

1 DNA-free CRISPR-Cas9 gene editing of tetraploid tomatoes using 2 protoplast regeneration

3

4 Chen-Tran Hsu¹, Yu-Hsuan Yuan¹, Po-Xing Zheng^{1,2}, Fu-Hui Wu¹, Qiao-Wei Cheng¹,
5 Yu-Lin Wu^{1,2}, Steven Lin³, Jin-Jun Yue⁴, Ying-Huey Cheng⁵, Shu-I Lin⁶, Ming-Che
6 Shih^{1,*}, Jen Sheen^{7,*}, Yao-Cheng Lin^{1,2,*,+} and Choun-Sea Lin^{1,*,+}

7

8 ¹ Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

9 ² Biotechnology Research Center in Southern Taiwan, Academia Sinica, Tainan,
10 Taiwan

11 ³ Institute of Biochemistry, Academia Sinica, Taipei, Taiwan

12 ⁴ Research Institute of Subtropical Forestry, Chinese Academy of Forestry,
13 Hangzhou, China

14 ⁵ Plant Pathology Division, Taiwan Agricultural Research Institute, Taichung, Taiwan

15 ⁶ Department of Horticulture and Landscape Architecture, National Taiwan University,
16 Taipei, Taiwan

17 ⁷ Department of Molecular Biology and Centre for Computational and Integrative
18 Biology, Massachusetts General Hospital, and Department of Genetics, Harvard
19 Medical School, Boston, MA 02114, USA

20 *Senior authors: mcshih@gate.sinica.edu.tw; sheen@molbio.mgh.harvard.edu;
21 yalin@sinica.edu.tw; cslin99@gate.sinica.edu.tw

22 +Corresponding authors:

23 Choun-Sea Lin

24 Agricultural Biotechnology Research Center, Academia Sinica
25 128 Academia Road, Section 2, Nangang, Taipei, 115201, Taiwan
26 Tel: +886 2 2787 2094
27 E-mail: cslin99@gate.sinica.edu.tw

28 Yao-Cheng Lin

29 Biotechnology Research Center in Southern Taiwan, Academia Sinica
30 No 100. Guiren 13th Road, Sec. 1, Guiren, Tainan City, 711010, Taiwan
31 Tel: +886 6 303 2312
32 Email: yalin@sinica.edu.tw

33

34 **ORCID IDs:** 0000-0001-5827-5865 (P-XZ); 0000-0002-7590-793X (M-CS); 0000-
35 0002-9390-795X (Y-CL); 0000-0001-9566-2952 (C-SL)

36 **Key words:** Virus resistant, DNA-free, microRNA synthesis, Ribonucleoprotein,
37 peptide hormone

38

39 **Short title**

40 DNA-free genome editing in tetraploid wild tomatoes

41

42 **One-sentence summary:**

43 DNA-free CRISPR-Cas9 genome editing in wild tomatoes creates stable and
44 inheritable diploid and tetraploid regenerants.

45

46 **Competing interests**

47 The authors declare that the research was conducted in the absence of any
48 commercial or financial relationships that could be construed as a potential conflict of
49 interest.

50

51 **Funding**

52 This research was supported by the Innovative Translational Agricultural Research
53 Program (AS-KPQ-107-ITAR-10; AS-KPQ-108-ITAR-10; AS-KPQ-109-ITAR-10; AS-
54 KPQ-110-ITAR-03) and Academia Sinica Institutional funding to Y-CL and C-SL, and
55 the Ministry of Science and Technology (105-2313-B-001-007-MY3; 108-2313-B-
56 001-011-; 109-2313-B-001-011-), Taiwan to C-SL. These funding bodies played no
57 role in the design of the study, collection, analysis or interpretation of data or in
58 writing the manuscript.

59

60 **Author contributions**

61 C-SL, Y-CL, JS, and M-CS conceived and designed the experiments. C-TH, and Y-
62 HY performed the CRISPR-Cas9 experiments. C-TH, Y-HY, Q-WC, J-JY, and F-HW
63 conducted the protoplast regeneration, cell biology, molecular biology, and targeted
64 mutagenesis experiments. SL conducted SpCas9 purification. Y-LW performed WGS
65 library preparation and qPCR analysis. P-XZ and Y-CL performed bioinformatics
66 analysis. Y-HC, C-TH, C-SL, Q-WC, and F-HW performed virus-related analysis. C-
67 TH performed cell biology. C-TH and S-IL performed grafting. JS, M-CS, Y-CL, and
68 C-SL wrote the manuscript with input from all co-authors. All authors read and
69 approved the final manuscript.

70

71 **Authors' emails:**

72 Chen-Tran Hsu: hightemp@gate.sinica.edu.tw
73 Yu-Hsuan Yuan: sharon11594@gate.sinica.edu.tw
74 Po-Xing Zheng: pxzheng@gate.sinica.edu.tw
75 Fu-Hui Wu: fuhuiwu@gate.sinica.edu.tw
76 Qiao-Wei Cheng: vivian40100@gate.sinica.edu.tw
77 Yu-Lin Wu: yulinwu@sinica.edu.tw
78 Steven Lin: stevenlin@gate.sinica.edu.tw
79 Jin-Jun Yue: yuejinjun@163.com

80 Ying-Huey Cheng: yhcheng@tari.gov.tw
81 Shu-I Lin: shuilin@ntu.edu.tw
82 Ming-Che Shih: mcsih@gate.sinica.edu.tw
83 Jen Sheen: sheen@molbio.mgh.harvard.edu
84 Yao-Cheng Lin: yalin@sinica.edu.tw
85 Choun-Sea Lin: cslin99@gate.sinica.edu.tw

86

87

88

89

90

91

92

93

94

95

96

97

98

99

100

101 **Abstract**

102 Wild tomatoes are important genomic resources for tomato research and breeding.
103 Development of a foreign DNA-free CRISPR-Cas delivery system has potential to
104 mitigate public concern about genetically modified organisms. Here, we established
105 a DNA-free protoplast regeneration and CRISPR-Cas9 genome editing system for
106 *Solanum peruvianum*, an important resource for tomato introgression breeding. We
107 generated mutants for genes involved in small interfering RNAs (siRNA) biogenesis,
108 *RNA-DEPENDENT RNA POLYMERASE 6* (*SpRDR6*) and *SUPPRESSOR OF*
109 *GENE SILENCING 3* (*SpSGS3*); pathogen-related peptide precursors,

110 *PATHOGENESIS-RELATED PROTEIN-1 (SpPR-1)* and *PROSYSTEMIN*
111 (*SpProsys*); and fungal resistance (*MILDEW RESISTANT LOCUS O, SpMlo1*) using
112 diploid or tetraploid protoplasts derived from *in vitro*-grown shoots. The ploidy level of
113 these regenerants was not affected by PEG-calcium-mediated transfection, CRISPR
114 reagents, or the target genes. By karyotyping and whole genome sequencing
115 analysis, we confirmed that CRISPR-Cas9 editing did not introduce chromosomal
116 changes or unintended genome editing sites. All mutated genes in both diploid and
117 tetraploid regenerants were heritable in the next generation. *spsgs3* null T_0
118 regenerants and *sprdr6* null T_1 progeny had wiry, sterile phenotypes in both diploid
119 and tetraploid lines. The sterility of the *spsgs3* null mutant was partially rescued, and
120 fruits were obtained by grafting to wild-type stock and pollination with wild-type pollen.
121 The resulting seeds contained the mutated alleles. Tomato yellow leaf curl virus
122 proliferated at higher levels in *spsgs3* and *sprdr6* mutants than in the wild type.
123 Therefore, this protoplast regeneration technique should greatly facilitate tomato
124 polyploidization and enable the use of CRISPR-Cas for *S. peruvianum* domestication
125 and tomato breeding.

126 **Introduction**

127 Tomato is an important vegetable crop, representing the sixth most economically
128 important crop worldwide (<http://www.fao.org/faostat/en/#data/QV>). Wild tomato
129 species are resistant to diverse biotic and abiotic stresses, and are often used for
130 tomato introgression breeding. De novo domestication of wild tomato was recently
131 achieved within a short period by gene editing using clustered regularly interspaced
132 short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) (Li et al.,
133 2018; Zsogon et al., 2018). Thus, CRISPR-Cas mutagenesis of wild tomato
134 represents a new strategy for tomato breeding and basic research.

135 Genome multiplication is a frequent occurrence during crop domestication.
136 Many of the most economically important crops are polyploid, including potato,
137 wheat, and cotton. Polyploidy conveys advantages in terms of genomic buffering,
138 viability, and environmental robustness (Van de Peer et al., 2021). Triploids can also
139 be used as seedless crops, such as watermelon and bananas. Thus, CRISPR-Cas-
140 edited tetraploid versions of crop species and their relatives represent important
141 materials for crop breeding in the face of rapid climate change caused by global
142 warming, among other challenges, as was recently demonstrated for tetraploid wild

143 rice (*Oryza alta*) (Yu et al., 2021). Therefore, it is important to establish a gene
144 editing platform for polyploid crops and related species.

145 The CRISPR-Cas system uses *Agrobacterium*-mediated stable transformation
146 to deliver DNA encoding Cas protein and single guide RNA (sgRNA) into the nuclei
147 of tomato cells. As an alternative approach, CRISPR ribonucleoprotein (RNP) or
148 plasmids harboring the Cas and sgRNA sequences can be introduced directly into
149 protoplasts using transient transfection, allowing recombinant DNA-free plants to be
150 regenerated to circumvent concerns about genetically modified organisms (GMOs)
151 (Woo et al., 2015; Andersson et al., 2018; Lin et al., 2018; Hsu et al., 2019; De Bruyn
152 et al., 2020; Hsu et al., 2021; Hsu et al., 2021; Yu et al., 2021). This protocol is
153 important for use with hybrids or plants with a long juvenile period and for vegetative
154 propagation because the transgenes from stable transformation (selection markers
155 and CRISPR reagent genes) cannot be removed from these crops by crossing. Also,
156 the progeny will be different from their heterozygous parental lines due to
157 segregation. The gene editing efficiency and specificity could be validated by
158 targeted sequencing (Woo et al., 2015; Nekrasov et al., 2017) or whole genome
159 sequencing (WGS) (Fossi et al., 2019; Hsu et al., 2021). Nevertheless, previous
160 analysis paid little attention to the overall chromosomal changes, especially in
161 polyploid regenerants (Fossi et al., 2019).

162 The protoplast regeneration gene editing system has two other major
163 advantages: (1) Gene-edited transformants derived from tissue-culture-based
164 *Agrobacterium*-mediated transformation are often chimeric, especially in
165 dicotyledons (Shimatani et al., 2017). If the transformant is an edited/wild-type (WT)
166 chimera and the edited allele occurs only in somatic cells (and not germ cells), edited
167 alleles cannot be passed on to the next generation (Zheng et al., 2020). In protoplast
168 regeneration, there is a low incidence of chimerism, and all mutated alleles detected
169 in the T₀ generation can be transmitted to the next generation (Lin et al., 2018; Hsu
170 et al., 2019; Hsu et al., 2021; Hsu et al., 2021). (2) The protoplast regeneration
171 system can be used to introduce many CRISPR reagents and donor DNAs into
172 plants for targeted insertion at the same time without the limitation of vector size
173 (Hsu et al., 2019; Hsu et al., 2021). In addition, the second transfer step can be
174 performed directly to obtain homozygous alleles in polyploids without self-fertilization
175 which is very useful for hybrid, long juvenile period, and sterile plants (Hsu et al.,

176 2019). However, the main bottleneck of this strategy is the difficulty of performing
177 protoplast regeneration.

178 Here, we established a diploid/allotetraploid protoplast regeneration protocol
179 for *S. peruvianum*, an important stress-resistant wild tomato, for use with CRISPR-
180 Cas-mediated genome editing. We targeted several genes for editing, including
181 *RNA-DEPENDENT RNA POLYMERASE6* (*SpRDR6*) and *SUPPRESSOR OF GENE*
182 *SILENCING3* (*SpSGS3*), two key genes in the plant RNA silencing pathway
183 (Mourrain et al., 2000) that mediate defense against tomato yellow leaf curl virus
184 (TYLCV) (Verlaan et al., 2013); *PATHOGENESIS-RELATED PROTEIN-1* (*SpPR-1*)
185 encoding the cysteine-rich secretory proteins antigen 5 and pathogenesis-related 1
186 protein (CAP)-derived peptide 1 (CAPE1) precursor (Chen et al., 2014) and
187 *PROSYSTEMIN* (*SpProslys*), two pathogen-resistance peptide precursors; and
188 *MILDEW RESISTANT LOCUS O* (*SpMlo1*) (Nekrasov et al., 2017). Targeting of
189 these genes, which was performed using two types of CRISPR reagents, plasmids
190 and RNPs, yielded diploid and tetraploid transgene-free lines. Stable genome
191 structures of ten plants, including one explant derived from stem cutting, three diploid
192 regenerants and six tetraploid of *SpProslys* or *SpMlo1* RNP transfection regenerants
193 were confirmed by WGS.

194

195 **Results**

196 **Protoplast regeneration in *S. peruvianum***

197 To obtain a high proportion of tetraploid protoplasts, we analyzed the genome sizes
198 of different explants (leaves and stems) using flow cytometry to determine the
199 proportion of tetraploid cells. In leaves, the ratio of diploid to tetraploid nuclei was 5:1
200 (Figure 1a), and in stems, the ratio was 1:1 (Figure 1b). The same ratio was detected
201 in protoplasts derived from stems (Figure 1c). Therefore, since stems had a higher
202 proportion of tetraploid cells, we used them in subsequent studies to increase the
203 proportion of tetraploid regenerated plants.

