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1 **Efficient transgene-free genome editing in plants in the T0 generation based on a co-editing**
2 **strategy**

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8 **Abstract**
9 Transgene-free genome editing of plants in the T0 generation is highly desirable but challenging,
10 especially in perennials and vegetatively propagated plants. Here, we investigated the co-editing
11 strategy for generating transgene-free, gene-edited plants via *Agrobacterium*-mediated transient
12 expression of cytosine base editor (CBE)/gRNA-Cas12a/crRNA-GFP *in planta*. Specifically,
13 CBE/gRNA was used to base edit the *ALS* gene to confer resistance to herbicide chlorsulfuron as
14 a selection marker, which has no negative effects on plant phenotypes; Cas12a/crRNA was used
15 for editing genes(s) of interest; GFP was used for selecting transgene-free transformants. Using
16 this approach, transgene-free genome-edited plants were efficiently generated for various genes
17 (either individual or multiplex) in tomato, tobacco, potato, and citrus in the T0 generation. The
18 biallelic/homozygous transgene-free mutation rates for target genes among herbicide-resistant
19 transformants ranged from 8% to 50%. Whole genome sequencing further confirmed transgene-
20 free and absence of off-target mutations in the edited plants. The co-editing strategy is efficient
21 for generating transgene-free, genome-edited plants in the T0 generation, thus being a potent tool
22 for plant genetic improvement.

23 **Main**
24 Transgene-free genome editing is highly desirable for plant genetic improvement. Cas9 and
25 Cas12a DNA, mRNA or ribonucleoprotein complex (RNP) were successfully used to generate
26 transgene-free plants [1, 2], which often require the transformation of embryogenic protoplasts.
27 However, the regeneration of plants from protoplasts remains technically challenging and/or
28 limited to specific plant species/genotypes [3]. Until now, most genome-edited plants were
29 generated through *Agrobacterium*-mediated transformation and are transgenic. Genome editing
30 via transgenic approaches not only causes regulatory and public concerns [4], but also can generate
31 new and off-target mutations in the next generation[5-7]. For annual crops such as rice, it is
32 relatively easy to obtain transgene-free, gene-edited plants by genetic segregation via
33 backcrossing or selfing [8]. However, for perennials and vegetatively propagated plants, it is
34 laborious and time-consuming to remove transgenes. Many crops lose traits of the parental
35 cultivars via backcrossing, owing to their heterozygous nature as hybrids. Furthermore, in some
36 plants, such as citrus and apple, the transgene cannot be removed through seed segregation once

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37 it is integrated into the plant genome because of their asexual reproduction nature through
38 apomixis[9, 10].

39 Even though genome editing via *Agrobacterium* results in transgenic plants, most T-DNAs used
40 for carrying the Cas/gRNA do not integrate into the host chromosome, but are present in the
41 nucleus, where they will be transcribed, leading to transient expression of the carried genes[11, 12].
42 The *Agrobacterium*-mediated transient expression was used for genome editing without
43 transgene integration into plant genomes on several occasions [13-16]. The main drawback of this
44 approach identified in previous studies is that the majority of transformants are wild type, and
45 most edited plants are mosaic/chimera or heterozygous and additional generations are needed to
46 identify transgene-free and homozygous/biallelic mutants. In addition, previous genome editing
47 through transient expression of Cas/gRNA constructs is usually performed without selection
48 pressure, making it difficult, laborious and time-consuming to differentiate edited plants from
49 unedited ones[17].

50 In this study, we aimed to generate transgene-free genome-edited plants by employing T-DNA
51 carrying CBE/gRNA-Cas12a/crRNA-GFP to co-edit the *ALS* gene, which encodes acetolactate
52 synthase, and gene(s) of interest. Herbicides, such as chlorsulfuron, kills plants by acting as the
53 inhibitors of acetolactate synthase. Mutation in the *ALS* genes using CBE confers resistance to
54 herbicides such as chlorsulfuron in diverse plant species [10, 15, 18-25], thus providing a useful
55 selection marker. The gene(s) of interest can be edited via Cas12a/crRNA, whereas GFP enables
56 screening of putative transgene-free (GFP-negative) transformants. In this study, we have
57 successfully used this co-editing strategy to efficiently generate transgene-free tomato, tobacco,
58 potato, and citrus in the T0 generation for various genes. It is anticipated that this strategy will
59 have broad applications in plant genetic improvements.

60 Results

61 Transgene-free genome editing of tomato in the first generation (T0) by co-editing of the 62 *ALS* gene and gene of interest

63 To test whether we can achieve transgene-free genome editing in the T0 generation by co-editing
64 of the *ALS* gene and gene of interest, we employed the model plant tomato (*Solanum*
65 *lycopersicum*) owing to its high efficacy in plant transformation and genome editing[26],
66 availability of high-quality genome sequences[27]. We first investigated if we could obtain
67 transgene-free, gene-edited tomato in the T0 generation by base-editing *SlALS1*
68 (Solyc03g044330) alone. Previous studies suggested such a possibility, but the putative
69 transgene-free plants were not confirmed by whole genome sequencing[10, 15, 28]. Here, we
70 constructed the CBE-Cas12a-GFP-SlALS1 construct to edit the *SlALS1* gene using CBE to target
71 the proline residue at position 186 (Pro186) (Fig. 1a). Our CBE-Cas12a-GFP construct also
72 contains a GFP expression cassette for screening putative transgene-free regenerants, and the
73 highly efficient, temperature-tolerant *ttLb*Cas12a [29] for editing gene of interest in downstream
74 studies (Fig. 1a; Extended Data Figure S1). In accordance with the results reported by Veillet et
75 al. [15], base editing of *SlALS1* enabled the generation of herbicide-resistant tomato transformants

