_116-14	WT	WT / WT	amp-seq
gRNA2_HS_116-15	WT	WT / WT	amp-seq
gRNA2_HS_116-16	WT	WT / WT	amp-seq
gRNA2_HS_116-17	WT	WT / WT	amp-seq
gRNA2_HS_116-18	WT	WT / WT	amp-seq
gRNA2_HS_116-19	WT	WT / WT	amp-seq
gRNA2_HS_116-20	WT	WT / WT	amp-seq
gRNA2_HS_116-21	WT	WT / WT	amp-seq
gRNA2_HS_116-22	WT	WT / WT	amp-seq
gRNA2_HS_116-23	WT	WT / WT	amp-seq
gRNA2_HS_116-24	WT	WT / WT	amp-seq

gRNA2_HS_116-25	WT	WT / WT	amp-seq
gRNA2_HS_116-26	WT	WT / WT	amp-seq
gRNA2_HS_116-27	WT	WT / WT	amp-seq
gRNA2_HS_116-28	WT	4bp del / WT	amp-seq
gRNA2_HS_116-29	WT	WT / WT	amp-seq
gRNA2_HS_116-30	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-31	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-32	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-33	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-34	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-35	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-36	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-38	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-39	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-40	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-41	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-42	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-43	Albino	11 bp del / 17bp del	amp-seq
gRNA2_HS_116-45	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-46	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-47	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-48	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-49	Albino	10bp del / 12 bp del	amp-seq

gRNA2_HS_116-50	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-51	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-52	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-53	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-54	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-56	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-57	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-58	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-59	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-60	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-61	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-65	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-72	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-79	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-80	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-84	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-85	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-86	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-90	Albino	17bp del / 11bp del	amp-seq
gRNA2_HS_116-91	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-92	Albino	4bp del / 4bp del	amp-seq
gRNA2_HS_116-94	WT	WT / WT	amp-seq
gRNA2_HS_116-95	WT	WT / WT	amp-seq

gRNA2_HS_116-96	WT	WT / WT	amp-seq
gRNA2_HS_116-97	WT	WT / WT	amp-seq
gRNA2_HS_116-98	WT	WT / WT	amp-seq
gRNA2_HS_116-99	WT	WT / WT	amp-seq
gRNA2_HS_116-100	WT	4bp del / WT	amp-seq
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gRNA2_HS_116-107	WT	WT / WT	amp-seq
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gRNA2_HS_116-112	WT	WT / WT	amp-seq
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gRNA2_HS_116-117	WT	WT / WT	amp-seq
gRNA2_HS_116-118	WT	WT / WT	amp-seq

gRNA2_HS_116-119	WT	WT / WT	amp-seq
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gRNA2_HS_116-122	WT	WT / WT	amp-seq
gRNA2_HS_116-123	WT	WT / WT	amp-seq
gRNA2_HS_116-124	WT	WT / WT	amp-seq
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gRNA2_HS_116-126	WT	WT / WT	amp-seq
gRNA2_HS_116-127	WT	WT / WT	amp-seq
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gRNA2_HS_116-132	WT	WT / WT	amp-seq
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gRNA2_HS_116-226	WT	WT / WT	amp-seq
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gRNA2_HS_116-244	WT	4bp del / WT	amp-seq

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gRNA2_HS_116-247	WT	WT / WT	amp-seq
gRNA2_HS_116-248	WT	WT / WT	amp-seq
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gRNA12_room_54-47	WT	WT / WT	Sanger
gRNA12_room_54-48	WT	WT / WT	Sanger
gRNA12_room_54-49	WT	17bp del / 36bp del	Sanger
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gRNA12_room_54-60	WT	WT / WT	Sanger

gRNA12_room_54-61	WT	17bp del / 36bp del	Sanger
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gRNA12_room_54-66	WT	WT / WT	Sanger
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gRNA12_room_54-70	WT	WT / WT	Sanger
gRNA12_room_54-71	WT	WT / WT	Sanger
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gRNA12_room_54-110	WT	3bp del / WT	Sanger
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gRNA12_room_54-145	WT	WT / WT	Sanger
gRNA12_room_54-146	WT	WT / WT	Sanger
gRNA12_room_54-148	WT	17bp del / 36bp del	Sanger
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gRNA12_room_54-183	WT	WT / WT	Sanger