204 Using a method previously published for *Nicotiana tabacum* (Lin et al., 2018),
205 we successfully isolated *S. peruvianum* protoplasts from *in vitro*-grown shoots. We
206 incubated the purified protoplasts in liquid medium consisting of half-strength
207 Murashige and Skoog medium (1/2 MS), 0.4 M mannitol, 3% sucrose, 1 mg/L

208 naphthaleneacetic acid (NAA), and 0.3 mg/L kinetin, pH 5.7, for 1 month in the dark,
209 leading to the formation of fine, sand-like calli (Figure 2a). Next, we subcultured
210 these calli in liquid medium containing 1/2 MS, 0.4 M mannitol, 3% sucrose, 2 mg/L
211 kinetin, and 0.3 mg/L Indole-3-acetic acid (IAA), pH 5.7, in the light (Figure 2b). After
212 one month, these white calli turned green and were transferred to solid medium (1/2
213 MS, 0.2 M mannitol, 3% sucrose, and 2 mg/L kinetin; Figure 2c). We transferred the
214 calli to fresh medium every month to induce the formation of small shoots (Figure 2d),
215 which were incubated in medium without plant growth regulators until adventitious
216 roots formed at the bottoms of the shoots (Figure 2e). Finally, we transferred the
217 rooted plants to pots (Figure 2f) and grew them in the greenhouse (Figure 2g). The
218 regenerated plants flowered (Figure 2h), fruited (Figure 2i), and produced seeds.

219

220 **Optimized protoplast regeneration protocol**

221 Compared to tobacco (Lin et al., 2018), *S. peruvianum* protoplasts take longer to
222 regenerate. According to our observations, the most important steps in the tomato
223 regeneration process are those in liquid culture: callus induction in the dark (the 1st
224 step) and callus proliferation in the light (the 2nd step). Therefore, we tested several
225 modifications to the composition of the culture medium to shorten the regeneration
226 time. The results indicated that zeatin and 6-Benzylaminopurine (BA) are the best
227 hormonal treatments for the two liquid culture steps (Figure S1), and zeatin is the
228 best cytokinin for the 3rd subculture step in solid medium (Figure S2).

229

230 **CRISPR-Cas9-targeted mutagenesis in *S. peruvianum***

231 We used this protoplast regeneration system to establish a method for CRISPR-
232 Cas9-targeted gene mutagenesis of *S. peruvianum*. First, we used plasmids as
233 CRISPR-Cas9 reagents for targeting mutagenesis of three important disease-
234 resistance-related genes: *SpSGS3*, *SpRDR6*, and *SpPR-1*.

235 In the *SpSGS3* experiment, we chose four target sites (Table 1), and the total
236 efficiency of mutagenesis was 8.3%. Based on sequencing results, mutations
237 occurred in all three target sites except GTAACAATGCTGGATCAGGC. Among
238 these, GCGCAATTGAATGGTTTACA was targeted the most effectively, and
239 mutations at this position were observed in all mutants (Table S1). *spsgs3#6*, #11,

240 and #13 are null mutants and *spsgs3#6* contains four mutated alleles. *SpSGS3#7*
241 also contains three mutated alleles and one non-mutated WT allele. A 68-bp
242 insertion from the vector was detected in *spsgs3#11*.

243 In the *SpRDR6* experiment, we selected two target sites (Table 1). Based on
244 the sequencing results, both target sites could be mutated by CRISPR-Cas9, with a
245 total mutation efficiency of 13.2%. TTAAAGCTGGGACCATTGCG gave the best
246 results, as all five mutant plants contained mutations at this target site. The mutation
247 TGCGAGGTCGAATTGAAACA was only identified in *SpRDR6#38* (Table S2). All
248 regenerated mutants were heterozygous, and *SpRDR6#38* had two mutated alleles
249 and at least one WT allele.

250 In the *SpPR-1* experiment, seven target sites were selected and used to
251 construct two vectors. These two constructs, harboring sgRNAs targeting seven
252 target sites, were co-transfected into protoplasts (Table 1). Among the 10
253 regenerated mutants, 4 contained fragment deletions, indicating that at least two
254 cleavages had occurred. Except for TGTCCGATCCAGTTGCCTAC and
255 CTATGATCCTGTAGGCAAC there were no mutations in the target sites; the five
256 other sgRNAs caused mutations at the expected positions. The mature CAPE1
257 peptide is derived from the C-terminal end of tomato *PR-1b*. *sppr-1#28*, #31, and
258 #52 were mutated only in the target sites located in CAPE1, all at
259 ATCCTGTAGGCAACTGGAT, resulting in a 5-bp deletion. All *SpPR-1* mutants were
260 null mutants except for *SpPR-1#72* (Table S3).

261 In the experiments with *N. tabacum* (Lin et al., 2018) and *SpSGS3* (Table S1),
262 the use of plasmid CRISPR reagent may still result in foreign DNA insertions.
263 Therefore, RNP is used as a CRISPR reagent to achieve DNA-free gene editing.
264 Here, we delivered two RNPs that target sites located in *SpProslys* to protoplasts and
265 regenerated the transfected protoplasts into plants. Upon sequencing of the 24
266 regenerated *SpProslys* plants, 11 showed target mutagenesis (45.8%, Table 1).
267 Prosystemin is a precursor of systemin, which is processed by phytaspase
268 (Beloshistov et al., 2018). The target site GGAGGATCACGCTTTGATGG is at the C
269 terminus of *SpProslys*, which is the position of systemin, and the mutations in lines
270 #5, #16, and #19 occurred only at this site (Table S4). Using two published *SIM1o1*
271 target sites (Nekrasov et al., 2017), we synthesized RNPs targeted to these sites *in*

272 *in vitro* and simultaneously delivered them into protoplasts. Of the regenerated calli and
273 plants, 63.6% showed targeted mutagenesis (Table 1, Table S5).

274

275 **Analysis of the genome sizes, phenotypes, and progeny of diploids and** 276 **tetraploids**

277 A higher proportion of tetraploid cells was observed in protoplasts derived from
278 diploid stems compared to leaf tissue (Figure 1). In addition, during target gene
279 genotyping, we observed that some mutants contained more than three alleles. For
280 example, *SpRDR6#38* contained three alleles (+1 bp, -7 bp, and WT, Table S2),
281 and its genome size was 4.40 ± 0.03 pg. Therefore, targeted mutant plants of
282 tetraploids can be obtained using this method. We performed karyotype analysis of
283 these regenerated plants (T_0 , sterile mutants) or their offspring (T_1) to confirm the
284 chromosome numbers (Figure 3). Except for a *SpPR-1* tetraploid without targeting
285 regenerant, we obtained diploid and tetraploid regenerated plants with or without
286 targeting mutations derived from plasmid CRISPR-Cas9 reagent-transfected
287 protoplasts (Figure 3, Table S6). Similar results were obtained for *SpProslys* RNP
288 transfection (Figure S3, Table S7). The ploidy of the plants that were regenerated
289 from transfected protoplasts is provided in Table S6 and S7. These results indicate
290 that most tetraploid plants were derived from tetraploid protoplasts from the explants
291 rather than by protoplast fusion caused by the presence of PEG-Ca⁺ in the
292 transfection medium.

293 In regenerated plants derived from *SpSGS3* transfection, the tetraploids had a
294 reduced seed set (Figure S4a). The seeds of tetraploids were larger than those of
295 diploids; this phenomenon was also observed in tetraploid regenerated plants
296 derived from transfection with other CRISPR reagents (Figure S4b). The tetraploid
297 plants grew more slowly than the diploid plants (Figure S4c). The leaf edges of
298 tetraploid plants were more rounded than those of the diploid plants (Figure S4c).

299 We subjected the offspring of *SpSGS3#7*, and #10 (Figure S5); *SpRDR6#6*,
300 #33, and #38 (Figure S6); and *sppr-1#52* and #61 (Figure S7) to target gene
301 sequencing. Except for *sppr-1#52*, which contained one mutant locus not present in
302 the parent, all other offspring had the same mutated locus as the parent. These

303 results demonstrate that these mutated loci can be transmitted to the next generation
304 in diploids and tetraploids.

305

306 **Stable genome structures in diploids and tetraploids**

307 To further confirm the stability of genome structure in regenerants, we performed
308 whole genome sequencing of ten samples, including one diploid plant propagated by
309 stem cutting (SpB), three diploids and six tetraploids derived from *SpProslys* or
310 *SpMlo1* RNP transfection (Table S7). Taking into account the different genome sizes
311 between diploid and tetraploid plants, each sample was sequenced to the anticipated
312 30x genome coverage. That is, 141-171 million pair-end reads were sequenced for
313 diploid plants and at least 252-373 million pair-end reads were sequenced for
314 tetraploid plants (Table S7).

315 Multiple analysis strategies were used to study the genome structures.
316 Despite the low mapping rate of both diploid and tetraploid samples at some
317 chromosome locations, sequencing coverage analysis did not show inconsistent
318 coverage changes between samples (Figure S8). Deletion of large chromosomal
319 segments, which were commonly seen in aneuploid cells (Musacchio and Salmon,
320 2007) cause allelic imbalance. By calculating heterozygous allele frequency of
321 sequenced plants, we did not identify abnormal allele frequency variations or loss of
322 heterozygosity (Figure 4a). A Bayesian approach to determine copy number
323 variations along chromosomes and compared between uneven sequencing depth of
324 samples did not identify abnormal copy number changes in sequenced plants
325 (Figure 4b). Taking these findings together, we concluded that there is no abnormal
326 chromosomal gain or loss in diploid and tetraploid plants. Neither the protoplast
327 regeneration process nor the CRISPR reagents caused detectible chromosomal
328 changes.

329

330 ***spsgs3* and *sprdr6* diploid and tetraploid null mutants show wiry phenotypes**

331 The regenerated plants containing a WT allele(s) produced flowers and fruits (Figure
332 2) with morphology and development similar to those of WT plants in the greenhouse.
333 Biallelic *spsgs3* mutants (carrying two distinct genome-edited alleles: *spsgs3#11*,
334 Figure 5; *spsgs3-6* and *spsgs3-13*; Figure S9a) had a wiry leaf phenotype and

335 abnormal flowers, which is similar to the previously reported *sgs3* domesticated
336 tomato mutants (Yifhar et al., 2012).

337 Among the six progeny of *SpSGS3#7*, two progeny harbored the mutated
338 alleles only (Figure S5); these plants also showed a wiry phenotype (*spsgs3#7-2*;
339 Figure S9b). A similar phenomenon was also observed in the *SpRDR6* regenerants.
340 Although all *SpRDR6* T₀ plants were heterozygous and contained WT *SpRDR6*
341 alleles in their genomes, no wiry phenotypes were observed. The *SpRDR6#33* and
342 *SpRDR6#38* offspring had wiry phenotypes (*sprdr6#33-G*, Figure 5b; *sprdr6#38-16*,
343 Figure S9b). The pollen of both null T₀ and T₁ mutated plants, including *SpSGS3* and
344 *SpRDR6* mutants, was abnormal (Figure S9c) and failed to produce seeds.

345 Because *AUXIN RESPONSE FACTOR3* (*ARF3*) and *ARF4* are the target
346 genes of trans-acting secondary siRNA3 (*TAS3*), whose biogenesis requires RDR6
347 (Marin et al., 2010), we investigated the transcript levels of these genes in WT,
348 *spsgs3* and *sprdr6* plants (Figure 5a). The *spsgs3* null mutants (T₀: *spsgs3#11* and
349 *spsgs3#13*; T₁: *spsgs3#7-1*) lacked *SpSGS3* expression. In contrast to the WT, the
350 transcript levels of *SpARF3* and *SpARF4* was increased in the *spsgs* mutants, not
351 only for null diploid mutants *spsgs3#11* and *spsgs3#13* but also for tetraploid mutant
352 *spsgs3#7-1* (Figure 5a). Similarly, the transcript levels of *SpARF3* and *SpARF4* were
353 also increased in the *SpRDR6* T₁ mutant *sprdr6#33-G* (Figure 5a).

354

355 **Tomato yellow leaf curl virus proliferation**

356 We evaluated the infectivity of TYLCV in the mutants by *in vitro* inoculation (Al
357 Abdallat et al., 2010). After 8 weeks *in vitro* inoculation, plant growth was severely
358 retarded (Figure S10) and leaf morphology changed in the T₁ diploid *spsgs3#11*
359 (Figure 6a) and the T₂ tetraploid *sprdr6#38-6* (Figure 6b). Compared to the WT, all of
360 the null mutants (*spsgs3/sprdr6* and diploid/tetraploid) showed higher levels of
361 TYLCV accumulation (Figure 6a, b, Figure S10).

362

363 **Grafting rescued the fertility of the *sgs3#11* null mutant**

364 We used WT pollen for hybridization, which failed to pollinate the fruits of the *spsgs3*
365 and *sprdr6* null mutants. Based on these results, these mutants could not produce

366 the substrate(s) needed for the development of male or female reproductive organs.
367 However, using grafting, the substrate(s) produced in WT stock was successfully
368 transported to the *spsgs3#11* scion (Figure 7a). Although there were no significant
369 differences in leaf (Figure 7b) or flower morphology (Figure 7c), *spsgs3#11* failed to
370 produce viable pollen (Figure S9c) and the pollen viability of *spsgs3#11* increased to
371 20% by grafting to the WT stock. Grafted *spsgs3#11* produced fruits (Figure 7d), but
372 non-grafted *spsgs3#11* did not. The fruits from *spsgs3#11* scions were smaller
373 (Figure 7e) and contained fewer seeds than the WT (Figure 7f). Genotyping
374 indicated that all of the progeny harbored *spsgs3#11* mutated alleles (Figure 7g).

375

376 Discussion

377 In addition to its use in wild tomatoes, CRISPR is also utilized in commercial
378 varieties of *S. lycopersicum*. Dozens of studies using this technique in tomato have
379 been published, most involving breeding trials for traits such as quality (fruit
380 architecture, color, metabolism, postharvest), anti-stress (biotic and abiotic stress),
381 and domestication (Li et al., 2018; Zsogon et al., 2018). These studies were
382 performed using several CRISPR platforms established in tomato, including (1) Cas9
383 (Brooks et al., 2014) and Cas12a (Bernabe-Orts et al., 2019), to generate DNA
384 double-strand breaks that are preferentially repaired by non-homologous end joining
385 to introduce target mutations; (2) precise modification of plant genomes using DNA
386 repair templates via homologous recombination (Cermak et al., 2015); and (3) the
387 cytidine base editor, an inactive Cas9 fusion with cytidine deaminase, which converts
388 cytosine to uracil without cutting DNA and introducing mutations (Shimatani et al.,
389 2017). Therefore, CRISPR is emerging as a powerful tool for tomato breeding.