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76 (Extended Data Figure S2). More than 20 chlorsulfuron-resistant lines without green
77 fluorescence were obtained, suggesting putative transgene-free genome editing. Consistently, the
78 *GFP* gene was not detected in 5 randomly selected GFP-negative lines with PCR (Fig. 1b). The
79 targeted nucleotides of *SlALS1* gene by CBE were within the digestion site of the restriction
80 enzyme *Sty*I (Fig. 1a), which enables identification of edited sequences by digestion. Editing of
81 the targeted nucleotides completely abolished digestion by *Sty*I in one line, but partially
82 abolished the digestion in four of the five tested lines (Fig. 1c), indicating homozygous/biallelic
83 mutations in both alleles of *SlALS1* in line L2 and mutations in one allele only in the other four
84 lines (L1, L3, L4, and L5). The mutations were confirmed by Sanger sequencing (Extended Data
85 Figure S3). These results showed that editing Pro186 in either one or two *SlALS1* alleles enables
86 herbicide resistance. To further confirm if the *SlALS1*-edited *GFP*-negative plants were indeed
87 transgene-free, we conducted whole genome sequencing of the edited line 2 and confirmed the
88 homozygous mutation of the *SlALS1* gene (Extended Data Figure S4). The sequence of the CBE-
89 Cas12a-GFP-SlALS1 construct was not found in the genome of the *SlALS1*-edited, *GFP*-
90 negative tomato plant. We also analyzed potential off-target genes. A total of 20 potential off-
91 target sites with up to 4 mismatches to the target site were identified and none of them were
92 edited (Extended Data Table S1), confirming the specificity of the base editing. These results
93 show that we can obtain transgene-free, gene-edited plants in the first generation through base-
94 editing of the *ALS* gene and selecting for chlorsulfuron-resistant and *GFP*-negative plants. This
95 prompted us to further explore herbicide-assisted transgene-free genome editing in the T0
96 generation for gene(s) of interest.

97 Co-editing of the *ALS* gene and gene(s) of interest has been suggested as a feasible approach to
98 generate transgene-free plants[10, 15]. Next, we tested this hypothesis by co-editing *SlALS1* by CBE
99 and *SlER* (Solyc08g061560) [30] by Cas12a in tomato using our CBE-Cas12a-GFP construct (Fig.
100 2a, Extended Data Figure S1). A total of 12 herbicide-resistant transformants without green
101 fluorescence were selected for genotyping. Of the 12 herbicide-resistant transformants, the
102 *SlALS1* gene was edited in all lines (Extended Data Figure S5). Five of these 12 herbicide-
103 resistant plants were edited in *SlER*, with 3 being biallelic mutants and 2 being heterozygous
104 mutants (Fig. 2b, Extended Data Figure S6). Consistent with the absence of green fluorescence,
105 the 3 biallelic lines did not contain the *GFP* gene, as indicated by PCR, suggesting transgene-
106 free (Fib. 2c). The 3 biallelic lines were further confirmed to be transgene-free by the absence of
107 the *ttLbCas12a* sequence (Fib. 2c). The phenotypes of the *SlER* biallelic mutants included
108 compact architecture, short petiole, densely clustered inflorescence, and enlarged SAM (Fig. 2d,
109 e), in agreement with a previous report [30].To test the heritability of the mutation, seeds of the
110 *sler-4* T0 plant were germinated, and the resulting seedlings were genotyped. The genotyping
111 analysis found that mutation in the *SlER* gene was indeed heritable, with the progeny being either
112 homozygous (inheriting one of two edited alleles) or biallelic (the same as their parent)
113 (Extended Data Figure S7a). Additionally, PCR analysis confirmed that the seedlings did not
114 contain *ttLbCas12a* or *GFP* (Extended Data Figure S7b), which was consistent with the absence
115 of green fluorescence in the *sler-4* seeds (Extended Data Figure S8).

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116 The co-editing strategy was also successful in generating transgene-free mutant lines for *SlRBL2*
117 (Solyc09g010880) and *SlRbohD* (Solyc03g117980). We obtained 5 biallelic/homozygous
118 mutants among 12 genotyped plants for *SlRBL2* (Extended Data Figure S9a). GFP observation
119 and PCR analysis of the *GFP* gene and *Cas12a* gene (Extended Data Figure S9b) demonstrated
120 that these 5 lines were transgene-free. For co-editing of *SlRbohD* with *SlALS1*, we tested one or
121 two crRNAs (Fig. 3a, b). When one crRNA was used to target *SlRbohD*, only 1 biallelic mutant
122 was generated (line 1) (Fig. 3c) among 12 non-GFP transformants. When two crRNAs targeting
123 two different sites of *SlRbohD* were used (1 of these 2 crRNAs was the same as aforementioned),
124 25% biallelic/homozygous mutations were achieved (Fig. 3c, d, Extended Data Figure S10),
125 suggesting that two crRNAs are more effective than one as reported previously [31, 32]. GFP
126 observation and PCR analysis of the *GFP* or *Cas12a* gene revealed that 3 lines, generated using
127 either one or two crRNAs, were transgene-free (Fig. 3e).

128 **Transgene-free, multiplex genome editing of tomato in the T0 generation**

129 Next, we investigated whether we could achieve transgene-free, multiplex genome editing of
130 tomato in the first generation. We performed co-editing of *SlEDS1* (Solyc06g071280) and
131 *SlPAD4* (Solyc02g032850), with *SlALS1*. *EDS1* and *PAD4* are required for plant immunity[33-35].
132 Among 18 non-GFP regenerants, 6 lines contained biallelic/homozygous edits for both *SlEDS1*
133 and *SlPAD4*, and 2 lines were biallelically edited in only *SlEDS1* but not *SlPAD4* (Fig. 4a,
134 Supplementary Information File 1). The edited lines were transgene-free based on GFP
135 observation and PCR analysis of the *GFP* and Cas*12a* genes (Fig. 4b). Similarly, we conducted
136 multiplex gene editing of *SlDMR6* (Solyc03g080190) [36] and *SlINVINH1* (Solyc12g099200) with
137 *SlALS1*. We obtained 3 biallelic/homozygous *SlDMR6/SlINVINH1* double mutants from 17 non-
138 GFP transformants (Fig. 4c, Supplementary Information File 2). We also obtained 4
139 homozygous/biallelic *slinvinh1* single mutants (Supplementary Information File 2). These edited
140 lines were transgene-free based on GFP observation and PCR analysis of the *GFP* and *Cas12a*
141 genes (Fig. 4d). Taken together, we can achieve transgene-free, multiplex gene editing of tomato
142 in the T0 generation efficiently.