gRNA12_room_54-184	WT	5bp del / WT	Sanger
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gRNA12_room_54-186	WT	WT / WT	Sanger
gRNA12_room_54-190	WT	9bp del / WT	Sanger
gRNA12_room_54-191	WT	15bp del / WT	Sanger
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gRNA12_room_54-193	WT	WT / WT	Sanger
gRNA12_room_54-194	WT	7bp del / 7bp del	Sanger
gRNA12_room_54-195	WT	WT / WT	Sanger
gRNA12_room_54-196	WT	WT / WT	Sanger
gRNA12_room_54-197	WT	10bp del / 10bp del	Sanger
gRNA12_room_54-198	WT	10bp del / WT	Sanger
gRNA12_room_54-199	WT	WT / WT	Sanger
gRNA12_room_54-200	WT	2bp del / 2bp del	Sanger
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gRNA12_room_54-212	WT	WT / WT	Sanger
gRNA12_room_54-213	WT	3bp del / WT	Sanger
gRNA12_room_54-214	WT	WT / WT	Sanger
gRNA12_room_54-215	WT	4bp del / 4bp del	Sanger
gRNA12_room_54-216	WT	WT / WT	Sanger

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gRNA12_room_54-223	WT	WT / WT	Sanger
gRNA12_room_54-224	WT	WT / WT	Sanger
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gRNA12_room_69-5	WT	5bp del / 24bp del	Sanger
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gRNA12_room_69-21	WT	5bp del / 24bp del	Sanger
gRNA12_room_69-22	WT	17bp del / WT	Sanger
gRNA12_room_69-23	WT	WT / WT	Sanger
gRNA12_room_69-28	WT	5bp del / 4bp del	Sanger

gRNA12_room_69-30	WT	29bp del / 29bp del	Sanger
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gRNA12_room_69-34	WT	5bp del / 5bp del	Sanger
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gRNA12_room_69-44	WT	5bp del / 15bp del	Sanger
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gRNA12_room_69-53	WT	15bp del / 15bp del	Sanger
gRNA12_room_69-55	WT	5bp del / 5bp del	Sanger
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gRNA12_room_69-80	WT	WT / WT	Sanger
gRNA12_room_69-87	WT	13bp del / 14bp del	Sanger
gRNA12_room_69-88	WT	11bp del / WT	Sanger
gRNA12_room_69-91	WT	WT / WT	Sanger
gRNA12_room_69-92	WT	5bp del / 10bp del	Sanger
gRNA12_room_69-93	WT	WT / WT	Sanger
gRNA12_room_69-94	WT	WT / WT	Sanger

gRNA12_room_69-95	WT	3bp del / 23bp del	Sanger
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gRNA12_room_69-108	WT	WT / WT	Sanger
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gRNA12_room_69-115	WT	WT / WT	Sanger
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gRNA12_room_69-124	WT	15bp del / 15bp del	Sanger
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gRNA12_room_69-129	WT	WT / WT	Sanger
gRNA12_room_69-130	WT	WT / WT	Sanger
gRNA12_room_69-131	WT	4bp del / 5bp del	Sanger
gRNA12_room_69-132	WT	WT / WT	Sanger
gRNA12_room_69-134	WT	WT / WT	Sanger
gRNA12_room_69-137	WT	4bp del / WT	Sanger
gRNA12_room_69-142	WT	15bp del / 5bp del	Sanger
gRNA12_room_69-148	WT	WT / WT	Sanger
gRNA12_room_69-149	WT	22bp del / 22bp del	Sanger
gRNA12_room_69-150	WT	WT / WT	Sanger
gRNA12_room_69-151	WT	WT / WT	Sanger
gRNA12_room_69-154	WT	11bp del / 11bp del	Sanger

gRNA12_room_69-157	WT	WT / WT	Sanger
gRNA12_room_69-158	WT	11bp del / WT	Sanger
gRNA12_room_69-159	WT	18bp del / WT	Sanger
gRNA12_room_69-163	WT	WT / WT	Sanger
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gRNA12_room_69-171	WT	3bp del / 5bp del	Sanger
gRNA12_room_69-172	WT	10bp del / 10bp del	Sanger
gRNA12_room_69-175	WT	5bp del / WT	Sanger
gRNA12_room_69-183	WT	WT / WT	Sanger
gRNA12_room_69-185	WT	5bp del / 5bp del	Sanger
gRNA12_room_69-188	WT	1bp del / WT	Sanger

Supplementary Table 2: Phenotype and genotype of the progenies from three individual TRV-infected plants. The plant ID column indicates an internal identifier for each plant sampled. The Phenotype column indicates whether the plant was green (WT) or albino. The Genotype (allele1 / allele2) column lists the alleles of the *AtPDS3* gene determined by amp-seq or Sanger Sequencing. WT is an abbreviation for wild type, bp is an abbreviation for base pair, and del is an abbreviation for deletion.