390 For commercial breeding, however, it is desirable to produce DNA-free plants
391 to avoid concerns about GMOs. Although there are several reports demonstrating
392 successful DNA-free genome editing via biolistic methods in many crops (Svitashev
393 et al., 2016; Liang et al., 2018; Banakar et al., 2020), protoplast regeneration
394 systems have higher efficiency. Many studies have been performed using RNPs and
395 plasmids to achieve DNA-free genome editing (Woo et al., 2015; Andersson et al.,
396 2018; Lin et al., 2018; Hsu et al., 2019; De Bruyn et al., 2020; Hsu et al., 2021; Hsu

397 et al., 2021; Yu et al., 2021). These reports indicate that it is possible to establish
398 protoplast regeneration platforms for tomato and various target crops/plants.

399 Tomato and related species have been important materials in the
400 development of protoplast isolation and regeneration techniques. Tomato protoplasts
401 were isolated by enzymatic digestion, and this landmark achievement allowed
402 sufficient amounts of protoplasts to be obtained for further application (Cocking,
403 1960). *S. peruvianum* was the first tomato-related species for which a protoplast
404 regeneration system was reported, and such systems have subsequently been
405 achieved in many tomato and wild tomato species (Kut and Evans, 1982). In this
406 study, we combined these techniques to achieve DNA-free genome editing of a wild
407 tomato. This method could be applied to other tomato-related species to facilitate
408 breeding.

409 Although protoplast regeneration was first reported 50 years ago (Takebe et
410 al., 1971), it still represents a major bottleneck in DNA-free genome editing. The
411 major issue is that various species have different regeneration capacities. Moreover,
412 no single protocol can be directly applied to all species efficiently because the
413 requirements for plant medium and regeneration are diverse (Kut and Evans, 1982)
414 and must be individually modified. Understanding how a cell is regenerated into a
415 complete plant is an important topic of scientific and agricultural research (Maher et
416 al., 2020), but information about this process is still limited. Such knowledge could be
417 applied to develop efficient tissue culture, gene transformation, and genome editing
418 system, tools that are important for *de novo* plant domestication (Li et al., 2018;
419 Zsogon et al., 2018; Maher et al., 2020; Yu et al., 2021). In this study, we assessed
420 the effects of plant growth regulators in the medium on protoplast regeneration. In
421 addition to the chemical approach, several genes encoding morphogenic regulators
422 have been identified and used to improve the efficiency of plant regeneration. It is
423 possible to control the expression of these genes to establish a non-tissue-culture
424 regeneration system for gene editing (Maher et al., 2020).

425 In addition to their roles in the domestication of wild species, polyploid crops
426 have other benefits, including larger plants (Chung et al., 2017), and higher yields
427 (Chen et al., 2018). In addition, triploid crop cultivars of species such as bananas
428 and watermelons can produce commercially desirable seedless fruits. Most previous
429 methods for chromosome multiplication have used colchicine. This procedure is

430 complicated and inefficient, producing regenerated plants with mixed cell populations
431 of various ploidy levels (Cola et al., 2014). Similar to haploid culture, in this report,
432 using isolated protoplasts from polyploid cells in explants for regeneration and gene
433 editing, we were able to obtain edited polyploid regenerated wild tomatoes without
434 colchicine treatment. This phenomenon has also been reported in other plant
435 species. In witloof chicory plants generated from CRISPR/Cas-edited protoplasts,
436 77.2% diploid and 21.5% tetraploid plants were produced and the remaining 1.3%
437 consisted of haploids, hexaploids, and mixoploids (De Bruyn et al., 2020). Therefore,
438 explants containing high proportions of polyploidized cells could be widely used for
439 protoplast regeneration for crop polyploidization. However, in this study, we found no
440 significant enlargement in the leaves or flowers of tetraploid versus diploid lines,
441 similar to the pattern reported for tetraploid tomatoes (Nilsson, 1950).

442 In addition to technological difficulties, the presumed mutagenicity of
443 protoplast regeneration is another reason why researchers are reluctant to use this
444 system as a gene editing platform. Indeed, whole genome sequencing has revealed
445 widespread genome instability in potatoes regenerated from protoplasts (Fossi et al.,
446 2019), which has increased the concerns about this technology. The original purpose
447 of protoplast regeneration was to use protoplast fusion to improve hybridization or as
448 a platform for mutagenesis. Since only successful cases of mutation or fusion have
449 been reported, and most such experiments have not been compared with other
450 tissue culture methods, many researchers have the impression that protoplast
451 regeneration readily leads to mutagenesis. In fact, other tissue culture technologies,
452 including multiple shoot proliferation (Lin et al., 2007) and somatic embryogenesis
453 (Lin et al., 2007), can also cause mutations. Although this study involved the use of
454 PEG-Ca²⁺ in the transfection process, which could promote cell fusion, non-
455 transfected tetraploid regenerated plants were also obtained. Based on our finding
456 that the proportion of tetraploid regenerated plants was similar to that of shoot
457 explants, we believe that the formation of polyploid regenerated plants was primarily
458 due to the presence of polyploid cells in the explants. In addition to protoplast
459 regeneration, there are also opportunities to obtain polyploid plants using other
460 tissue culture technologies (Chung et al., 2017). In an *Agrobacterium*-mediated
461 transformation experiment in tomato, the rate of tetraploid transgenic plants ranged
462 from 24.5% to 80% and depended on both the genotype and the transformation

463 procedure (Ellul et al., 2003). In *Arabidopsis* T-DNA insertion mutagenesis, large-
464 scale genomic rearrangements have occurred (Pucker et al., 2021). Therefore, we
465 believe that protoplast regeneration is an excellent tool for gene editing as well as
466 other transgenic platforms.

467 Unlike the previous report of widespread genome changes in the
468 autotetraploid potato (Fossi et al., 2019), the whole genome sequencing analysis in
469 this study does not identify aneuploidy and abnormal chromosomal changes in either
470 diploid or tetraploid regenerants. Chromosomes in the autotetraploid genome, such
471 as cultivated potato, were derived from merging of two different chromosome sets
472 (Van de Peer et al., 2021). On the other hand, tetraploid plants in this study, which
473 were derived from chromosome doubling, contained the two identical sets of
474 chromosomes. As the tissue culture steps caused a certain level of cell stresses,
475 pairing of non-homologous chromosomes in the autotetraploid genomes (Fossi et al.,
476 2019) likely has a higher probability of incorrect chromosome pairing than in the
477 allotetraploid genomes (Hsu et al., 2019; Yu et al., 2021). Incorrect chromosome
478 segregation during mitosis in the autotetraploid cells likely has a higher probability of
479 evading the spindle-assembly checkpoint (Musacchio and Salmon, 2007).
480 Furthermore, by analyzing changes in the allele frequency and copy number
481 variation, we confirmed that the CRISPR-Cas9 editing did not introduce large scale
482 chromosomal changes and unintended genome editing sites (Hsu et al., 2021).

483 In this study, all tetraploid and diploid *spsgs3* and *sprdr6* null mutants had wiry
484 phenotypes, similar to other microRNA biogenesis null mutants in tomatoes (Yifhar
485 et al., 2012; Brooks et al., 2014). *sgs3* and *rdr6* null mutants show various
486 phenotypes in different species. *N. benthamiana* *spsgs3* and *sprdr6* mutants have a
487 wiry flower morphology and sterile phenotype, but their leaves are similar to those of
488 the WT (Hsu et al., 2021). The *Arabidopsis* *sgs3* mutant shows no significant
489 phenotype (Adenot et al., 2006). Therefore, we would like to discover ways to
490 improve the fertility of these mutants.

491 Grafting is a traditional agricultural tool that is used to control flowering,
492 improve fruit quality, and increase resistance to biotic and abiotic stress (Haroldsen
493 et al., 2012). In *N. benthamiana*, gene silencing was transmitted with 100% efficiency
494 in a unidirectional manner from silenced stocks to non-silenced scions expressing
495 the corresponding transgene (Palauqui et al., 1997). In this study, a mutant of

496 *SpSGS3*, an RNA silencing-related gene, was used as a scion and grafted onto
497 RNA-silenced normal wild-type rootstock. The fertility of *spsgs3#11* scions was
498 rescued, and they produced seeds with mutated alleles. In *Arabidopsis*, more than
499 3,000 mobile genes have been identified. The mRNA from these genes could be
500 transported long distance, including *SGS3* mRNA (Thieme et al., 2015). In addition
501 to mRNA, organellar DNA, proteins, and plant growth regulators can also move
502 across graft unions (Haroldsen et al., 2012). Whether these mobile substances were
503 also involved in rescuing the fertility of the *spsgs3#11* scions or whether grafting with
504 wild-type plants could rescue other sterile mutants of mobile RNA requires further
505 investigation.

506

507 **Conclusions**

508 To obtain tetraploid *S. peruvianum* DNA-free genome-edited plants, we used
509 *in vitro*-grown shoots, which contain high proportions of tetraploid cells, as explants
510 for protoplast isolation and regeneration. The medium components were optimized,
511 and genome-edited regenerants were obtained within 6 months. This is the first
512 study in *S. peruvianum* describing the use of both RNP and plasmid CRISPR
513 reagents for DNA-free genome editing, yielding a targeted mutagenesis efficiency of
514 60% without the need for marker gene selection. Diploid and tetraploid heritable
515 mutants were obtained for all pathogen-related genes targeted in this study,
516 including *SpSGS3*, *SpRDR6*, *SpPR-1*, *SpProsys*, and *SpMlo1*, and the expected
517 phenotypes were obtained. In comparative whole genome sequencing analysis,
518 protoplast derived CRISPR-Cas9 edited plants, either diploid or tetraploid, showed
519 stable genome structure. The proliferation of TYLCV, an important viral disease of
520 tomato, was increased in *spsgs3* and *sprdr6* null mutants. The reproductive growth
521 defect of the *SpSGS3* mutant was successfully rescued by grafting with WT stock.
522 The protocols and materials described in this study will be useful for tomato breeding.

523

524 **Materials and methods**

525 **Plant materials**

526 Sterile *S. peruvianum* plantlets were propagated by cutting and growing them in half-
527 strength Murashige and Skoog (1/2 MS) medium supplemented with 30 mg/L
528 sucrose and 1% agar, pH 5.7. The plantlets were incubated in a 26°C culture room
529 (12 h light/12 h in dark, light intensity of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The plantlets were cut and
530 subcultured in fresh medium monthly.

531

532 **Protoplast isolation and transfection**

533 Protoplast isolation and transfection of *S. peruvianum* were performed following our
534 previously published method with minor modifications (Hsu et al., 2019). Protoplasts
535 were isolated from the stems and petioles of *in vitro*-grown plantlets. Five or more
536 stems (approximately 5 cm/each, total 0.2-0.25 g) were used to isolate roughly 1 \times
537 10^6 protoplasts. These materials were placed in a 6-cm glass Petri dish with 10 mL
538 digestion solution [1/4 Murashige and Skoog (MS) liquid medium containing 1%
539 cellulose and 0.5% macerozyme, 3% sucrose, and 0.4 M mannitol, pH 5.7] and cut
540 into 0.5-cm-wide strips longitudinally. The material was incubated at room
541 temperature in the dark overnight. The digested solution was diluted in 10 mL W5
542 (154 mM NaCl, 125 mM CaCl_2 , 5 mM KCl, 2 mM MES, and 5 mM glucose) solution
543 and filtered through a 40- μm nylon mesh. The sample was centrifuged at low speed
544 ($360 \times g$) for 3 min to collect the protoplasts. The protoplasts were purified in 20%
545 sucrose solution and washed three times with W5 solution. The protoplasts were
546 transferred to transfection buffer (1/2 MS medium supplemented with 3% sucrose,
547 0.4 M mannitol, 1 mg/L NAA, and 0.3 mg/L kinetin, pH 5.7) and adjusted to a
548 concentration of 3×10^5 cells/mL.

549 The protoplasts were transfected with plasmids by PEG-mediated transfection
550 (Woo et al., 2015; Lin et al., 2018). A 400- μL sample (1.2×10^5 protoplasts) was
551 combined with 40 μL of CRISPR reagent (DNA: 20-40 μg ; RNP: 10 μg) and mixed
552 carefully. The same volume (440 μL) of PEG solution was added to the sample,
553 mixed, and incubated for 30 min. To end the reaction, 3 mL of W5 was added, and
554 the sample was mixed well. Transfected protoplasts were collected by centrifugation

555 at 360 × g for 3 min. The protoplasts were washed with 3 mL of W5 and centrifuged
556 at 360 × g for 3 min. The target sites are shown in Table 1.

557

558 **CRISPR/Cas reagents**

559 The SpCas9 vector for dicot transformation (pYLCRISPR/Cas9P35S-N) (Ma et al.,
560 2015) was isolated using a Plasmid Midi-prep kit (Bio-Genesis). Preparation of Cas9
561 protein and sgRNA and Cas9 RNP nucleofection were performed according to
562 Huang et al., 2020. Cas9 RNP complexes were assembled immediately before
563 nucleofection by mixing equal volumes of 40 μM Cas9 protein and 88.3 μM sgRNA
564 at a molar ratio of 1:2.2 and incubating at 37°C for 10 min.

565

566 **Protoplast regeneration**

567 Pooled protoplast DNA was used as a template to amplify the target genes for
568 validation by sequencing. The putatively edited protoplasts were transferred to 5-cm-
569 diameter Petri dishes containing 3 mL 1/2 MS liquid medium supplemented with 3%
570 sucrose, 0.4 M mannitol, 1 mg/L NAA, and 0.3 mg/L kinetin for plant regeneration.
571 Calli formed from the protoplasts after 1 month of incubation in the dark. The calli
572 were subcultured in a 9-cm-diameter Petri dish containing fresh medium with
573 cytokinin for 3-4 weeks in the light. Calli that had turned green were transferred to
574 solid medium containing the same plant growth regulators. The explants were
575 subcultured every 4 weeks until shoots formed after several subcultures. The shoots
576 were subcultured in solid rooting medium (HB1: 3 g/L Hyponex No. 1, 2 g/L tryptone,
577 20 g/L sucrose, 1 g/L activated charcoal, and 10 g/L Agar, pH 5.2) for adventitious
578 roots formation.