143 **Transgene-free genome editing of tobacco in the T0 generation**

144 Next, we tested whether the co-editing strategy could be used to generate transgene-free plants in
145 other plant species. We first investigated the model plant tobacco (*Nicotiana tabacum*) by co-
146 editing *NtPDS* with *NtALS* (Fig. 5a). It is noteworthy that *N. tabacum* contains two *PDS* genes,
147 *NtPDS1* and *NtPDS2*. Thus, we designed one crRNA targeting a conserved region of both genes.
148 Over 20 chlorsulfuron-resistant tobacco plants showing albino phenotype were obtained (Fig. 5a).
149 Among them, 7 albino plants did not display obvious green fluorescence (Fig. 5a). We further
150 confirmed the absence of *GFP* and *Cas12a* in three albino, non-fluorescent lines by PCR (Fig.
151 5b, Extended Data Figure S11). As expected, the chlorsulfuron-resistant tobacco plant contained
152 mutation in the *NtALS* gene, while the wild type *N. tabacum* did not contain the mutation in the
153 *NtALS* gene (Fig. 5c, Extended Data Figure S12a). Genotyping of *NtPDS* in this line confirmed

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154 editing of both *NtPDS1* and *NtPDS2* genes, which was responsible for the albino phenotype (Fig.
155 5c, Extended Data Figure S12b).

156 Transgene-free genome editing of potato in the T0 generation

157 Furthermore, we investigated the feasibility of achieving transgene-free genome editing in potato,
158 a vegetatively propagated crop with a tetraploid genome, using the co-editing strategy. We aimed
159 to co-edit *StDMR6*, a disease susceptibility gene [36, 37], together with *StALS*, with a single crRNA
160 that targets a conserved region in the first exon of four StDMR6 alleles. A total of 15 GFP-
161 negative shoots regenerated from chlorsulfuron-containing media were genotyped and 10 were
162 found to be wild-type (WT) at *StDMR6*, while 5 carried heterozygous edits. However, no tetra-
163 allelic *StDMR6* mutants were observed, even in transgenic lines. The genotyping of a
164 representative edited line revealed that it was transgene-free with 2 of the 4 *StDMR6* alleles
165 edited (Fig. 5d, e, Extended Data Figure S13). These results suggest that our strategy can
166 generate transgene-free, gene-edited potato in the T0 generation, but the generation of tetra-
167 allelic mutants needs further optimization.

168 Transgene-free genome editing of citrus in the T0 generation

169 Lastly, we aimed to achieve transgene-free genome editing of citrus in the T0 generation. Many
170 tree plants, like citrus, have a long juvenile period, which makes it challenging to remove foreign
171 DNA fragments when transgenic approaches are used for genome editing. We previously
172 succeeded in obtaining transgene-free *ALS*-edited citrus through transient expression of CBE[10].
173 Here, we co-edited the TAL Effector Binding Element (EBE) region in the promoter of the citrus
174 canker susceptibility gene *LOB1*[38-41] with citrus *ALS* using our PBE-Cas12a-GFP-LOBP
175 construct (Extended Data Figure S14). We utilized nCas9-PBE, a variant of CBE that has a
176 unique 5 nucleotide editing window, resulting in targeting the proline residue at position 188
177 (Pro188) of *CsALS* only, which is equivalent to the proline residue at position 186 (Pro186) of
178 *SlALS* [42]. In the presence of chlorsulfuron, 107 pummelo (*Citrus maxima*) shoots were generated.
179 Among them, 4 shoots were GFP-positive (Fig. 6a), and 103 shoots were GFP-negative (Fig. 6a).
180 Based on genotyping of the *CsALS* and EBE_{PthA4}-LOBP, and PCR analysis of the *nptII* gene (Fig.
181 6b), four transgene-free, EBE_{PthA4}-LOBP-edited citrus lines were generated and subjected to
182 downstream analyses. For the *CsALS* site, the four transgenic genome-edited and four transgene-
183 free genome-edited lines contained homozygous/biallelic mutations (Extended Data Figures. S15
184 and S16). Intriguingly, among the four transgenic citrus genome-edited lines, for the EBE_{PthA4}-
185 LOBP site, $Pum_{GFP}1$, $Pum_{GFP}2$ and $Pum_{GFP}4$ were chimeric, but without wild type sequences,
186 and $Pum_{GFP}3$ was wild type (Extended Data Fig. S15). Among the four transgene-free genome-
187 edited lines, for the EBE_{PthA4}-LOBP site, $Pum_{NoGFP}1$, $Pum_{NoGFP}2$, $Pum_{NoGFP}3$ and $Pum_{NoGFP}4$
188 contained biallelic, heterozygous, homozygous, and heterozygous mutations, respectively (Fig.
189 6c, Extended Data Fig. S16). As expected, biallelic/homozygous mutants and chimeric mutants
190 without wild type sequence in the EBE_{PthA4}-LOBP site demonstrated canker resistance and did
191 not show any canker symptoms after inoculation with *Xcc*, regardless of being transgenic or
192 transgene-free (Fig. 6d). Wild type pummelo showed typical canker symptoms, such as

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193 hyperplasia and hypertrophy (Fig. 6d). As a positive control, we inoculated wild type and
194 genome-edited lines with XccΔpthA4:dLOB1.5. dLOB1.5 is a designed TALE, which binds to a
195 different region from the target EBE_{PthA4}-LOBP site in the promoter region of *CsLOB1*, thus
196 activating *LOB1* expression to cause canker symptoms [41]. Sanger sequencing results indicated
197 that the dLOB1.5 binding sites were intact among the tested Pummelo plants (Extended Data Fig.
198 S17). Consequently, XccΔpthA4:dLOB1.5 caused typical canker symptoms in wild type and all
199 EBE_{PthA4}-LOBP-edited lines (Fig. 6d). Taken together, the mutation of EBE_{PthA4}-LOBP
200 conferred Pummelo canker resistance, consistent with previous studies [39, 41, 43]. Importantly, two
201 transgene-free plants, $Pum_{NoGFP}1$ and $Pum_{NoGFP}3$, were resistant to *Xcc* infection (Fig. 6).