Sample	Reads count	Unmapped reads count	Mapping rate (%)	Coverage
WT-rep1	331996121	2122433	99.36	817.69
WT-rep2	314298698	1969352	99.37	774.20
gRNA2_HS_116-2	315840117	1628611	99.48	778.87
gRNA2_HS_116-32	357227432	2397598	99.33	879.55
gRNA2_HS_116-43	265745567	1688428	99.36	654.55

Supplementary Table 3: Whole genome sequencing read count and mapping rate of WT control and albino mutant plants.

573

Sample	GATK	Strealk2	GATK+Strealk2	Filter with WT	Filter by depth > 30	Manually check
WT	40852	24406	NA	NA	NA	NA
albino-plant2	36346	20659	16296	28	25	5
albino-plant32	36342	20991	16629	35	30	5
albino-plant43	36989	21928	17111	80	71	4

574 **Supplementary Table 4: WGS variant detection of WT control and albino mutant plants using**

575 **GATK and Strelka2.**

576

DNA	Chromosome	Location	Direction	Mismatches	Variants
TTcATtAGGCAAATTCaCtGC	chr1	1799061	+	4	No SNP/Indel
TTGATAAGGaAgATTCGtCtC	chr1	21688156	+	4	No SNP/Indel
TTGATAAGcCAtATTCtCCcC	chr1	28196564	+	4	No SNP/Indel
TTGATcAGGaAAATTCGCgGa	chr1	29172329	-	4	No SNP/Indel
TTGATcAGGCAAATTCGgaGC	chr2	2183162	+	3	No SNP/Indel
aaGcTAAGGCAAATcCGCCGC	chr2	10574134	-	4	No SNP/Indel
TTGATAAGaacAtTTCGCCGC	chr2	11679602	-	4	No SNP/Indel
gTcATAAGtCAcATTCGCCGC	chr2	12173059	+	4	No SNP/Indel
TTGATAAGtCAAAgTCtaCGC	chr2	13554186	-	4	No SNP/Indel
TTGATActGCAAAgTCGCCtC	chr2	16402169	+	4	No SNP/Indel
TacAgAAGGCAAATTCcCCGC	chr3	381121	-	4	No SNP/Indel
TTGATAAtGCAAcTTCtCCGt	chr3	527026	-	4	No SNP/Indel
TTcATcAGGCAtATTCGCCGt	chr3	13200296	-	4	No SNP/Indel
TTGgTAAGGCAAATTCttaGC	chr4	8560337	+	4	No SNP/Indel
gTGAaAAcGCAAATTCGCgGC	chr5	3918356	+	4	No SNP/Indel
TTGATAAGtCAAAaTCcCCaC	chr5	10937249	-	4	No SNP/Indel
TTGATAAtGCAAATTacCCGt	chr5	18593314	-	4	No SNP/Indel
TTGATAAGGCAAtcTCtaCGC	chr5	26138716	+	4	No SNP/Indel

577 **Supplementary Table 5: Off-target editing analysis using Cas-OFFinder.** The DNA column
578 contains the sequence of the potential off-target site, with lower case letters indicating a
579 mismatch compared to the actual target site. The Chromosome, Location, and Direction
580 columns indicate where in the *Arabidopsis* genome that potential off-target site is located. The
581 mismatch column indicates the number of mismatches in the potential off-target site relative to

582 the actual target site sequence. The Variants column lists the off-target editing result for that
583 site, with every off-target site analyzed as wild type (No SNP/Indel).