579

580 **Analysis of the genotypes of regenerated plants**

581 Two pairs of primers were designed to amplify the sgRNA-targeted DNA region for
582 each target gene. The PCR conditions were 94°C for 5 min, 35 cycles of denaturing
583 (94°C for 30 s), annealing (55°C for 30 s), and polymerization (72°C for 30 s),
584 followed by an extension reaction at 72°C for 3 min. The PCR product was
585 sequenced by Sanger sequencing to confirm mutagenesis. The multiple sequences

586 derived from mutated regenerated plants were bioinformatically separated using Poly
587 Peak Parser (<http://yosttools.genetics.utah.edu/PolyPeakParser/>; (Hill et al., 2014))
588 or further confirmed by sequential T/A cloning and sequencing. The primer
589 sequences are listed in Table S7.

590

591 **Estimation of genome size**

592 Fresh leaves were finely chopped with a new razor blade in 250 μ L isolation buffer
593 (200 mM Tris, 4 mM $MgCl_2 \cdot 6H_2O$, and 0.5% Triton X-100) and mixed well (Dolezel et
594 al., 2007). The mixture was filtered through a 40- μ m nylon mesh, and the filtered
595 suspensions were incubated with a DNA fluorochrome (50 μ g/mL propidium iodide
596 containing RNase A). The samples were analyzed using a MoFlo XDP Cell Sorter
597 (Beckman Coulter Life Science) and an Attune NxT Flow Cytometer (Thermo Fisher
598 Scientific). Chicken erythrocyte (BioSure) was used as an internal reference.

599

600 **Whole genome sequencing**

601 Leaves of *S. peruvianum* regenerates were harvested and genomic DNA was
602 extracted using two independent protocols. A nuclei isolation protocol (Sikorskaite et
603 al., 2013) was used on the wild type (SpB) sample to recover higher quality and
604 quantity of DNA samples. Briefly, nuclei were extracted by 36mM sodium bisulfite,
605 0.35M Sorbitol, 0.1M Tris-base, 5mM EDTA, 2M NaCl, 2% (w/v) CTAB, and 2 ml 5%
606 N-lauroylsarcosine sodium salt. The genomic DNA was then extracted by
607 chloroform-isoamyl alcohol (24:1), ethanol precipitation, and further cleaned up by
608 DNeasy Blood & Tissue Kit (69504, Qiagen) and AMPure (Beckman Coulter). The
609 other nine samples used the chloroform-isoamyl alcohol (24:1) for DNA extraction,
610 followed with Zymo Genomic DNA Clean & Concentrator-25 (D4064, Zymo), and
611 Zymo OneStep PCR Inhibitor Removal Kit (D6030, Zymo) to obtain high quality
612 genomic DNA. DNA integrity was checked using the D1000 Screen Tape on the
613 Agilent TapeStation 4150 System with DIN value > 8. Genomic DNA were sheared
614 using a Covaris E220 sonicator (Covaris) and paired- end sequencing libraries were
615 constructed by the NEBNext Ultra DNA Library Prep Kit II for Illumina (E7370S,
616 NEB). DNA libraries were validated again on the Agilent TapeStation 4150, and were
617 quantified by qPCR (E7630, NEB). The 2 \times 150 bp paired-end sequencing with

618 average insert size of 700 bp was performed by Welgene Biotech on an Illumina
619 NovaSeq 6000 platform.

620

621 **WGS data analysis**

622 Since there was no assembled *S. peruvianum* genome, high quality Illumina reads
623 were mapped to the *S. lycopersicum* Heinz 1706 reference genome (SL4.0)
624 (Hosmani et al., 2019) by the GPU-based NVIDIA Clara Parabricks package
625 (NVIDIA). To determine the variant frequency, we used the deep learning-based
626 Google DeepVariant (Yun et al., 2021) with 'WGS model' to identify variants. All
627 samples were then combined by GLnexus (Yun et al., 2021) to perform 'joint
628 genotype calling' using 'DeepVariant' model to combine samples. We then calculated
629 the heterozygous allele frequency by dividing the read depth of the heterozygous
630 allele (labeled as 0/1 by GLnexus) over the total read depth of the variant. A large
631 chromosomal region with heterozygous allele frequency lower than 0.5 indicated
632 either the chromosome region with low recombination rate or deletion of the
633 chromosome fragments. To determine CNVs between samples, we used the
634 cn.mops pipeline (Klambauer et al., 2012) to analyze mapped Illumina reads. To
635 minimize the effects of repetitive sequence regions, we set the segment size to
636 3,000 bp and minimum number of segments as 10 to identify high confidence CNVs.

637

638 **Quantitative real-time PCR (RT-qPCR)**

639 Expression of four genes was analysed using real-time PCR. These genes
640 were: *SpSGS3*, *SpARF3*, *SpARF4*, and *SpRDR6*. Transcripts of all four genes were
641 profiled with three biological replications and each with at least three technical
642 replications using the RNA samples of regenerants. RT-qPCR was carried out in 96-
643 well optical reaction plates using the iQ™ SYBR® Green Supermix (Bio-Rad). The
644 reference gene Actin and gene-specific primers for the RT-qPCR are listed
645 in Supplementary Table S8.

646

647 **Acknowledgments**

648 We thank Te-Chang Hsu and the AS-BCST Bioinformatics Core for the
649 computational support and Miranda Loney and Plant Editors for English editing.
650 Experiments and data analysis were performed in part using the confocal
651 microscope at the Division of Instrument Service of Academia Sinica with the
652 assistance of Shu-Chen Shen. We thank IPMB Flow Cytometry Analysis and Sorting
653 Service of Academia Sinica for flow cytometry analysis. We thank Ruei-Shiuan
654 Wang for genomic DNA preparation, Yu-Jung Cheng for tissue culture, Wei-Fong
655 Hung for genotyping, Ting-Li Wu and Jheng-Yang Ou for figure preparation and
656 Song-Bin Chang for karyotyping.

657

658 **Competing interests**

659 The authors declare that the research was conducted in the absence of any
660 commercial or financial relationships that could be construed as a potential conflict of
661 interest.

662

663 **Funding**

664 This research was supported by the Innovative Translational Agricultural Research
665 Program (AS-KPQ-107-ITAR-10; AS-KPQ-108-ITAR-10; AS-KPQ-109-ITAR-10; AS-
666 KPQ-110-ITAR-03) and Academia Sinica Institutional funding to Y-CL and C-SL, and
667 the Ministry of Science and Technology (105-2313-B-001-007-MY3; 108-2313-B-
668 001-011-; 109-2313-B-001-011-), Taiwan to C-SL. These funding bodies played no
669 role in the design of the study, collection, analysis or interpretation of data or in
670 writing the manuscript.

671

672 **Author contributions**

673 C-SL, Y-CL, JS, and M-CS conceived and designed the experiments. C-TH, and Y-
674 HY performed the CRISPR-Cas9 experiments. C-TH, Y-HY, Q-WC, J-JY, and F-HW

675 conducted the protoplast regeneration, cell biology, molecular biology, and targeted
676 mutagenesis experiments. SL conducted SpCas9 purification. Y-LW performed WGS
677 library preparation and qPCR analysis. P-XZ and Y-CL performed bioinformatics
678 analysis. Y-HC, C-TH, C-SL, Q-WC, and F-HW performed virus-related analysis. C-
679 TH performed cell biology. C-TH and S-IL performed grafting. JS, M-CS, Y-CL, and
680 C-SL wrote the manuscript with input from all co-authors. All authors read and
681 approved the final manuscript.

682

683 **Data Availability Statement**

684 The Illumina sequencing reads generated for this study have been deposited at
685 NCBI under BioProject PRJNA768623.

686 ([https://dataview.ncbi.nlm.nih.gov/object/PRJNA768623?reviewer=m0ufmjvjdqsj9evn](https://dataview.ncbi.nlm.nih.gov/object/PRJNA768623?reviewer=m0ufmjvjdqsj9evnf4ek6qtl8l)
687 [f4ek6qtl8l](https://dataview.ncbi.nlm.nih.gov/object/PRJNA768623?reviewer=m0ufmjvjdqsj9evnf4ek6qtl8l))

688

689 **Tables**

690 Table 1. CRISPR-Cas9 target sites and mutagenesis efficiencies.

691

Reagent	Target Gene	Target Site		Mutation (%)
Plasmid	<i>SpSGS3</i>	ATTCCCCCCAGGATAAAAAGC	GCGCAATTGAATGGTTTACA	8.3 (6/72)
		GTTCCCTCCTGCTCTGAAGAA	GTAACAATGCTGGATCAGGC	
	<i>SpRDR6</i>	TTAAAGCTGGGACCATTGCG	TGCGAGGTCGAATTGAAACA	13.2 (5/38)
	<i>SpPR-1</i>	CCAGGAGAGAATCTTGCCAA	CTGAATTGTGGGTGGCGGAG	13.9 (10/72)
		GGGCTCGTTGCAACAACGGA	TCTTGCAACTATGATCCTGT	
		ACTATGATCCTGTAGGCAAT	GATCCTGTAGGCAATTGGGT	
		GTAGGCAATTGGGTCGGACA		
	RNP	<i>SpProSys</i>	TCATGGTGAAGTTTCACCTT	GGAGGATCACGCTTTGATGG
<i>SpMlo1</i>		GGTGTACCTGTGGTGGAGAC	GTACAAAGTTAATCAAGAAT	63.6 (14/22)

692 **Figure legends**

693 **Figure 1. Flow cytometric analysis of the nuclear DNA contents of *S.***
694 ***peruvianum* tissues.** The genome sizes of (a) leaves, (b) stems (c), and
695 protoplasts derived from stems. X: fluorescence density; Y: count. Chicken
696 erythrocyte nuclei (CEN: 2.5 Gb) were used as the calibration standard. The
697 bar indicates the area used for counting nuclei. 2C: diploid; 4C: tetraploid. The
698 number in brackets after the ploidy is the percentage of each different ploidy
699 level versus the total counts.

700

701 **Figure 2. Regeneration of *S. peruvianum* protoplasts.** (a) Protoplasts
702 incubated in 1/2 Murashige and Skoog (MS) medium supplemented with 3%
703 sucrose, 0.4 M mannitol, 1 mg/L naphthaleneacetic acid (NAA), and 0.3 mg/L
704 kinetin, pH 5.7 liquid medium for 1 month. (b) Calli subcultured in 1/2 MS
705 medium supplemented with 3% sucrose, 0.4 M mannitol, 2 mg/L kinetin, and
706 0.3 mg/L Indole-3-acetic acid (IAA), pH 5.7 liquid medium in the light. (c) Calli
707 subcultured in 1/2 MS medium supplemented with 3% sucrose, 0.4 M
708 mannitol, 2 mg/L kinetin, pH 5.7, solid medium. (d) Shoot bud formation after
709 two subcultures in 2 mg/L kinetin solid medium. (e) Adventitious root
710 formation in plant growth regulator-free 1/2 MS solid medium supplemented
711 with 3% sucrose. (f) Regenerated plants after 1 month of growth in a pot. (g)
712 Regenerated plants grown in the field. (h) Flowers of a regenerated plant. (i)
713 Fruits of a regenerated plant. Throughout, bars = 1 cm.

714

715 **Figure 3. Karyotypes of *S. peruvianum* plants regenerated from**
716 **protoplasts.** Gray font: null mutant. Black font: heterozygous or wild-type.
717 Underline: $4n$. Bars = 5 μ m.

718

719 **Figure 4. Stable genome structures in plants regenerated from stem**
720 **cutting and protoplasts.** (a) Heterozygous allele frequency of WGS samples.
721 The heterozygous allele frequency was attained by dividing the read depth of
722 the heterozygous allele (labeled as 0/1 by GLnexus) by the total read depth of

723 the variant. Heterozygous frequency is plotted using 10-kb chromosome
724 window size on the X axis. A value of heterozygous allele frequency 0.5
725 indicates the frequency of the heterozygous genotype (0/1) from the
726 DeepVariant is 0.5, regardless the ploidy level. (b) Copy number variation
727 (CNV) of WGS samples. CNV was predicted as 3kb fragment size with
728 minimum 10 fragments. Predicted CNV is plotted using 30 bins per
729 chromosome on the X axis. Dot colors indicate the CNV density per bin. A
730 value of zero on the Y axis indicates no copy number change was detected.
731 Values above zero indicate copy number gain and below zero indicate copy
732 number loss.

733

734 **Figure 5. Gene expression and phenotypic profiles of *S. peruvianum***
735 ***sgs3* and *rdr6* mutants.** (a) RT-qPCR analysis of auxin response regulator
736 genes (*SpSGS3*, *SpARF3*, *SpARF4* and *SpRDR6*) in the wild type and
737 protoplast-derived regenerants. T₀: regenerated plants derived from
738 protoplasts. T₁: seedlings derived from T₀ plants. (b) Phenotypes of *spsgs3*
739 and *sprdr6* mutants. Bars = 1 cm.

740

741 **Figure 6. Symptoms and TYLCV proliferation on *in vitro*-cultured *S. peruvianum***
742 **plants inoculated with the infectious TYLCV clone.** (a) Diploid wild type and
743 *spsgs3*#11 mutant. (b) Tetraploid regenerated plant (#24) and *sprdr6*#38-6
744 mutant. Gray: null mutant. Black: Un-edited tetraploid regenerated plant (#24)
745 or the wild type. Underline: 4n. Bars = 1 cm.

746

747 **Figure 7. Growth of a sterile *spsgs3* #11 plant grafted with wild-type stock.** (a)
748 Grafted plant. Gray: null mutant. Leaves (b), flowers (c), and fruit of *spsgs3*
749 #11 scion. Mature fruit (e) and seeds (f) of wild-type stock (left) and *spsgs3*
750 #11 scion (right). (g) Results of Sanger sequencing of the seedling derived
751 from *spsgs3* #11 scion fruit, which is heterozygous, harboring *spsgs3*#11
752 mutated alleles mixed with the wild-type allele. Bars = 1 cm.