202 **Whole genome sequencing analysis of edited lines confirms transgene-free genome editing**
203 **without off-target mutations**

204 To further confirm whether the putative transgene-free genome-edited lines were indeed
205 transgene-free, we conducted whole genome sequencing. For tomato, we sequenced six
206 transgene-free lines that were edited for *SlRbohD* (#1, #2, #8), *SlER* (#4), *SlEDS1/SlPAD4* (#8,
207 #18), as well as two transgenic control lines, EPGFP and *SlRbohD* (#6) (Extended Data Table
208 S2). The sequencing coverage ranged from 28 × to 59 ×. Genomic analysis confirmed that the
209 construct DNA was integrated into the genome of the transgenic control lines EPGFP and
210 *SlRbohD* (#6) (Extended Data Table S2), as evidenced by the presence of construct reads
211 (Extended Data Figure S18, Extended Data Table S2). In contrast, genomic analysis of the
212 *SlRbohD* (#1, #2, #8), *SlER* (#4), *SlEDS1/SlPAD4* (#18) lines found no construct DNA
213 (Extended Data Table S2). Intriguingly, *SlEDS1/SlPAD4* line 8 contained 281 reads matching
214 construct sequences despite being GFP-negative and PCR-negative for GFP and Cas12a (Fig.
215 4b). Genomic analysis of the edited lines confirmed genome editing, as demonstrated by Sanger
216 sequencing results. For instance, *SlRbohD* edited line #1 contained biallelic mutations of -4/-8,
217 whereas #8 contained homozygous mutations of 7 bp deletion at *SlRbohD* (Extended Data
218 Figures. S19-S20), consistent with previous Sanger sequencing results (Fig. 3c, Extended Data
219 Figure S10). We searched for potential off-target sites of the crRNA targeting *SlRbohD*, *SlER*,
220 *SlEDS1*, *SlPAD4*, *SlINVINH1*, and *SlRBL2* genes using the CRISPR P v2.0 program and Cas-
221 OFFinder program. Whole genome sequencing analyses or Sanger sequencing of PCR amplicons
222 of the homologous sites showed no off-target mutations (Extended Data Table S3). Similarly,
223 whole genome sequencing analysis of the GFP-negative, *LOB1*-edited citrus line $Pum_{NoGFP}3$
224 (Extended Data Table S2) found no construct DNA in its genome. Furthermore, whole genome
225 sequencing analysis indicated that $Pum_{NoGFP}3$ harbored heterozygous *CsALS* and homozygous
226 mutant EBE_{PthA4}-LOBP, which was consistent with Sanger sequencing results (Fig. 6c). In
227 addition, off-targets were analyzed in $Pum_{NoGFP}3$ based on whole genome sequencing data. In the
228 case of mismatch number<=5 for crRNA, there were eight potential off-targets (Extended Data
229 Figure. S21). The off-target sites were visualized using IGV software version 2.15.4 (Robinson
230 et al., 2011), and no off-target mutations were identified.

231 **Discussion**

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232 In this study, we have developed a potent genome editing toolkit to generate transgene-free
233 genome-edited plants in the T0 generation by co-editing the *ALS* gene and gene(s) of interest
234 through *Agrobacterium*-mediated transient expression. It was successfully used for genome
235 editing of tomato and tobacco (annuals), citrus (a perennial tree crop), and potato (a vegetatively
236 propagated tetraploid crop). The biallelic/homozygous mutation rates for target genes among
237 herbicide-resistant transformants in our study ranged from 8% to 50%, which is comparable to
238 the genome editing efficacy using Cas/gRNA DNA, mRNA, or ribonucleoproteins [1, 2]. The
239 efficient identification of biallelic/homozygous mutants resulted from co-editing of the *ALS* gene,
240 which provides a useful and practical selection marker as a gain-of-function against sulfonylurea
241 herbicides. Importantly, precise editing by CBE targets the proline residue, for instance, Pro186
242 in tomato or Pro188 in citrus, which probably disrupts the recognition and binding of the
243 herbicides without affecting ALS function [44]. Consistently, *ALS* mutants at the proline residue
244 did not show any phenotypical changes in our study. Natural mutations of the *ALS* gene are
245 prevalent in plant species without affecting plant phenotypes (except herbicide resistance) and
246 fitness [45-49]. Thus, editing of the *ALS* gene as a selection marker will not negatively affect genetic
247 improvement of crops and their commercialization.

248 The co-editing strategy has multiple advantages in generating transgene-free genome-edited
249 plants: 1) The co-editing strategy ensures transgene-free genome editing by transient expression
250 of Cas/gRNA and removing of stable transformants using GFP as an indicator. Transiently
251 expressed Cas/gRNA eventually degrades, thus mimicking the approaches that use Cas/gRNA
252 DNA in transgene-free genome editing via transformation of protoplasts[50]. Genotyping of
253 construct components such as GPF, Cas12a, or *nptII* genes and whole genome sequencing of
254 putative transgene-free lines indeed confirm the absence of foreign genes in 87.5% (7 of 8)
255 putative transgene-free lines. Intriguingly, the *sleds1/slpad4* line 8 contained 281 reads matching
256 construct sequences whereas the two transgenic lines contained 1830 and 2716 construct reads.
257 This is consistent with previous reports that small DNA fragments from transformed plasmids
258 are sometimes inserted at both on-target and off-target sites in host cells, even though at low
259 frequencies [51]. Consequently, whole genome sequencing is required to verify the edited lines to
260 be transgene-free. "Transgene-free" is a prerequisite for commercialization of genetically
261 modified organisms. Transgenic crops are under robust and strict regulations in different
262 countries and regions, [4] and cause negative public reception, which impedes their
263 commercialization despite superior traits. 2) Transgene-free lines without T-DNA eliminate the
264 potential disruption of gene functions at the insertion site caused by T-DNA in *Agrobacterium*-
265 mediated stable transformation [52]. Thus, this approach is not only suitable for crop genetic
266 improvements, but also provides advantages in genetic studies. 3) Off-target mutations are
267 another critical factor for consideration during genetic improvement by genome editing. No off-
268 target mutations were identified in our genome-edited lines. This probably results from the short
269 functional time of Cas/gRNA during transient expression, as suggested by previous studies [10, 53].
270 Similarly, transient expression of Cas/gRNA DNA, mRNA, and RNP in embryogenic protoplasts,
271 calli, or immature embryo cells has been reported to generate transgene-free plants without