584

Plasmid	Description
pMK003	Cloning vector for PaqCI Golden Gate assembly of ISDra2 guides into ISDra2 plant expression vector
pMK025	Cloning vector for PaqCI Golden Gate assembly of ISYmu1 guides into ISYmu1 plant expression vector
pMK024	Cloning vector for PaqCI Golden Gate assembly of ISAam1 guides into ISAam1 plant expression vector
pMK026	ISDra2 g12 cloned into pMK003
pMK027	ISDra2 g13 cloned into pMK003
pMK028	ISDra2 g8 cloned into pMK003
pMK029	ISDra2 g14 cloned into pMK003
pMK030	ISDra2 g4 cloned into pMK003
pMK031	ISDra2 g15 cloned into pMK003
pMK032	ISDra2 g17 cloned into pMK003
pMK033	ISDra2 g18 cloned into pMK003
pMK034	ISDra2 g5 cloned into pMK003
pMK035	ISDra2 g20 cloned into pMK003
pMK036	ISDra2 g22 cloned into pMK003
pMK037	ISDra2 g1 cloned into pMK003
pMK038	ISDra2 g2 cloned into pMK003
pMK039	ISDra2 g3 cloned into pMK003
pMK040	ISDra2 g16 cloned into pMK003

pMK041	ISDra2 g19 cloned into pMK003
pMK042	ISDra2 g9 cloned into pMK003
pMK043	ISDra2 g21 cloned into pMK003
pMK044	ISDra2 g11 cloned into pMK003
pMK045	ISDra2 g7 cloned into pMK003
pMK060	ISYmu1 g1 cloned into pMK025
pMK061	ISYmu1 g2 cloned into pMK025
pMK062	ISYmu1 g3 cloned into pMK025
pMK063	ISYmu1 g4 cloned into pMK025
pMK064	ISYmu1 g5 cloned into pMK025
pMK065	ISYmu1 g7 cloned into pMK025
pMK066	ISYmu1 g8 cloned into pMK025
pMK067	ISYmu1 g9 cloned into pMK025
pMK068	ISYmu1 g10 cloned into pMK025
pMK070	ISYmu1 g12 cloned into pMK025
pMK050	ISAam1 g11 cloned into pMK024
pMK051	ISAam1 g12 cloned into pMK024
pMK052	ISAam1 g13 cloned into pMK024
pMK053	ISAam1 g14 cloned into pMK024
pMK054	ISAam1 g15 cloned into pMK024
pMK055	ISAam1 g16 cloned into pMK024
pMK056	ISAam1 g17 cloned into pMK024

pTW2065	ISYmu1 gRNA2 TRV2 Architecture_A
pTW2066	ISYmu1 gRNA2 TRV2 Architecture_B
pTW2082	ISYmu1 gRNA12 TRV2 Architecture_B

585 **Supplementary Table 6: Plasmids used in this study.** The Plasmid column lists the name of
586 each plasmid used. The Description column provides a TnpB and gRNA description of each
587 plasmid used, along with the intermediate plant expression vector that was used to create it.

Oligo name	Oligo sequence	Oligo description
MK_29476	TCAAgcgttggagcatataacaga	cloning ISDra2 gRNA_20_bp_gRNA_12_Top
Trevor_28675	GGCCtctgttatatgctccaacgc	cloning ISDra2 gRNA_20_bp_gRNA_12_Bottom
MK_29477	TCAAttacgaattgatgaccatat	cloning ISDra2 gRNA_20_bp_gRNA_1_Top
Trevor_28677	GGCCatatggtcatcaattcgtaa	cloning ISDra2 gRNA_20_bp_gRNA_1_Bottom
MK_29478	TCAAaaggcaaattcgccgcagaa	cloning ISDra2 gRNA_20_bp_gRNA_2_Top
Trevor_28679	GGCCttctgcggcgaatttgcctt	cloning ISDra2 gRNA_20_bp_gRNA_2_Bottom
MK_29479	TCAAtaaagagaggaaattgcagg	cloning ISDra2 gRNA_20_bp_gRNA_13_Top
Trevor_28681	GGCCcctgcaatttcctctcttta	cloning ISDra2 gRNA_20_bp_gRNA_13_Bottom
MK_29480	TCAAtggtctcactttccgaatta	cloning ISDra2 gRNA_20_bp_gRNA_8_Top
Trevor_28683	GGCCtaattcggaaagtgagacca	cloning ISDra2 gRNA_20_bp_gRNA_8_Bottom
MK_29481	TCAAtcacattaagcctagaaact	cloning ISDra2 gRNA_20_bp_gRNA_3_Top
Trevor_28685	GGCCagtttctaggcttaatgtga	cloning ISDra2 gRNA_20_bp_gRNA_3_Bottom
MK_29482	TCAAggtagagctgataagatata	cloning ISDra2 gRNA_20_bp_gRNA_14_Top
Trevor_28687	GGCCtatatcttatcagctctacc	cloning ISDra2 gRNA_20_bp_gRNA_14_Bottom
MK_29483	TCAAcccaagttctccaaataaat	cloning ISDra2 gRNA_20_bp_gRNA_4_Top
Trevor_28689	GGCCatttatttggagaacttggg	cloning ISDra2 gRNA_20_bp_gRNA_4_Bottom
MK_29484	TCAAaaaattggattaatgtgcac	cloning ISDra2 gRNA_20_bp_gRNA_15_Top
Trevor_28691	GGCCgtgcacattaatccaatttt	cloning ISDra2 gRNA_20_bp_gRNA_15_Bottom
MK_29485	TCAAcaatacaaataaatacatgc	cloning ISDra2 gRNA_20_bp_gRNA_17_Top
Trevor_28693	GGCCgcatgtatttatttgtattg	cloning ISDra2 gRNA_20_bp_gRNA_17_Bottom
MK_29486	TCAAcaattcaagctaattataga	cloning ISDra2 gRNA_20_bp_gRNA_18_Top
Trevor_28695	GGCCtctataattagcttgaattg	cloning ISDra2 gRNA_20_bp_gRNA_18_Bottom
MK_29487	TCAAtactattaaatgtcaaaatc	cloning ISDra2 gRNA_20_bp_gRNA_16_Top