753

754 **Supplemental Figures**

755 **Figure S1. Effects of cytokinins on callus induction (1st subculture) and**
756 **callus proliferation (2nd subculture).** The effects of cytokinins [kinetin, zeatin,
757 6-(γ,γ -Dimethylallylamino)purine (2ip), and 6-Benzylaminopurine (BA)] during
758 these two stages were investigated separately. Different cytokinins were
759 added during callus induction [1st subculture, 1/2 Murashige and Skoog (MS)
760 medium supplemented with 3% sucrose, 0.4 M mannitol, pH 5.7 liquid
761 medium supplemented with 0.2 mg/L cytokinin and 1 mg/L NAA]. Kinetin
762 yielded the fewest calli, and the three other cytokinins led to better callus
763 induction. During callus proliferation [2nd subculture, 1/2 MS medium
764 supplemented with 3% sucrose, 0.4 M mannitol, pH 5.7 liquid medium
765 supplemented with 2 mg/L cytokinin and 0.3 mg/L Indole-3-acetic acid (IAA)],
766 the addition of zeatin, 2ip, and BA caused the callus to grow and turn green.
767 Inclusion of 2ip during callus induction yielded the same number of cells as
768 the other cytokinin treatments, but the cell clusters were smaller and did not
769 grow easily when directly transferred to callus proliferation medium in the light.
770 Therefore, zeatin and BA are the best treatments for liquid culture. Bar = 1 cm.

771

772 **Figure S2. Effects of cytokinins on callus in solid medium (3rd**
773 **subculture).** Calli from media containing different cytokinins (2nd subculture)
774 were transferred to solid medium containing the same cytokinin (3rd
775 subculture). Cytokinin in the medium had a strong effect on callus growth
776 (Figure S4). Regardless of the callus induction medium used, browning of the
777 callus occurred in solid medium supplemented with kinetin. Callus derived
778 from 2ip callus induction medium proliferated only in 2ip solid medium. BA
779 and zeatin had similar effects on callus growth, but calli on zeatin medium
780 showed more greening. We therefore identified zeatin as the most suitable
781 cytokinin for use in solid medium. Bar = 1 cm.

782

783 **Figure S3. Flow cytometric analysis of the nuclear DNA contents of**
784 **tetraploid plants regenerated from *SpProsys* RNP-transfected**
785 **protoplasts.** The number of regenerated plants is shown at the top left of

786 each panel. Gray font: null mutant. The genome sizes are shown at the top
787 right. The results are derived from three technical repeats. Unit: pg. Un-edited:
788 The *SpProsys* sequences are similar to the wild type. Chicken erythrocyte
789 nuclei (CEN: 2.5 Gb) were used as the calibration standard. The bar indicates
790 the area used to count nuclei. The genome sizes of all seven regenerants
791 were measured by flow cytometry, including two un-edited, three
792 heterozygous, and two biallelic plants that were tetraploid. Both tetraploid and
793 diploid regenerants (Table S7) derived from *SpProSys* RNP transfections
794 flowered normally, and no distinctive phenotype was observed. Bar = 1 cm.

795

796 **Figure S4. Phenotypes of diploid and tetraploid plants regenerated from**
797 **protoplasts transfected with CRISPR reagents.** Underline: $4n$. Bars = 1 cm.
798 *SpSGS3#10*, *SpSGS3#7* and *SpRDR6#38* contained mutated alleles. (a) the
799 fruits of diploid and tetraploids regenerated from transfected protoplasts. (b)
800 T_1 seeds of the heterozygous diploid (*SpSGS3#10*) and tetraploid
801 (*SpSGS3#7* and *SpRDR6#38*) mutants. (c) 1.5-month-old T_1 seedling derived
802 from T_0 transfected protoplast regenerated plants.

803

804 **Figure S5. Progeny analysis of *SpSGS3*.** Underlined regenerated plant
805 name: tetraploid. Red font: mutated nucleotide. Green/blue font: sequences
806 shown in the green/blue boxes in the Sanger sequencing results. WT: wild
807 type. M: mutant. WT:M: wild type/mutant ratio based on Sanger sequencing
808 results. No.: number of progeny in this ratio. (a) *SpSGS3#7* T_1 progeny
809 analysis. The allele sequences in the GTTCCTCCTGCTCTGAAGAA target
810 site are listed; 0–3 mutated alleles were identified. This regenerated plant was
811 shown to be allotetraploid. (b) The PCR product of the *spsgs3#7-2* null mutant
812 was subjected to T/A cloning, and the clones were subjected to Sanger
813 sequencing (GCGCAATTGAATGGTTTACA target site and
814 ATTCCCCCAGGATAAAAGC target site). Three types of mutated alleles
815 were identified. (c) Analysis of diploid *SpSGS3#10* T_1 progeny.

816

817 **Figure S6. Progeny analysis of *SpRDR6*.** Underlined regenerated plant
818 name: tetraploid. Red font: mutated nucleotides. Blue font: sequences shown
819 in blue boxes in the Sanger sequencing results. (a) *SpRDR6#6-2* genotyping.
820 Top: allele sequences. Middle: The Sanger sequencing results indicate the
821 presence of multiple peaks after TTAAGCT. Bottom: The T/A cloning results
822 demonstrate that *SpRDR6#6-2* contains a mutated allele (M) similar to
823 *SpRDR6#6*. (b) RT-PCR product of the *sprdr6#33-G* null mutant. The result
824 indicates that *sprdr6#33-G* is a homozygous null mutant. The mutated allele
825 can still generate a transcript. (c) Genotyping of the *sprdr6#38-6* null mutant.
826 Top: The allele sequences of *SpRDR6#38*. Middle: Sanger sequencing
827 results of *sprdr6#38-6* genomic DNA. Bottom: The M1 and M2 mutated alleles
828 identified by T/A cloning without wild-type alleles.

829

830 **Figure S7. Progeny analysis of *SpPR-1*.** Red font: mutated nucleotide(s).
831 Blue/green font: sequences shown in blue/green boxes in the Sanger
832 sequencing results. (a) Progeny analysis of *sppr-1#52*. Top: allele sequences.
833 Middle: Sanger sequencing results of different genotypes. Multiple peaks are
834 shown in heterozygous lines (M1M2, M1M3, M2M3). No.: number of progeny
835 of each genotype. Bottom: M3 sequence identified by T/A cloning. (b)
836 Progeny analysis of *sppr-1#61*. Top: allele sequences. Middle: *SpPR-1*
837 genomic PCR products of *sppr-1#61* progeny. The genotypes of individual
838 progeny were determined based on DNA size and are shown below the image.
839 Sanger sequencing results for the LL and SS genotypes.

840

841 **Figure S8. Illumina sequencing coverage for the tomato SL4.0 genome**
842 **assembly.** The Illumina PE reads were mapped by BWA and the sequencing
843 depth was calculated in 10kb window size. Coverage is plotted using 30 bins
844 per chromosome on the X axis. Black dashed line: median of the sequencing
845 coverage of each chromosome.

846

847 **Figure S9. Phenotypes of the *spsgs3* and *sprdr6* null mutants.** Underlined
848 regenerated plant name: tetraploid. Wiry phenotypes of T₀ diploid *spsgs3* null

849 mutants #6 and #13. Bar = 1 cm. (b) Wiry phenotypes of T₁ tetraploid
850 *spsgs3#7-2* and *sprdr6#38-16*. Bar = 1 cm. (c) Alexander staining of wild-type
851 and *spsgs3#11* pollen. Bar = 50 μm.

852

853 **Figure S10. Symptoms and TYLCV proliferation on *in vitro*-cultured *S.***
854 ***peruvianum* plants inoculated with the infectious TYLCV clone.** Gray: null
855 mutant. Underline: 4*n*. Bars = 1 cm. *SpRDR6#2* and *SpSGS3#24* were non-
856 mutated protoplast regenerated plants. Line 1, 7: *SpRDR6#2*; 2, 8: *sprdr6#38-*
857 6; 3, 9: Wild type; 4, 10: *spsgs3#11*; 5, 11: *SpSGS3#24*; 6, 12: *spsgs3#7-2*

858

859 Supplemental Tables

860 **Table S1. *SpSGS3* gene sequences of the *SpSGS3* mutants.** Gray: null
861 mutant. Underline: 4*n*. Red font: mutated nucleotide(s). Number in brackets:
862 length of nucleotide sequence. -: deletion. +: insertion.

863

864 **Table S2. *SpRDR6* gene sequences of the *SpRDR6* mutants.** Underline:
865 4*n*. Red: mutated nucleotide(s). -: deletion. +: insertion.

866

867 **Table S3. *SpPR-1* gene sequences of the *SpPR-1* mutants.** Gray: null
868 mutant. Underline: 4*n*. Red: mutated nucleotide(s). Number in brackets:
869 length of nucleotide sequence. -: deletion. +: insertion.

870

871 **Table S4. *SpProsys* gene sequences of the *SpProsys* mutants.** Gray: null
872 mutant. Underline: 4*n*. Red: mutated nucleotide(s). Number in brackets:
873 length of nucleotide sequence. -: deletion. +: insertion.

874

875 **Table S5. *SpMlo1* gene sequences of the *SpMlo1* mutants.** Gray: null
876 mutant. Underline: 4*n*. Red: mutated nucleotide(s). Number in brackets:
877 length of nucleotide sequence. -: deletion. +: insertion.

878

879 **Table S6. Karyotypes of plants regenerated from protoplasts transfected**
880 **with CRISPR reagents.** WT: the target gene sequences are un-edited, like
881 the wild type. *: the genome size was determined by flow cytometry.

882

883 **Table S7. Overview of Illumina WGS sequencing, mapping rate and SRA**
884 **number.**

885

886 **Table S8. Primers used in these studies.**

887

888

889 Literature Cited

890 **Adenot X, Elmayan T, Laouressergues D, Boutet S, Bouche N,**
891 **Gascioli V, Vaucheret H** (2006) DRB4-dependent TAS3 trans-acting siRNAs
892 control leaf morphology through AGO7. *Curr Biol* **16**: 927-932

893 **Al Abdallat AM, Al Debei HS, Asmar H, Misbeh S, Quraan A,**
894 **Kvarnheden A** (2010) An efficient in vitro-inoculation method for Tomato
895 yellow leaf curl virus. *Virol J* **7**: 84

896 **Andersson M, Turesson H, Olsson N, Falt AS, Ohlsson P,**
897 **Gonzalez MN, Samuelsson M, Hofvander P** (2018) Genome editing in

898 potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiol Plant* **164**: 378-
899 384

900 **Banakar R, Schubert M, Collingwood M, Vakulskas C,**
901 **Eggenberger AL, Wang K** (2020) Comparison of CRISPR-Cas9/Cas12a
902 Ribonucleoprotein Complexes for Genome Editing Efficiency in the Rice
903 Phytoene Desaturase (OsPDS) Gene. *Rice (N Y)* **13**: 4

904 **Beloshistov RE, Dreizler K, Galiullina RA, Tuzhikov AI,**
905 **Serebryakova MV, Reichardt S, Shaw J, Taliansky ME, Pfannstiel J,**
906 **Chichkova NV, Stintzi A, Schaller A, Vartapetian AB** (2018) Phytaspase-
907 mediated precursor processing and maturation of the wound hormone
908 systemin. *New Phytol* **218**: 1167-1178

909 **Bernabe-Orts JM, Casas-Rodrigo I, Minguet EG, Landolfi V,**
910 **Garcia-Carpintero V, Gianoglio S, Vazquez-Vilar M, Granell A, Orzaez D**
911 (2019) Assessment of Cas12a-mediated gene editing efficiency in plants.
912 *Plant Biotechnol J* **17**: 1971-1984

913 **Brooks C, Nekrasov V, Lippman ZB, Van Eck J** (2014) Efficient gene
914 editing in tomato in the first generation using the clustered regularly
915 interspaced short palindromic repeats/CRISPR-associated9 system. *Plant*
916 *Physiol* **166**: 1292-1297

917 **Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF** (2015) High-
918 frequency, precise modification of the tomato genome. *Genome Biol* **16**: 232

919 **Chen EG, Tsai KL, Chung HH, Chen JT** (2018) Chromosome
920 Doubling-Enhanced Biomass and Dihydrotanshinone I Production in *Salvia*
921 *miltiorrhiza*, A Traditional Chinese Medicinal Plant. *Molecules* **23**: 3106

922 **Chen YL, Lee CY, Cheng KT, Chang WH, Huang RN, Nam HG,**
923 **Chen YR** (2014) Quantitative peptidomics study reveals that a wound-induced
924 peptide from PR-1 regulates immune signaling in tomato. *Plant Cell* **26**: 4135-
925 4148

926 **Chung HH, Shi SK, Huang B, Chen JT** (2017) Enhanced Agronomic
927 Traits and Medicinal Constituents of Autotetraploids in *Anoectochilus*
928 *formosanus* Hayata, a Top-Grade Medicinal Orchid. *Molecules* **22**: 1907

929 **Cocking EC** (1960) A Method for the Isolation of Plant Protoplasts and
930 Vacuoles. *Nature* **187**: 962-963

931 **Cola GPA, Marques AM, Damasceno S, Carvalho CR, Clarindo WR**
932 (2014) In Vitro Polyploidization in *Solanum lycopersicum* Mill. 'Santa Cruz
933 Kada Gigante'. *Cytologia* **79**: 351-358

934 **De Bruyn C, Ruttink T, Eeckhaut T, Jacobs T, De Keyser E,**
935 **Goossens A, Van Laere K** (2020) Establishment of CRISPR/Cas9 Genome
936 Editing in Witloof (*Cichorium intybus* var. *foliosum*). *Frontiers in Genome*
937 *Editing* **2**: 604876

938 **Ellul P, Garcia-Sogo B, Pineda B, Rios G, Roig LA, Moreno V** (2003)
939 The ploidy level of transgenic plants in *Agrobacterium*-mediated
940 transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is
941 genotype and procedure dependent. *Theor Appl Genet* **106**: 231-238

942 **Fossi M, Amundson K, Kuppu S, Britt A, Comai L** (2019)
943 Regeneration of *Solanum tuberosum* Plants from Protoplasts Induces
944 Widespread Genome Instability. *Plant Physiol* **180**: 78-86

945 **Haroldsen VM, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ,**
946 **Chi-Ham CL, Labavitch JM, Bennett AB, Powell AL** (2012) Mobility of
947 Transgenic Nucleic Acids and Proteins within Grafted Rootstocks for
948 Agricultural Improvement. *Front Plant Sci* **3**: 39

949 **Hill JT, Demarest BL, Bisgrove BW, Su YC, Smith M, Yost HJ** (2014)
950 Poly peak parser: Method and software for identification of unknown indels
951 using sanger sequencing of polymerase chain reaction products. *Dev Dyn*
952 **243**: 1632-1636