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272 causing off-target mutations[1, 2, 54]. 4) This co-editing strategy can produce transgene-free, gene-
273 edited plants in the T0 generation. Generation of transgene-free genome-edited plants in the T0
274 generation bypasses the need to remove transgenes in future generations by genetic separation
275 via backcrossing or selfing. The removal of transgenes is not feasible for many crops which are
276 asexually propagated, or highly heterozygous, or have long juvenility, such as grape, citrus,
277 potato and banana. Generation of transgene-free plants in the T0 generation significantly
278 expedites the genetic improvement of crops. For example, transgene-free citrus was generated
279 within 6 months using our approach. However, it takes approximately 20 years to generate new
280 citrus varieties using traditional breeding approach [55]. Lastly, our strategy is based on
281 *Agrobacterium*-mediated delivery of CRISPR components into recipient plant tissues such as
282 cotyledons, leaves, and epicotyls. Hence, it can be easily adopted because *Agrobacterium*-
283 mediated transformation is one of the most widely used and convenient methods. Transformation
284 of embryogenic protoplasts with Cas/gRNA RNP[56], or DNA[50] has successfully generated
285 transgene-free, genome-edited plants. However, plant regeneration from protoplasts is
286 technically challenging. Noticeably, the regenerated plants from protoplasts are prone to
287 somaclonal variations and genome instability[57, 58]. In addition, regeneration from protoplasts is
288 not accessible to many plant species, especially monocots. Another method to generate
289 transgene-free edits is to transiently deliver plasmids, mRNA, or RNPs directly into callus cells
290 or immature embryos through biolistic particle bombardment[3, 59, 60]. However, due to low
291 efficiency and no selection, a huge amount of work must be done on tissue culture, regeneration,
292 genotyping, and selection of edited plants from unedited plants. It is not surprising that the co-
293 editing strategy may also aid in the enrichment and selection of edited protoplasts, callus cells or
294 immature embryos achieved through means other than *Agrobacterium*.

295 In sum, we have developed an efficient transgene-free genome editing methodology based on
296 *Agrobacterium*-mediated transient expression for plants. As *Agrobacterium*-mediated
297 transformation works for many plant species, we anticipate that this approach has broad
298 applications in genetic improvements and genetic studies of plants. It is particularly useful for
299 perennials and vegetatively propagated plants to generate transgene-free, gene-edited plants in
300 the T0 generation.

301 **Methods**

302 **Making the CBE-Cas12a-GFP construct**

303 The CBE plasmid with GFP [10] was digested with *Pme*I/*Rsr*II and the vector backbone was
304 retained. The Cas12a-D156R[29] fragment was recovered by digesting Hybrid-D156R-PDS-
305 LOB1-A containing tt*Lb*Cas12a[29] with *Pme*I/*Rsr*II. These two fragments were then ligated to
306 form CBE-Cas12a-partial. CBE-Cas12a-partial was then digested with *Rsr*II. The other half of
307 Cas12a was PCR-amplified using primers Cas12half-F1/Cas12half-R1 (Extended Data Table S4)
308 with tt*Lb*Cas12a[29] as the template. The amplicon was then In-fusion cloned with the *Rsr*II-
309 digested CBE-Cas12a-partial to create CBE-Cas12a-GFP. The final construct was confirmed
310 through Sanger sequencing.

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311 **Making CBE-gRNA-Cas12a-crRNA-GFP constructs**

312 CBE-Cas12a-GFP was digested with *Aar*I and ligated with annealed primers for *NtALS* or
313 *CsNLS* or *SlALS1* or *StALS* (Extended Data Table S4) with compatible ends. A construct named
314 PUC57-mini-crRNA was synthesized to drive crRNA expression (Extended Data Fig. S22). The
315 crRNA array is flanked by ribozymes, Hammerhead (HH) and hepatitis delta virus (HDV), for
316 precise processing[61]. Primers for single crRNA were annealed and ligated to *Bsm*BI-digested
317 PUC57-mini-crRNA. For multiplexing, multiple HH-DR-HDV units were PCR amplified from
318 the synthesized plasmid PUC57-HDV-HH-DR (Extended Data Fig. S22). The PCR products
319 were ligated and cloned into *Bsm*BI-digested PUC57-mini-crRNA through GoldenGate cloning.
320 The whole crRNA cassette, including the promoter and terminator, was PCR amplified using
321 primers Mini-F1/Mini-R1 and cloned into the *Sbf*I-digested CBE-Cas12a-GFP-ALS constructs
322 using In-Fusion cloning (Takara Bio). All constructs were confirmed by sequencing.

323 **Making the GFP-p1380N-ttLbCas12a:LOBP1-PBE:ALS construct**

324 Using pUC-NosT-crRNA:LOBP as template[43] , the fragment containing AtU6-26 promoter, the
325 coding sequence of hammerhead ribozyme (HH) and crRANA-LOBP1 was PCR-amplified using
326 primers AtU6-5-*Xho*I (5´-AGGTCTCGAGTCGTTGAACAACGGAAACTCGA CTTGCC-3´)
327 and CrRNA-LBDP1-phos (5´-phosphorylated- AAGGCAAAAGGGGTTTATAT
328 AGAATCTACACTTAGTAGAAATTAga -3´), and the fragment containing the coding
329 sequence of hepatitis delta virus ribozyme (HDV) and NosT was PCR-amplified using primers
330 HDV-5-Phos (5´-phosphorylated- GGCCGGCATGGTCCCAGCCTCCTCGCT - 3´) and NosT-
331 3-*Asc*I (5´-ACCTGGGCCCGGCGCGCCGATCTAGTAACATAGATGA-3´). *Xho*I-cut AtU6-
332 26-HH-crRNA-LOBP1 and *Asc*I-cut HDV-NosT were inserted into *Xho*I-*Asc*I-cut pUC-NosT-
333 MCS to build pUC-NosT-crRNA:LOBP1 through three-way ligation, in which the vector and
334 two DNA fragments were ligated together in one step. pUC-NosT-MCS contained *Eco*RI-NosT-
335 *Xho*I-*Asc*I-*Xba*I-*Pme*I for cloning, as described before [62]. Subsequently, the *Eco*RI-NosT-
336 crRNA:LOBP1-NosT-*Asc*I-*Xba*I-*Pme*I fragment was cloned into *Eco*RI-*Pme*I-cut GFP-p1380N-
337 ttLbCas12a to generate GFP-p1380N- ttLbCas12a:LOBP1-*Asc*I-*Xba*I-*Pme*I (Extended Data
338 Figure S14). GFP-p1380N-ttLbCas12a was constructed previously [63].