Trevor_28697	GGCCgattttgacatttaatagta	cloning ISDra2 gRNA_20_bp_gRNA_16_Bottom
MK_29488	TCAAtacccatcctaaagtatggg	cloning ISDra2 gRNA_20_bp_gRNA_5_Top
Trevor_28699	GGCCcccatactttaggatgggta	cloning ISDra2 gRNA_20_bp_gRNA_5_Bottom
MK_29489	TCAAgagcttaacttggtagagta	cloning ISDra2 gRNA_20_bp_gRNA_19_Top
Trevor_28701	GGCCtactctaccaagttaagctc	cloning ISDra2 gRNA_20_bp_gRNA_19_Bottom
MK_29490	TCAAttgtcagctttcttatggat	cloning ISDra2 gRNA_20_bp_gRNA_20_Top
Trevor_28703	GGCCatccataagaaagctgacaa	cloning ISDra2 gRNA_20_bp_gRNA_20_Bottom
MK_29491	TCAAagctttgaaccggtttcttc	cloning ISDra2 gRNA_20_bp_gRNA_9_Top
Trevor_28705	GGCCgaagaaaccggttcaaagct	cloning ISDra2 gRNA_20_bp_gRNA_9_Bottom
MK_29492	TCAAgttgattaacttgtactacc	cloning ISDra2 gRNA_20_bp_gRNA_21_Top
Trevor_28707	GGCCggtagtacaagttaatcaac	cloning ISDra2 gRNA_20_bp_gRNA_21_Bottom
MK_29493	TCAAtaacttgtactacctcatcc	cloning ISDra2 gRNA_20_bp_gRNA_22_Top
Trevor_28709	GGCCggatgaggtagtacaagtta	cloning ISDra2 gRNA_20_bp_gRNA_22_Bottom
MK_29494	TCAAcgaaaactgaagaacacata	cloning ISDra2 gRNA_20_bp_gRNA_11_Top
Trevor_28711	GGCCtatgtgttcttcagttttcg	cloning ISDra2 gRNA_20_bp_gRNA_11_Bottom
MK_29495	TCAAaaattcaacatctttctcta	cloning ISDra2 gRNA_20_bp_gRNA_7_Top
Trevor_28713	GGCCtagagaaagatgttgaattt	cloning ISDra2 gRNA_20_bp_gRNA_7_Bottom
MK_29758	TCAAttacgaattgatgacc	cloning ISYmu1 gRNA_16_bp_gRNA_1_Top
MK_29759	GGCCggtcatcaattcgtaa	cloning ISYmu1 gRNA_16_bp_gRNA_1_Bottom
MK_29760	TCAAaaggcaaattcgccgc	cloning ISYmu1 gRNA_16_bp_gRNA_2_Top
MK_29761	GGCCgcggcgaatttgcctt	cloning ISYmu1 gRNA_16_bp_gRNA_2_Bottom
MK_29762	TCAAtcacattaagcctaga	cloning ISYmu1 gRNA_16_bp_gRNA_3_Top
MK_29763	GGCCtctaggcttaatgtga	cloning ISYmu1 gRNA_16_bp_gRNA_3_Bottom
MK_29764	TCAAcccaagttctccaaat	cloning ISYmu1 gRNA_16_bp_gRNA_4_Top