953 **Hosmani PS, Flores-Gonzalez M, van de Geest H, Maumus F,**
954 **Bakker LV, Schijlen E, van Haarst J, Cordewener J, Sanchez-Perez G,**
955 **Peters S, Fei Z, Giovannoni JJ, Mueller LA, Saha S** (2019) An improved de
956 novo assembly and annotation of the tomato reference genome using single-
957 molecule sequencing, Hi-C proximity ligation and optical maps. *bioRxiv*:
958 767764

959 **Hsu C-T, Lee W-C, Cheng Y-J, Yuan Y-H, Wu F-H, Lin C-S** (2021)
960 Genome Editing and Protoplast Regeneration to Study Plant–Pathogen
961 Interactions in the Model Plant *Nicotiana benthamiana*. *Frontiers in Genome*
962 *Editing* **2**: 627803

963 **Hsu CT, Cheng YJ, Yuan YH, Hung WF, Cheng QW, Wu FH, Lee LY,**
964 **Gelvin SB, Lin CS** (2019) Application of Cas12a and nCas9-activation-
965 induced cytidine deaminase for genome editing and as a non-sexual strategy
966 to generate homozygous/multiplex edited plants in the allotetraploid genome
967 of tobacco. *Plant Mol Biol* **101**: 355-371

968 **Hsu CT, Yuan YH, Lin YC, Lin S, Cheng QW, Wu FH, Sheen J, Shih**
969 **MC, Lin CS** (2021) Efficient and Economical Targeted Insertion in Plant
970 Genomes via Protoplast Regeneration. *CRISPR J* **4**: 752-760

971 **Klambauer G, Schwarzbauer K, Mayr A, Clevert D-A, Mitterecker A,**
972 **Bodenhofer U, Hochreiter S** (2012) cn.MOPS: mixture of Poissons for
973 discovering copy number variations in next-generation sequencing data with a
974 low false discovery rate. *Nucleic Acids Res.* **40**: e69

975 **Kut SA, Evans DA** (1982) Plant regeneration from cultured leaf
976 explants of eight wild tomato species and two related *Solanum* species. In
977 *Vitro* **18**: 593-598

978 **Li T, Yang X, Yu Y, Si X, Zhai X, Zhang H, Dong W, Gao C, Xu C**
979 (2018) Domestication of wild tomato is accelerated by genome editing. *Nat*
980 *Biotechnol* **36**: 1160-1163

981 **Liang Z, Chen K, Zhang Y, Liu J, Yin K, Qiu JL, Gao C** (2018)
982 Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in
983 vitro transcripts or ribonucleoproteins. *Nat Protoc* **13**: 413-430

984 **Lin C-S, Kalpana K, Chang W-C, Lin N-S** (2007) Improving Multiple
985 Shoot Proliferation in Bamboo Mosaic Virus-free *Bambusa oldhamii* Munro
986 Propagation by Liquid Culture. *HortScience* **42**: 1243-1246

987 **Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, Cheng QW, Wu FH,**
988 **Hsiao HC, Zhang Y, Zhang R, Chang WJ, Yu CT, Wang W, Liao LJ, Gelvin**
989 **SB, Shih MC** (2018) Application of protoplast technology to CRISPR/Cas9

- 990 mutagenesis: from single-cell mutation detection to mutant plant regeneration.
991 *Plant Biotechnol J* **16**: 1295-1310
- 992 **Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H,**
993 **Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X,**
994 **Dong Z, Liu YG** (2015) A Robust CRISPR/Cas9 System for Convenient,
995 High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Mol*
996 *Plant* **8**: 1274-1284
- 997 **Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, Voytas**
998 **DF** (2020) Plant gene editing through de novo induction of meristems. *Nat*
999 *Biotechnol* **38**: 84-89
- 1000 **Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H,**
1001 **Nussaume L, Crespi MD, Maizel A** (2010) miR390, Arabidopsis TAS3
1002 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an
1003 autoregulatory network quantitatively regulating lateral root growth. *Plant Cell*
1004 **22**: 1104-1117
- 1005 **Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-**
1006 **B, Jouette D, Lacombe A-M, Nikic S, Picault N, Rémoüé K, Sanial M, Vo**
1007 **T-A, Vaucheret H** (2000) Arabidopsis SGS2 and SGS3 Genes Are Required
1008 for Posttranscriptional Gene Silencing and Natural Virus Resistance. *Cell* **101**:
1009 533-542
- 1010 **Musacchio A, Salmon ED** (2007) The spindle-assembly checkpoint in
1011 space and time. *Nat Rev Mol Cell Biol* **8**: 379-393
- 1012 **Nekrasov V, Wang C, Win J, Lanz C, Weigel D, Kamoun S** (2017)
1013 Rapid generation of a transgene-free powdery mildew resistant tomato by
1014 genome deletion. *Sci Rep* **7**: 482
- 1015 **Nilsson E** (1950) Some Experiments with Tetraploid Tomatoes.
1016 *Hereditas* **36**: 181-204
- 1017 **Palauqui JC, Elmayan T, Pollien JM, Vaucheret H** (1997) Systemic
1018 acquired silencing: transgene-specific post-transcriptional silencing is
1019 transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J*
1020 **16**: 4738-4745

- 1021 **Pucker B, Kleinbolting N, Weisshaar B** (2021) Large scale genomic
1022 rearrangements in selected Arabidopsis thaliana T-DNA lines are caused by
1023 T-DNA insertion mutagenesis. BMC Genomics **22**: 599
- 1024 **Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H,**
1025 **Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H, Nishida K,**
1026 **Ariizumi T, Kondo A** (2017) Targeted base editing in rice and tomato using a
1027 CRISPR-Cas9 cytidine deaminase fusion. Nat Biotechnol **35**: 441-443
- 1028 **Sikorskaite S, Rajamaki ML, Baniulis D, Stanys V, Valkonen JP**
1029 (2013) Protocol: Optimised methodology for isolation of nuclei from leaves of
1030 species in the Solanaceae and Rosaceae families. Plant Methods **9**: 31
- 1031 **Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A**
1032 (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein
1033 complexes. Nat Commun **7**: 13274
- 1034 **Takebe I, Labib G, Melchers G** (1971) Regeneration of whole plants
1035 from isolated mesophyll protoplasts of tobacco. Die Naturwissenschaften **58**:
1036 318-320
- 1037 **Thieme CJ, Rojas-Triana M, Stecyk E, Schudoma C, Zhang W,**
1038 **Yang L, Minambres M, Walther D, Schulze WX, Paz-Ares J, Scheible WR,**
1039 **Kragler F** (2015) Endogenous Arabidopsis messenger RNAs transported to
1040 distant tissues. Nat Plants **1**: 15025
- 1041 **Van de Peer Y, Ashman TL, Soltis PS, Soltis DE** (2021) Polyploidy:
1042 an evolutionary and ecological force in stressful times. Plant Cell **33**: 11-26
- 1043 **Verlaan MG, Hutton SF, Ibrahim RM, Kormelink R, Visser RG,**
1044 **Scott JW, Edwards JD, Bai Y** (2013) The Tomato Yellow Leaf Curl Virus
1045 resistance genes Ty-1 and Ty-3 are allelic and code for DFDGD-class RNA-
1046 dependent RNA polymerases. PLoS Genet **9**: e1003399
- 1047 **Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, Kim H, Kim SG,**
1048 **Kim ST, Choe S, Kim JS** (2015) DNA-free genome editing in plants with
1049 preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol **33**: 1162-
1050 1164

1051 **Yifhar T, Pekker I, Peled D, Friedlander G, Pistunov A, Sabban M,**
1052 **Wachsman G, Alvarez JP, Amsellem Z, Eshed Y** (2012) Failure of the
1053 tomato trans-acting short interfering RNA program to regulate AUXIN
1054 RESPONSE FACTOR3 and ARF4 underlies the wiry leaf syndrome. *Plant*
1055 *Cell* **24**: 3575-3589

1056 **Yu H, Lin T, Meng X, Du H, Zhang J, Liu G, Chen M, Jing Y, Kou L,**
1057 **Li X, Gao Q, Liang Y, Liu X, Fan Z, Liang Y, Cheng Z, Chen M, Tian Z,**
1058 **Wang Y, Chu C, Zuo J, Wan J, Qian Q, Han B, Zuccolo A, Wing RA, Gao**
1059 **C, Liang C, Li J** (2021) A route to de novo domestication of wild allotetraploid
1060 rice. *Cell* **184**: 1156-1170 e1114

1061 **Yun T, Li H, Chang P-C, Lin MF, Carroll A, McLean CY** (2021)
1062 Accurate, scalable cohort variant calls using DeepVariant and GLnexus.
1063 *Bioinformatics* **36**: 5582-5589

1064 **Zheng N, Li T, Dittman JD, Su J, Li R, Gassmann W, Peng D,**
1065 **Whitham SA, Liu S, Yang B** (2020) CRISPR/Cas9-Based Gene Editing
1066 Using Egg Cell-Specific Promoters in Arabidopsis and Soybean. *Front Plant*
1067 *Sci* **11**: 800

1068 **Zsogon A, Cermak T, Naves ER, Notini MM, Edel KH, Weini S,**
1069 **Freschi L, Voytas DF, Kudla J, Peres LEP** (2018) De novo domestication of
1070 wild tomato using genome editing. *Nat Biotechnol* **36**: 1211-1216

1071

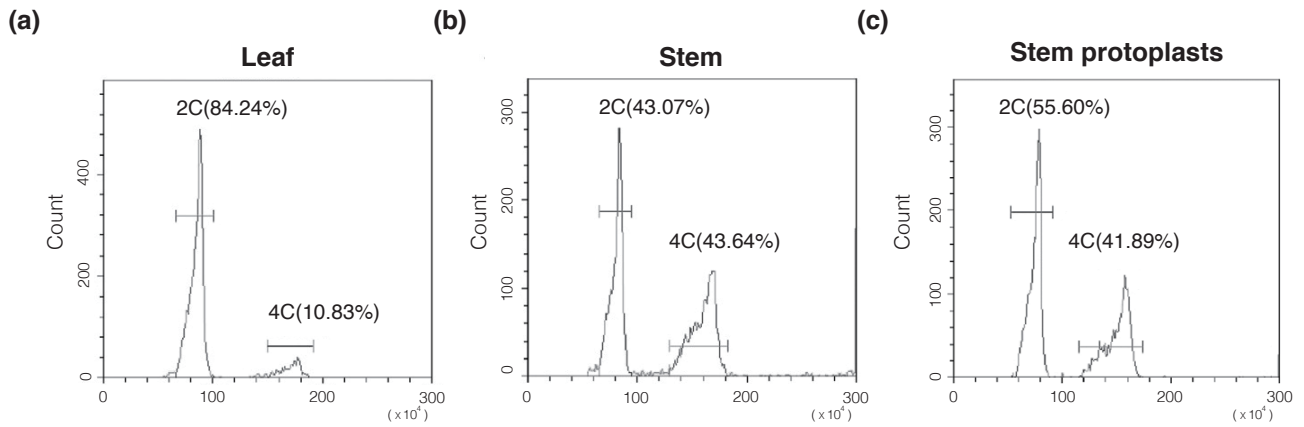


Figure 1. Flow cytometric analysis of the nuclear DNA contents of *S. peruvianum* tissues.

The genome sizes of (a) leaves, (b) stems (c), and protoplasts derived from stems. X: fluorescence density; Y: count. Chicken erythrocyte nuclei (CEN: 2.5 Gb) were used as the calibration standard. The bar indicates the area used for counting nuclei. 2C: diploid; 4C: tetraploid. The number in brackets after the ploidy is the percentage of each different ploidy level versus the total counts.

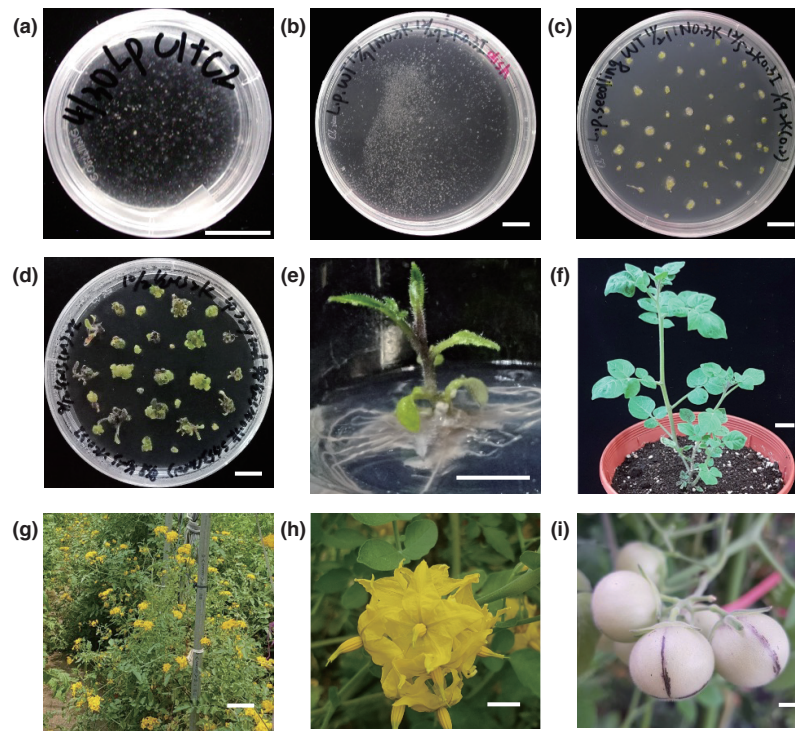


Figure 2. Regeneration of *S. peruvianum* protoplasts.

(a) Protoplasts incubated in 1/2 Murashige and Skoog (MS) medium supplemented with 3% sucrose, 0.4 M mannitol, 1 mg/L naphthaleneacetic acid (NAA), and 0.3 mg/L kinetin, pH 5.7 liquid medium for 1 month. **(b)** Calli subcultured in 1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, 2 mg/L kinetin, and 0.3 mg/L Indole-3-acetic acid (IAA), pH 5.7 liquid medium in the light. **(c)** Calli subcultured in 1/2 MS medium supplemented with 3% sucrose, 0.4 M mannitol, 2 mg/L kinetin, pH 5.7, solid medium. **(d)** Shoot bud formation after two subcultures in 2 mg/L kinetin solid medium. **(e)** Adventitious root formation in plant growth regulator-free 1/2 MS solid medium supplemented with 3% sucrose. **(f)** Regenerated plants after 1 month of growth in a pot. **(g)** Regenerated plants grown in the field. **(h)** Flowers of a regenerated plant. **(i)** Fruits of a regenerated plant. Throughout, bars = 1 cm.