339 From vector CmYLCV-A3A-RAD51-nCas9 [10], the CmYLCV promoter was amplified using
340 primer CmYLCV-5-*Hin*dIII-*Sbf*I-*Asc*I (5′-AGGTAAGCTTCCTGCAGGCGCG
341 CCAGATTTGCCTTTTCAATTTCAGAAAGA-3′) and CmYLCV-3-*Bam*HI (5´-AGGTGGAT
342 CCAGCTTAGCTCTTACCTGTTTTCGTCGT-3′). *Hin*dIII-*Bam*HI-cut CmYLCV was cloned
343 into *Hin*dIII-*Bam*HI-cut pnCas9-PBE vector from Addgene (Addgene plasmid #98164) to build
344 pCmYLCV-nCas9-PBE. To produce GFP-p1380N-CmYLCV-nCas9-PBE, the *Sbf*I-*Eco*RI-cut
345 CmYLCV-nCas9-PBE fragment was ligated with the *Sbf*I-*Eco*RI-cut GFP-p1380N-Cas9
346 construct [40]

347 From 35S-SpCas9p:DunLOBP [43], the AtU6-26 promoter was amplified again using AtU6-26-5-
348 *Xho*I and AtU6-26-3-phos (5′-phosphorylated-AATCACTACTTCGACTCTAGCTGT-3′), and
349 the sgRNA-ALSBE-NosT was PCR-amplified using sgRNA-ALSBE-P (5′-phosphorylated-

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350 GcaggtcccgcggaggatgatGTTTTAGAGCTAGAAATAGCAAGT-3′) and NosT-3-*Spe*I. Through
351 three-way ligation, *Xho*I-cut AtU6-26 and *Spe*I-digested sgRNA-ALSBE-NosT were inserted
352 into *Xho*I-*Xba*I-treated pUC-NosT-MCS to construct pUC-NosT-AtU6-26-sgRNA-ALSBE.
353 Finally, the *Eco*RI-NosT-AtU6-26-sgRNA-ALSBE-NosT-*Pme*I fragment from pUC-NosT-
354 AtU6-26-sgRNA-ALSBE were cloned into *Eco*RI-*Pme*I-cut GFP-p1380N-CmYLCV-nCas9-
355 PBE to build GFP-p1380N-PBE:ALS (Extended Data Figure S14). The *Asc*I-*Pme*I-cut
356 CmYLCV-nCas9-PBE:ALS fragment from GFP-p1380N-PBE:ALS was clone into *Asc*I-*Pme*I-
357 cut vector GFP-p1380N-ttLbCas12a:LOBP1-*Asc*I-*Xba*I-*Pme*I to form GFP-p1380N-
358 ttLbCas12a:LOBP1-PBE:ALS (Extended Data Figure S14). All constructs were confirmed by
359 sequencing.

360 **Plant transformation**

361 The final constructs were transformed into either the *Agrobacterium* strain AGL1 (for tomato
362 and potato) or EHA105 (for tobacco and citrus). For tomato transformation (cultivar
363 Moneymaker), we followed the described protocol [64] with modifications. After co-cultivating the
364 cotyledon explants on co-cultivation medium for three days, the explants were subcultured on a
365 Murashige and Skoog (MS) regeneration medium with 2 mg/L zeatin riboside and 350 mg/L
366 Timentin (for Agrobacterium elimination) at 30°C for 6 to 10 days. The explants were then
367 subcultured on the same regeneration medium with 2 mg/L zeatin riboside, 350 mg/L Timentin,
368 and 110 nM herbicide chlorsulfuron to select chlorsulfuron-resistant calli and shoots. The calli
369 showing green fluorescence were discarded, while the non-fluorescent calli were kept as
370 potential transgene-free, gene-edited transformants for further culture on chlorsulfuron-
371 containing regeneration media. A similar protocol was used for tobacco transformation, using
372 young sterile tobacco leaf discs as explants, and a regeneration medium containing 1 mg/L 6-
373 Benzylaminopurine (BAP), 0.1 mg/L Naphthalene acetic acid (NAA), 350 mg/L Timentin, and
374 250 nM herbicide chlorsulfuron. For potato transformation, the tetraploid cultivar Atlanta
375 plantlets were purchased from the University of Wisconsin and the University of Idaho. A
376 similar protocol was used for potato transformation. Potato leaves were used as explants for
377 transformation. The regeneration medium for potato transformation contains 1 mg/L Zeatin, 2
378 mg/L Gibberellic acid (GA), 350 mg/L Timentin, 100 nM herbicide chlorsulfuron. For citrus
379 transformation, we followed the protocol we developed previously[10]. Plants were grown at room
380 temperature (22 °C - 25 °C) with a 16-hour light/8-hour dark cycle. After rooting, the plants
381 were transferred to a glasshouse.

382 **Canker symptom assay in citrus**

383 Wild type, transgenic and transgene-free Pummelo plants were grown in a greenhouse at the
384 Citrus Research and Education Center, University of Florida. Prior to *Xcc* treatment, all plants
385 were trimmed to generate new shoots. Leaves of similar age were infiltrated with either *Xcc* or
386 *Xcc*ΔpthA4:dLOB1.5 (5×10^8 CFU/mL) using needleless syringes. At five days post inoculation
387 (DPI), canker symptoms were observed and photographed.

388 **Microscopy analysis**

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389 An Omax camera was installed to a Zeiss Stemi SV11 dissecting microscope for photographing
390 GFP fluorescence. Under illumination of the Stereo Microscope Fluorescence Adapter
391 (NIGHTSEA), GFP fluorescence was visualized. Subsequently, the samples were photographed
392 with the Omax Toupview software connected to the Omax camera.

393 **Genomic DNA extraction and genotyping**

394 Genomic DNA was extracted from plant leaves with a cetyltrimethylammonium bromide
395 (CTAB)-based genomic DNA extraction protocol we described previously[39, 65]. Detection of
396 edits in the target genes was performed via PCR amplification of fragments spanning gRNAs or
397 crRNAs, followed by cloning of PCR products into a cloning vector (Zero Blunt™ TOPO™
398 PCR Cloning Kit, Invitrogen) and Sanger sequencing. At least 10 clones for each gene from each
399 plant were subjected to Sanger sequencing. Primers were designed for the detection of *GFP*
400 fragment near the T-DNA right border, and *Cas12a* fragment near the T-DNA left border in the
401 edited plant lines.

402 **Whole genome sequencing and data analysis**

403 The 150-bp paired-end reads whole genome sequencing data were generated using the Illumina
404 NovaSeq 6000 platform by Novogene. The raw reads were filtered using Fastp version 0.22.0 to
405 remove low-quality reads. On average, more than 20.6 and 48.4Gb of high-quality data were
406 generated for each citrus Pummelo and tomato plant sample, respectively. The high-quality
407 paired-end short genomic reads were mapped to the reference genomes of citrus Pummelo (C.
408 maxima) or tomato using Bowtie2 software version 2.2.6 [66]. The mutations (single nucleotide
409 polymorphisms, deletions, and insertions) in the gene-edited plant genomes were generated using
410 the SAMtools package version 1.2 [67] and Deepvariant program version 1.4.0 [68]. The mutations
411 were filtered based on quality and sequence depth, and the target site mutations were visualized
412 using IGV software version 2.15.4 [69]. The off-target sites were predicted using CRISPR-P 2.0 [70]
413 and the Cas-OFFinder program [71] and aligning target sequence with whole genome using blast
414 program. Based on the mapping results, mutations of off-target sites were detected using the
415 SAMtools package version 1.2 and deepvariant program version 1.4.0.