MK_29765	GGCCatttggagaacttggg	cloning ISYmu1 gRNA_16_bp_gRNA_4_Bottom
MK_29766	TCAAtacccatcctaaagta	cloning ISYmu1 gRNA_16_bp_gRNA_5_Top
MK_29767	GGCCtactttaggatgggta	cloning ISYmu1 gRNA_16_bp_gRNA_5_Bottom
MK_29770	TCAAaaattcaacatctttc	cloning ISYmu1 gRNA_16_bp_gRNA_7_Top
MK_29771	GGCCgaaagatgttgaattt	cloning ISYmu1 gRNA_16_bp_gRNA_7_Bottom
MK_29772	TCAAtggtctcactttccga	cloning ISYmu1 gRNA_16_bp_gRNA_8_Top
MK_29773	GGCCtcggaaagtgagacca	cloning ISYmu1 gRNA_16_bp_gRNA_8_Bottom
MK_29774	TCAAagctttgaaccggttt	cloning ISYmu1 gRNA_16_bp_gRNA_9_Top
MK_29775	GGCCaaaccggttcaaagct	cloning ISYmu1 gRNA_16_bp_gRNA_9_Bottom
MK_29776	TCAAtaacttgtactacctc	cloning ISYmu1 gRNA_16_bp_gRNA_10_Top
MK_29777	GGCCgaggtagtacaagtta	cloning ISYmu1 gRNA_16_bp_gRNA_10_Bottom
MK_29780	TCAAgcgttggagcatataa	cloning ISYmu1 gRNA_16_bp_gRNA_12_Top
MK_29781	GGCCttatatgctccaacgc	cloning ISYmu1 gRNA_16_bp_gRNA_12_Bottom
MK_29818	TCACcaattcatctggtatc	cloning ISAam1 gRNA_16_bp_gRNA_11_Top
MK_29819	GGCCgataccagatgaattg	cloning ISAam1 gRNA_16_bp_gRNA_11_Bottom
MK_29820	TCACccaagaacaagcctta	cloning ISAam1 gRNA_16_bp_gRNA_12_Top
MK_29821	GGCCtaaggcttgttcttgg	cloning ISAam1 gRNA_16_bp_gRNA_12_Bottom
MK_29822	TCACgttttgtcctcttctc	cloning ISAam1 gRNA_16_bp_gRNA_13_Top
MK_29823	GGCCgagaagaggacaaaac	cloning ISAam1 gRNA_16_bp_gRNA_13_Bottom
MK_29824	TCACcaatttacctatctta	cloning ISAam1 gRNA_16_bp_gRNA_14_Top
MK_29825	GGCCtaagataggtaaattg	cloning ISAam1 gRNA_16_bp_gRNA_14_Bottom
MK_29826	TCACcacataattgaaaaga	cloning ISAam1 gRNA_16_bp_gRNA_15_Top
MK_29827	GGCCtcttttcaattatgtg	cloning ISAam1 gRNA_16_bp_gRNA_15_Bottom
MK_29828	TCACaatttgttacacaact	cloning ISAam1 gRNA_16_bp_gRNA_16_Top

MK_29829	GGCCagttgtgtaacaaatt	cloning ISAam1 gRNA_16_bp_gRNA_16_Bottom
MK_29830	TCACtttgtgtggtatttaa	cloning ISAam1 gRNA_16_bp_gRNA_17_Top
MK_29831	GGCCttaaataccacacaaa	cloning ISAam1 gRNA_16_bp_gRNA_17_Bottom

589 **Supplementary Table 7: Oligos used for cloning gRNA sequences into intermediate TnpB plant**
590 **expression vectors.** The Oligo name column is an internal name for each oligo used. The Oligo
591 sequence column lists the sequence of each oligo used for cloning the gRNA. The Oligo
592 description column provides details for cloning each TnpB gRNA target site.