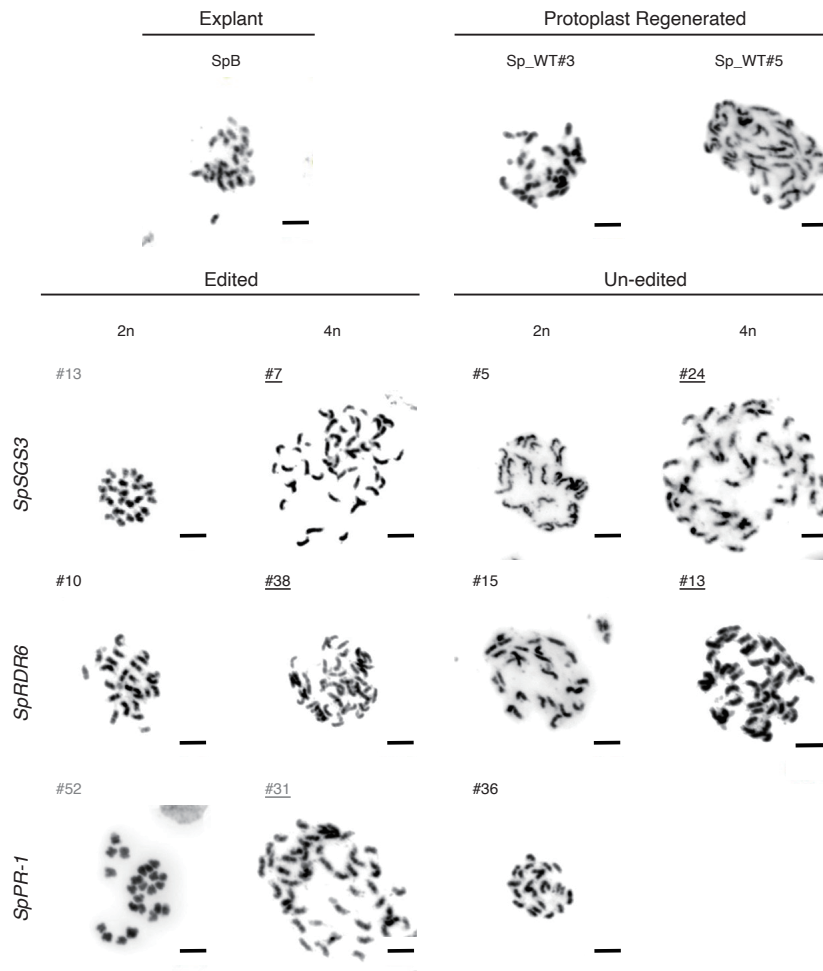


Figure 3. Karyotypes of *S. peruvianum* plants regenerated from protoplasts.
Gray font: null mutant. Black font: heterozygous or wild-type. Underline: 4n. Bars = 5 µm.

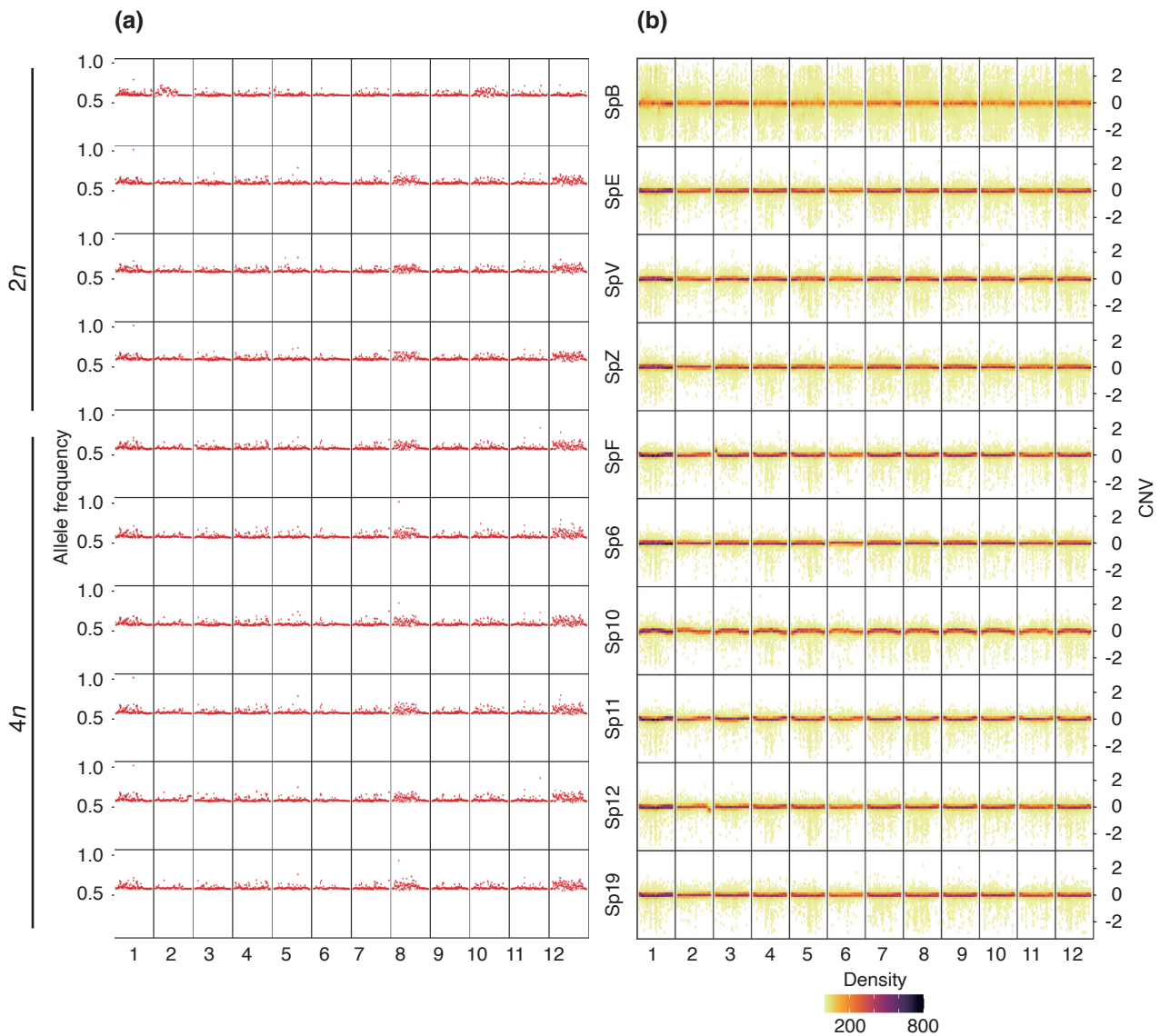


Figure 4. Stable genome structures in plants regenerated from stem cutting and protoplasts.

(a) Heterozygous allele frequency of WGS samples. The heterozygous allele frequency was attained by dividing the read depth of the heterozygous allele (labeled as 0/1 by GLnexus) by the total read depth of the variant. Heterozygous frequency is plotted using 10-kb chromosome window size on the X axis. A value of heterozygous allele frequency 0.5 indicates the frequency of the heterozygous genotype (0/1) from the DeepVariant is 0.5, regardless the ploidy level. **(b)** Copy number variation (CNV) of WGS samples. CNV was predicted as 3kb fragment size with minimum 10 fragments. Predicted CNV is plotted using 30 bins per chromosome on the X axis. Dot colors indicate the CNV density per bin. A value of zero on the Y axis indicates no copy number change was detected. Values above zero indicate copy number gain and below zero indicate copy number loss.

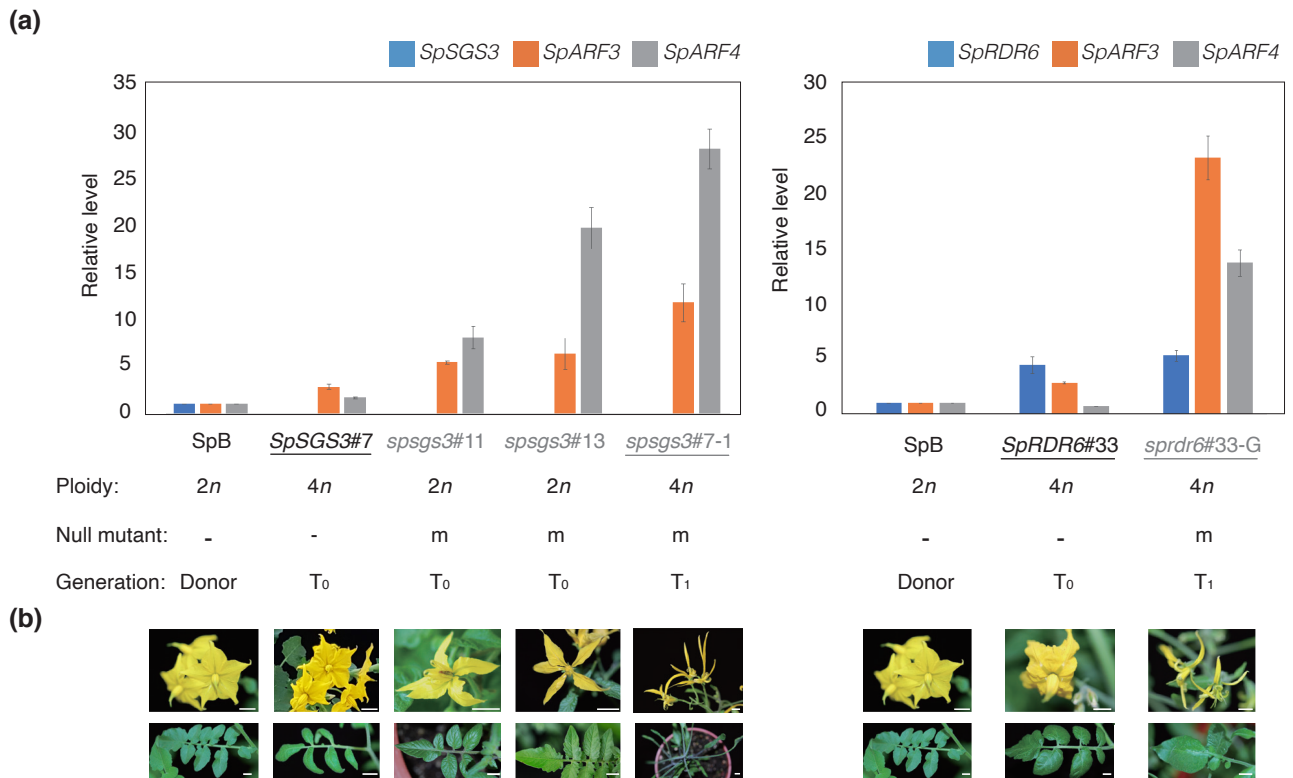


Figure 5. Gene expression and phenotypic profiles of *S. peruvianum* *sgs3* and *rdr6* mutants.

(a) RT-qPCR analysis of auxin response regulator genes (*SpSGS3*, *SpARF3*, *SpARF4* and *SpRDR6*) in the wild type and protoplast-derived regenerants. T₀: regenerated plants derived from protoplasts. T₁: seedlings derived from T₀ plants. **(b)** Phenotypes of *spsgs3* and *sprdr6* mutants. Bars = 1 cm.

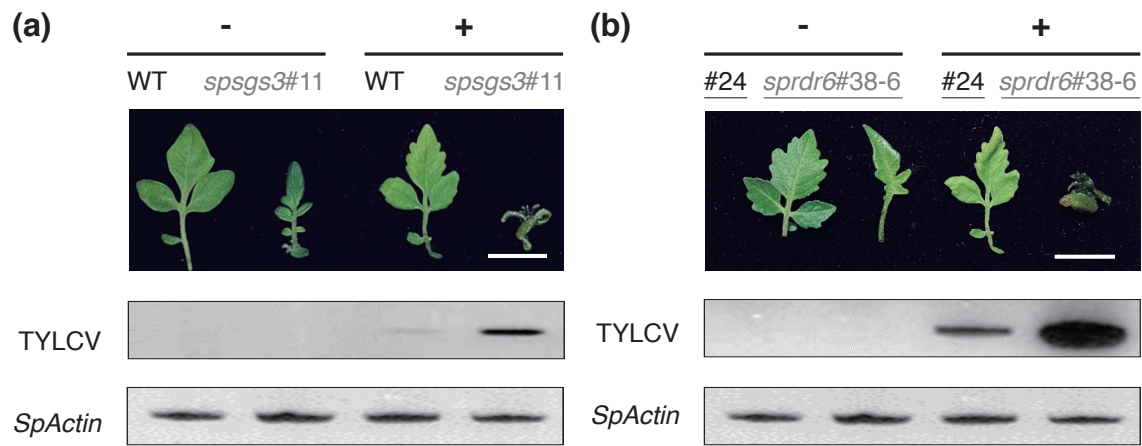


Figure 6. Symptoms and TYLCV proliferation on *in vitro*-cultured *S. peruvianum* plants inoculated with the infectious TYLCV clone.

(a) Diploid wild type and *spsgs3#11* mutant. **(b)** Tetraploid regenerated plant (#24) and *sprdr6#38-6* mutant. Gray: null mutant. Black: Un-edited tetraploid regenerated plant (#24) or the wild type. Underline: 4n. Bars = 1 cm.