416 **Figure legends**

417 **Fig. 1. Establishment of herbicide-assisted transgene-free genome editing system. a,** The
418 CBE-Cas12a-GFP-SlALS1 construct used in the generation of transgene-free, *SlALS1*-edited
419 tomato. The gRNA for *SlALS1* is boxed. The targeted nucleotides (CC) are highlighted in yellow.
420 CsVMV, *Cassava vein mosaic virus* promoter; U6, citrus U6 promoter; CmYLCV, *Cestrum*
421 *yellow leaf curling virus* promoter; CBE, cytosine base editor; T, terminator. For GFP, Nos
422 terminator; for SlALS gRNA, poly (T) terminator; for CBE and Cas12a, HSP 18.2 terminator.
423 RB, T-DNA right border; LB, T-DNA left border. **b,** PCR amplification of *GFP* in the
424 regenerated chlorsulfuron-resistant tomato lines with or without green fluorescence. **c,** *SlALS1*
425 gene genotyping of chlorsulfuron-resistant tomato regenerants through restriction enzyme
426 digestion of PCR amplicons with *Sty*I. PCR amplicons spanning the *SlALS1* gRNA region were

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427 subjected to restriction enzyme digestion with *Sty*I. Editing of the targeted nucleotides abolishes
428 the *Sty*I recognition site, resulting in resistance to *Sty*I digestion. Bottom text: *SlALS1* genotypes
429 in the edited lines were confirmed by Sanger sequencing.

430 **Fig. 2. Transgene-free gene editing in the first generation (T0) in tomato. a,** CBE-Cas12a-
431 GFP-SlALS1-SlER construct used in the generation of transgene-free, *SlALS1*-edited, *SlER*-
432 edited tomato. CsVMV, *Cassava vein mosaic virus* promoter; U6, U6 promoter; CmYLCV,
433 *Cestrum yellow leaf curling virus* promoter; HH, ribozyme Hammerhead; DR, direct repeat;
434 HDV, ribozyme hepatitis delta virus, CBE, cytosine base editor; T, terminator. For GFP, Nos
435 terminator; for *SlALS* gRNA, poly (T) terminator; for *SlER* crRNA, poly (T) terminator followed
436 by HSP 18.2 terminator; for CBE and Cas12a, HSP 18.2 terminator. RB, T-DNA right border;
437 LB, T-DNA left border. **b,** The *SlER* genotypes of the edited lines without green fluorescence. **c,**
438 PCR amplification of *GFP* and *Cas12a* from the biallelic mutants in b. **d, e,** Phenotypes of a
439 representative transgene-free, *SlER*-edited line *sler-4*.

440 **Fig. 3. Efficient transgene-free gene editing of tomato in the T0 generation with 2 crRNAs.**
441 **a,** Construct scheme showing 1 crRNA targeting *SlRbohD*. Other parts of the construct are not
442 shown. **b,** Construct scheme showing 2 crRNAs targeting *SlRbohD*. **c,** The *SlRbohD* genotypes
443 of the transgene-free, homozygous/biallelic edited lines without green fluorescence. **d,**
444 Comparison of rate of transgene-free, homozygous/biallelic mutants using 1 crRNA and 2
445 crRNAs. **e,** PCR amplification of *GFP* and *Cas12a* in the lines shown in c.

446 **Fig. 4. Transgene-free, multiplex gene editing of tomato in the first generation. a,**
447 Generation of transgene-free, biallelic/homozygous double mutants of tomato for *SlEDS1* and
448 *SlPAD4*. **b,** PCR amplification of *GFP* and *Cas12a* from the edited *sleds1/slpad4* mutant lines
449 from a. **c,** Generation of transgene-free, biallelic/homozygous double mutants for *SlDMR6* and
450 *SlINVINH1*. **d,** PCR amplification of *GFP* and *Cas12a* from the edited *sldmr6/slinvinh1* mutant
451 lines from c.

452 **Fig.5: Transgene-free gene editing in the first generation (T0) in tobacco and potato. a-c,**
453 Co-editing of *NtALS* and *NtPDS* in *Nicotiana tabacum*. **a**, Albino phenotype with or without
454 green fluorescence. Regenerants were selected on herbicide chlorsulfuron-containing media.
455 Upper: transgenic albino tobacco plant; lower: transgene-free albino tobacco plant. **b,**
456 Confirmation of transgene-free gene editing. PCR amplification of *GFP* and *Cas12a* in WT,
457 non-transgenic (NT), and transgenic (T) plants. **c,** Genotypes of *NtALS*, *NtPDS* genes in a
458 transgene-free, albino tobacco line from **a**. d & e, Transgene-free gene editing in potato**. d,** PCR
459 amplification of *GFP* and *Cas12a* from a regenerated potato line 9 and control transgenic plant. **e,**
460 Genotype of line 9 at *StDMR6*. crRNAs are underlined. 1 crRNA was used for *StDMR6* editing.

461 **Fig. 6: Transgene-free gene editing in the first generation (T0) in pummelo (*Citrus maxima*).**
462 **a,** GFP fluorescence was observed in transgenic Pummelo plants, whereas wild type and
463 transgene-free plants did not exhibit any GFP signal. **b**, Using a pair of primers Npt-Seq-5 and
464 35T-3PCR, wild type, transgenic and transgene-free Pummelo plants were analyzed. The wild
465 type Pummelo and plasmid GFP-p1380N-ttLbCas12a:LOBP1-EBE:ALS were used as controls.