593

Plasmid	PCR template	Primer 1	Primer 2	Reaction #
pTW2065	pDK3888	gaatttcctttaccattgacgtcagtgtcgttggtagcat	tgtaatccatCTCGTTAACTCGGGTAAGTGATACA	1
pTW2065	pMK061	AGTTAACGAGatggattacaaggatgatgatgataagga	CCAACTGAGCTACGGgcggcgaatttgccttTTGA	2
pTW2065	pDK3888	aggcaaattcgccgcCCGTAGCTCAGTTGGTTAGAGC	TGGTCACCTGTAATTCACACGTGGTGGTGGTGGTGGT	3
pTW2066	pDK3888	gaatttcctttaccattgacgtcagtgtcgttggtagcat	tgtaatccatCTCGTTAACTCGGGTAAGTGATACA	1
pTW2066	pMK061	AGTTAACGAGatggattacaaggatgatgatgataagga	TCTAACCAACTGAGCTACGGgtcccattcgccatgcc	2
pTW2066	pDK3888	catggcgaatgggacCCGTAGCTCAGTTGGTTAGAGC	TGGTCACCTGTAATTCACACGTGGTGGTGGTGGTGGT	3
pTW2082	pDK3888	gaatttcctttaccattgacgtcagtgtcgttggtagcat	tgtaatccatCTCGTTAACTCGGGTAAGTGATACA	1
pTW2082	pMK070	AGTTAACGAGatggattacaaggatgatgatgataagga	TCTAACCAACTGAGCTACGGgtcccattcgccatgcc	2
pTW2082	pDK3888	catggcgaatgggacCCGTAGCTCAGTTGGTTAGAGC	TGGTCACCTGTAATTCACACGTGGTGGTGGTGGTGGT	3

594 **Supplementary Table 8: Cloning TRV Cargo Architecture_A and Architecture_B plasmids.** The
595 Plasmid column lists the name of each plasmid created. The PCR template column lists the
596 name of each plasmid used for PCR DNA template. The Primer 1 and Primer 2 columns provide
597 the sequence used to amplify fragments using the corresponding PCR template. The reaction
598 column indicates the individual reaction for each PCR. The three PCR reactions, along with the
599 restriction enzyme digested pDK3888, were used for NEB Hifi assembly to create the TRV2
600 vector listed in the Plasmid column.

601

TnpB	Guide	F primer	R primer	Sequencing type
ISDra2	gRNA1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaccaaattacgttgagatgcatggtc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgctcttttgttgcttacgaaacatgt	amp-seq
ISDra2	gRNA2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaagcagttgtgagttaagttggaga	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttgtcttaagcgcttgagaagtgg	amp-seq
ISDra2	gRNA3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTatgtcttcagtcttgtgctacacacc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaaacggaacaaacaagaacctaacct	amp-seq
ISDra2	gRNA4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtctcttcatgttcttgttggaagtcgg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgtgctggtaggacatctgggaagt	amp-seq
ISDra2	gRNA5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgacgtcaggaagaacatggtcatttg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTagaacatttcagcgctaatgctacaa	amp-seq
ISDra2	gRNA7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtagcttcaaatgagggaccaggctt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtgtagaggacgacatggttcacagtt	amp-seq
ISDra2	gRNA8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcacaaacctctgttgcttgaagc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcattgggtacctaggagctgtaac	amp-seq
ISDra2	gRNA9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgcgctaaactttataaaccctgatg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtatgattgccagtgagataaagattc	amp-seq
ISDra2	gRNA11	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaatcgagattacatgcaggtctta	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccaacactgtatccaactaagtattc	amp-seq
ISDra2	gRNA12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaaccgacccgagaagagatttg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtggaatacacacatttgtacaacca	amp-seq
ISDra2	gRNA13	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtggagttctcgaaatttcatctagtg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcggaaggatgcagataaactagca	amp-seq
ISDra2	gRNA14	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtctgtaagtccaaaactcataccct	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcttccaacaagaacatgaagagagt	amp-seq
ISDra2	gRNA15	ACACTCTTTCCCTACACGACGCTCTTCCGATCTacagtggttgaactattagttagtgg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcaaaatctataattagcttgaattgatcaatac	amp-seq
ISDra2	gRNA16	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgtgaggttagtaactatgatgcatgt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaagacaaatgaccatgttcttcctg	amp-seq
ISDra2	gRNA17	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTacagtggttgaactattagttagtgg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcctcaaacattcatcagttctttgat	amp-seq
ISDra2	gRNA18	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTacagtggttgaactattagttagtgg	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcctcaaacattcatcagttctttgat	amp-seq