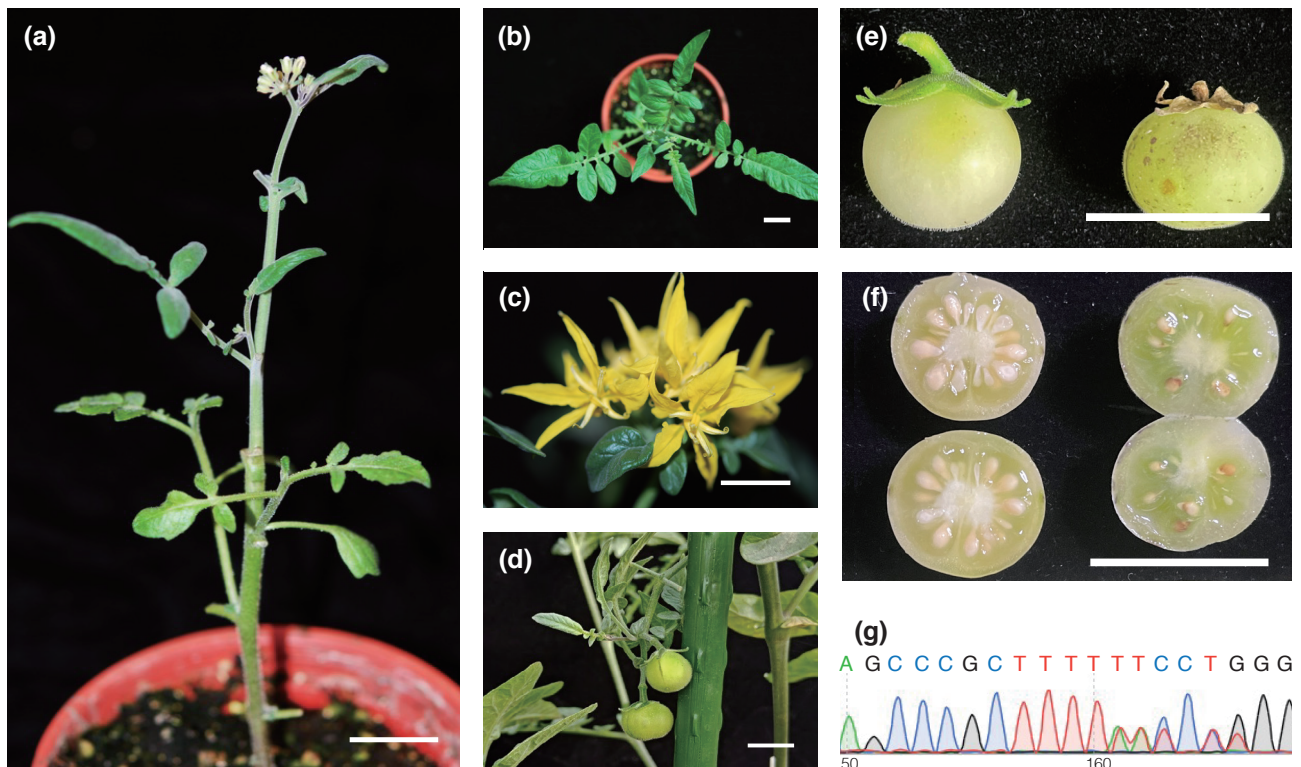


Figure 7. Growth of a sterile *spsgs3* #11 plant grafted with wild-type stock.

(a) Grafted plant. Gray: null mutant. Leaves (b), flowers (c), and fruit of *spsgs3*#11 scion. Mature fruit (e) and seeds (f) of wild-type stock (left) and *spsgs3*#11 scion (right). (g) Results of Sanger sequencing of the seedling derived from *spsgs3*#11 scion fruit, which is heterozygous, harboring *spsgs3*#11 mutated alleles mixed with the wild-type allele. Bars = 1 cm.

Parsed Citations

Adenot X, Elmayan T, Lauressergues D, Boutet S, Bouche N, Gasciolli V, Vaucheret H (2006) DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Curr Biol* 16: 927-932

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Al Abdallat AM, Al Debei HS, Asmar H, Misbeh S, Quraan A, Kvarnheden A (2010) An efficient in vitro-inoculation method for Tomato yellow leaf curl virus. *Virology* 7: 84

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Andersson M, Turesson H, Olsson N, Falt AS, Ohlsson P, Gonzalez MN, Samuelsson M, Hofvander P (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiol Plant* 164: 378-384

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Banakar R, Schubert M, Collingwood M, Vakulskas C, Eggenberger AL, Wang K (2020) Comparison of CRISPR-Cas9/Cas12a Ribonucleoprotein Complexes for Genome Editing Efficiency in the Rice Phytoene Desaturase (OsPDS) Gene. *Rice (N Y)* 13: 4

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Beloshistov RE, Dreizler K, Galiullina RA, Tuzhikov AI, Serebryakova MV, Reichardt S, Shaw J, Taliansky ME, Pfannstiel J, Chichkova NV, Stintzi A, Schaller A, Vartapetian AB (2018) Phytaspase-mediated precursor processing and maturation of the wound hormone systemin. *New Phytol* 218: 1167-1178

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Bernabe-Orts JM, Casas-Rodrigo I, Minguet EG, Landolfi V, Garcia-Carpintero V, Gianoglio S, Vazquez-Vilar M, Granell A, Orzaez D (2019) Assessment of Cas12a-mediated gene editing efficiency in plants. *Plant Biotechnol J* 17: 1971-1984

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Brooks C, Nekrasov V, Lippman ZB, Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol* 166: 1292-1297

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. *Genome Biol* 16: 232

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Chen EG, Tsai KL, Chung HH, Chen JT (2018) Chromosome Doubling-Enhanced Biomass and Dihydroshikonin I Production in *Salvia miltiorrhiza*, A Traditional Chinese Medicinal Plant. *Molecules* 23: 3106

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Chen YL, Lee CY, Cheng KT, Chang WH, Huang RN, Nam HG, Chen YR (2014) Quantitative peptidomics study reveals that a wound-induced peptide from PR-1 regulates immune signaling in tomato. *Plant Cell* 26: 4135-4148

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Chung HH, Shi SK, Huang B, Chen JT (2017) Enhanced Agronomic Traits and Medicinal Constituents of Autotetraploids in *Anoectochilus formosanus* Hayata, a Top-Grade Medicinal Orchid. *Molecules* 22: 1907

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Cocking EC (1960) A Method for the Isolation of Plant Protoplasts and Vacuoles. *Nature* 187: 962-963

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Cola GPA, Marques AM, Damasceno S, Carvalho CR, Clarindo WR (2014) In Vitro Polyploidization in *Solanum lycopersicum* Mill. 'Santa Cruz Kada Gigante'. *Cytologia* 79: 351-358

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

De Bruyn C, Ruttink T, Eeckhaut T, Jacobs T, De Keyser E, Goossens A, Van Laere K (2020) Establishment of CRISPR/Cas9 Genome Editing in Witloof (*Cichorium intybus* var. *foliosum*). *Frontiers in Genome Editing* 2: 604876

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ellul P, Garcia-Sogo B, Pineda B, Rios G, Roig LA, Moreno V (2003) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent. *Theor Appl Genet* 106: 231-238

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Fossi M, Amundson K, Kuppu S, Britt A, Comai L (2019) Regeneration of *Solanum tuberosum* Plants from Protoplasts Induces Widespread Genome Instability. *Plant Physiol* 180: 78-86

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Haroldsen VM, Szczerba MW, Aktas H, Lopez-Baltazar J, Odias MJ, Chi-Ham CL, Labavitch JM, Bennett AB, Powell AL (2012) Mobility of Transgenic Nucleic Acids and Proteins within Grafted Rootstocks for Agricultural Improvement. *Front Plant Sci* 3: 39

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hill JT, Demarest BL, Bisgrove BW, Su YC, Smith M, Yost HJ (2014) Poly peak parser: Method and software for identification of unknown indels using sanger sequencing of polymerase chain reaction products. *Dev Dyn* 243: 1632-1636

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hosmani PS, Flores-Gonzalez M, van de Geest H, Maumus F, Bakker LV, Schijlen E, van Haarst J, Cordewener J, Sanchez-Perez G, Peters S, Fei Z, Giovannoni JJ, Mueller LA, Saha S (2019) An improved de novo assembly and annotation of the tomato reference genome using single-molecule sequencing, Hi-C proximity ligation and optical maps. bioRxiv: 767764

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hsu C-T, Lee W-C, Cheng Y-J, Yuan Y-H, Wu F-H, Lin C-S (2021) Genome Editing and Protoplast Regeneration to Study Plant-Pathogen Interactions in the Model Plant *Nicotiana benthamiana*. *Frontiers in Genome Editing* 2: 627803

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hsu CT, Cheng YJ, Yuan YH, Hung WF, Cheng QW, Wu FH, Lee LY, Gelvin SB, Lin CS (2019) Application of Cas12a and nCas9-activation-induced cytidine deaminase for genome editing and as a non-sexual strategy to generate homozygous/multiplex edited plants in the allotetraploid genome of tobacco. *Plant Mol Biol* 101: 355-371

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Hsu CT, Yuan YH, Lin YC, Lin S, Cheng QW, Wu FH, Sheen J, Shih MC, Lin CS (2021) Efficient and Economical Targeted Insertion in Plant Genomes via Protoplast Regeneration. *CRISPR J* 4: 752-760

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Klambauer G, Schwarzbauer K, Mayr A, Clevert D-A, Mitterecker A, Bodenhofer U, Hochreiter S (2012) cn.MOPS: mixture of Poissons for discovering copy number variations in next-generation sequencing data with a low false discovery rate. *Nucleic Acids Res.* 40: e69

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Kut SA, Evans DA (1982) Plant regeneration from cultured leaf explants of eight wild tomato species and two related *Solanum* species. *In Vitro* 18: 593-598

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Li T, Yang X, Yu Y, Si X, Zhai X, Zhang H, Dong W, Gao C, Xu C (2018) Domestication of wild tomato is accelerated by genome editing. *Nat Biotechnol* 36: 1160-1163

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Liang Z, Chen K, Zhang Y, Liu J, Yin K, Qiu JL, Gao C (2018) Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in vitro transcripts or ribonucleoproteins. *Nat Protoc* 13: 413-430

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lin C-S, Kalpana K, Chang W-C, Lin N-S (2007) Improving Multiple Shoot Proliferation in Bamboo Mosaic Virus-free *Bambusa oldhamii* Munro Propagation by Liquid Culture. *HortScience* 42: 1243-1246

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, Cheng QW, Wu FH, Hsiao HC, Zhang Y, Zhang R, Chang WJ, Yu CT, Wang W, Liao LJ, Gelvin SB, Shih MC (2018) Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. *Plant Biotechnol J* 16: 1295-1310

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X, Dong Z, Liu YG (2015) A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Mol Plant* 8: 1274-1284

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, Voytas DF (2020) Plant gene editing through de novo induction of meristems. *Nat Biotechnol* 38: 84-89

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A (2010) miR390, *Arabidopsis* TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. *Plant Cell* 22: 1104-1117

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, Jouette D, Lacombe A-M, Nikic S, Picault N, Rémoúé K, Sanial M, Vo T-A, Vaucheret H (2000) *Arabidopsis* SGS2 and SGS3 Genes Are Required for Posttranscriptional Gene Silencing and Natural Virus Resistance. *Cell* 101: 533-542

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Musacchio A, Salmon ED (2007) The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* 8: 379-393

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nekrasov V, Wang C, Min J, Lanz C, Weigel D, Kamoun S (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Sci Rep* 7: 482

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nilsson E (1950) Some Experiments with Tetraploid Tomatoes. *Hereditas* 36: 181-204

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Palauqui JC, Elmayan T, Pollien JM, Vaucheret H (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J* 16: 4738-4745

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Pucker B, Kleinbolting N, Weisshaar B (2021) Large scale genomic rearrangements in selected *Arabidopsis thaliana* T-DNA lines are caused by T-DNA insertion mutagenesis. *BMC Genomics* 22: 599

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H, Nishida K, Ariizumi T, Kondo A (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nat Biotechnol* 35: 441-443

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Sikorskaite S, Rajamaki ML, Baniulis D, Stanys V, Valkonen JP (2013) Protocol: Optimised methodology for isolation of nuclei from leaves of species in the Solanaceae and Rosaceae families. *Plant Methods* 9: 31

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat Commun* 7: 13274

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Takebe I, Labib G, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Die Naturwissenschaften* 58: 318-320

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Thieme CJ, Rojas-Triana M, Stecyk E, Schudoma C, Zhang W, Yang L, Minambres M, Walther D, Schulze WX, Paz-Ares J, Scheible WR, Kragler F (2015) Endogenous *Arabidopsis* messenger RNAs transported to distant tissues. *Nat Plants* 1: 15025

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Van de Peer Y, Ashman TL, Soltis PS, Soltis DE (2021) Polyploidy: an evolutionary and ecological force in stressful times. *Plant Cell* 33: 11-26

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Verlaan MG, Hutton SF, Ibrahim RM, Kormelink R, Visser RG, Scott JW, Edwards JD, Bai Y (2013) The Tomato Yellow Leaf Curl Virus resistance genes Ty-1 and Ty-3 are allelic and code for DFDGD-class RNA-dependent RNA polymerases. *PLoS Genet* 9: e1003399

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat Biotechnol* 33: 1162-1164

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Yifhar T, Pekker I, Peled D, Friedlander G, Pistunov A, Sabban M, Wachsman G, Alvarez JP, Ansellem Z, Eshed Y (2012) Failure of the tomato trans-acting short interfering RNA program to regulate AUXIN RESPONSE FACTOR3 and ARF4 underlies the wiry leaf syndrome. *Plant Cell* 24: 3575-3589

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Yu H, Lin T, Meng X, Du H, Zhang J, Liu G, Chen M, Jing Y, Kou L, Li X, Gao Q, Liang Y, Liu X, Fan Z, Liang Y, Cheng Z, Chen M, Tian Z, Wang Y, Chu C, Zuo J, Wan J, Qian Q, Han B, Zuccolo A, Wang RA, Gao C, Liang C, Li J (2021) A route to de novo domestication of wild allotetraploid rice. *Cell* 184: 1156-1170 e1114

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Yun T, Li H, Chang P-C, Lin MF, Carroll A, McLean CY (2021) Accurate, scalable cohort variant calls using DeepVariant and GLnexus. *Bioinformatics* 36: 5582-5589

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zheng N, Li T, Dittman JD, Su J, Li R, Gassmann W, Peng D, Whitham SA, Liu S, Yang B (2020) CRISPR/Cas9-Based Gene Editing Using Egg Cell-Specific Promoters in *Arabidopsis* and Soybean. *Front Plant Sci* 11: 800

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Zsogon A, Cermak T, Naves ER, Notini MM, Edel KH, Weini S, Freschi L, Voytas DF, Kudla J, Peres LEP (2018) De novo domestication of wild tomato using genome editing. *Nat Biotechnol* 36: 1211-1216

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)