ISDra2	gRNA19	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaagtaggctattgagttcacagttt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTatgaaaccacaacaaccacatg	amp-seq
ISDra2	gRNA20	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcttggtagagtagttagcatgctga	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccctggacaagtatacaattaatcag	amp-seq
ISDra2	gRNA21	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgttgcagcttatgacacacaataag	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaatctttatctcactggcaatcatag	amp-seq
ISDra2	gRNA22	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgttgcagcttatgacacacaataag	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaatctttatctcactggcaatcatag	amp-seq
ISYmu1	gRNA1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaccaaattacgttgagatgcatggtc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgctcttttgttgcttacgaaacatgt	amp-seq
ISYmu1	gRNA2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaagcagttgtgagttaagttggaga	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttgtcttaagcgctgagaagtgg	amp-seq
ISYmu1	gRNA3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTatgtcttcagtcttgtgctacacacc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaaacggaacaaacaagaacctaacct	amp-seq
ISYmu1	gRNA4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtctcttcatgttcttgttggaagtcgg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgtgctggtaggacatctgggaagt	amp-seq
ISYmu1	gRNA5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgacgtcaggaagaacatggtcatttg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTagaacatttcagcgctaatgctacaa	amp-seq
ISYmu1	gRNA7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtagcttcaaatgagggaccaggctt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaagtacttctgttttgtgtaatctcc	amp-seq
ISYmu1	gRNA8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcacaaacctctgttgcttgaagc	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcattgggtacctaggagctgtaac	amp-seq
ISYmu1	gRNA9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgcgctaaactttataaaccctgatg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtatgattgccagtgagataaagattc	amp-seq
ISYmu1	gRNA10	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgaatctttatctcactggcaatcata	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgttgcagcttatgacacacaataag	amp-seq
ISYmu1	gRNA12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaaccgacccgagaagagatttg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtggaatacacacatttgtacaacca	amp-seq
ISAam1	gRNA11	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTagtttcttactcactaatggaagcac	ACACTCTTTCCCTACACGACGCTCTTCCGATCTaaggagcttcaggatatcgactacaa	amp-seq
ISAam1	gRNA12	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgcaggtgaatttcttatttatgcagt	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaaggttacttctgcaaaaaacaaca	amp-seq
ISAam1	gRNA13	ACACTCTTTCCCTACACGACGCTCTTCCGATCTcgtctggaccaagaaagttgt	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcagaaacgtgagatgtcaaatctgt	amp-seq
ISAam1	gRNA14	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgttaagttgttattgtgttctctgg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgaaaatatgtaaaccagtctcatacc	amp-seq

ISAam1	gRNA15	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgctgcgtctcctgtttctctac	ACACTCTTTCCCTACACGACGCTCTTCCGATCTccggtggaagcaaatcagac	amp-seq
ISAam1	gRNA16	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtgcaaggaggaggagtatttgg	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtatccgattccgctagtttgc	amp-seq
ISAam1	gRNA17	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaccaaattacgttgagatgcatggtc	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtggtcttgtttggttttcagacc	amp-seq
ISYmu1	gRNA12	tgttacacaacttattatgattgggct	ggaagtagccgataacaaaatggag	Sanger

Supplementary Table 9: Primers used for amp-seq or Sanger sequencing. The TnpB and Guide columns indicate the site being targeted for each TnpB. The F primer and R primer columns list the oligo sequences used to amplify genomic DNA for amp-seq or Sanger Sequencing.

oligo name	Oligo sequence	Description
SP9238	TGTATAGACTGTTTGAGATCGGCG	RT-PCR primer to check for the presence/absence of TRV in mutant albino plants
SP9239	GTAATAACGCTTACGTAGGCGAGG	RT-PCR primer to check for the presence/absence of TRV in mutant albino plants
IPP2 RT-PCR Fw	GTATGAGTTGCTTCTCCAGCAAAG	RNA control for RT-PCR
IPP2 RT-PCR Rev	GAGGATGGCTGCAACAAGTGT	RNA control for RT-PCR

Supplementary Table 10: Oligos for RT-PCR. The name of each oligo is listed in the Oligo name column. Each oligo sequence is provided in the Oligo sequence column, with a description of what the oligos were used for in the Description column.

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