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466 M, 1kb DNA ladder. **c**, Sanger sequencing analysis of GFP-negative lines by PCR amplification
467 and cloning of *LOB1* promoter. **d**, Canker-resistance in the transgenic and transgene-free
468 Pummelo plants. Five days post Xanthomonas citri subsp. citri (Xcc) inoculation, citrus canker
469 symptoms were observed on wild type Pummelo, transgenic $Pum_{GFP}3$, transgene-free $Pum_{NoGFP}1$
470 and $Pum_{NoGFP}3$, whereas no canker symptoms were observed on other LOBP-edited Pummelo
471 plants, which could be attributed to 100% mutation rates in $Pum_{GFP}1$, $Pum_{GFP}2$, $Pum_{GFP}4$,
472 $Pum_{NoGFP}2$ and $Pum_{NoGFP}4$. As expected, *XccpthA4:Tn5*(dCsLOB1.5) caused canker symptoms
473 on all plants. dCsLOB1.5 induces *LOB1* to cause canker symptoms by recognizing a different
474 region from EBE_{PthA4}-TII LOBP. GFP-positive lines: $Pum_{GFP}1$ to $Pum_{GFP}4$. GFP-negative lines:
475 $Pum_{NoGFP}1$ to $Pum_{NoGFP}4$.

476 **Data availability**

477 The raw reads of genome resequencing for pumelo plants were deposited in the NCBI Bioproject
478 database under the accession number PRJNA931434. The reference genome of pumelo was
479 downloaded from public citrus genome database CPBD: Citrus Pan-genome to Breeding
480 Database (http://citrus.hzau.edu.cn/index.php). The raw reads of genome resequencing for
481 tomato plants were deposited in the NCBI Bioproject database under the accession number
482 PRJNA931572. The reference genome of tomato was downloaded from public tomato genome
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485

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648 **Author Contributions**

649 X.H., H.J. and N.W. conceptualized and designed the experiments. X.H., H.J., and Y.W.
650 performed the experiments. J.X. and J.W. performed bioinformatics. X.H., H.J. and N.W. wrote
651 the manuscript with input from all co-authors.

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652 **Competing interests.** N. W., H. J. and X. H. filed a PCT patent application based on the results
653 reported in this paper. All other authors declare no competing financial interests.

654 **Correspondence and requests** for materials should be addressed to N. Wang.

655

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Fig. 1

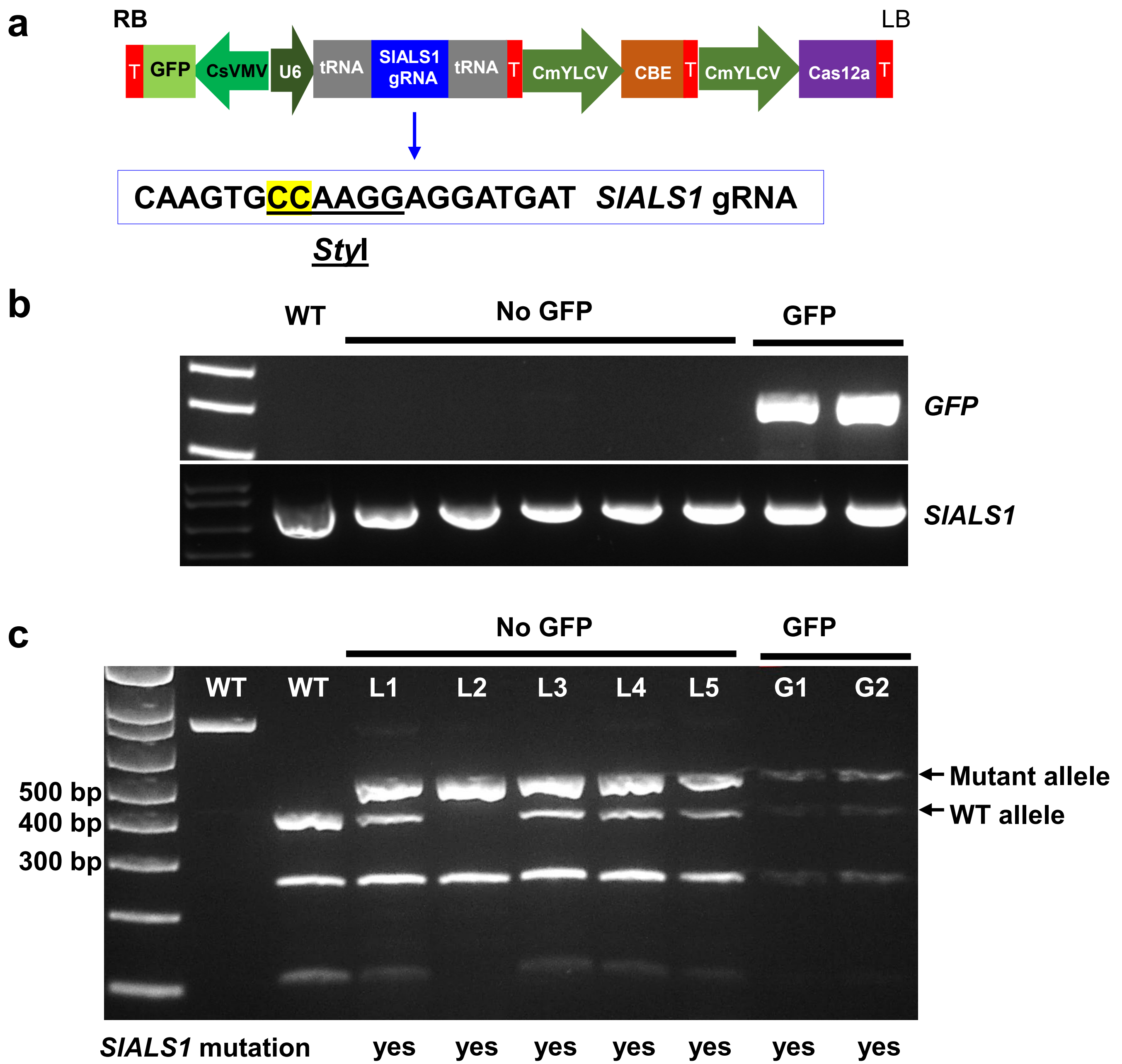

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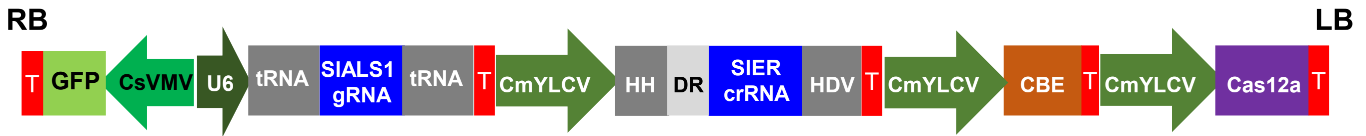
Fig. 2

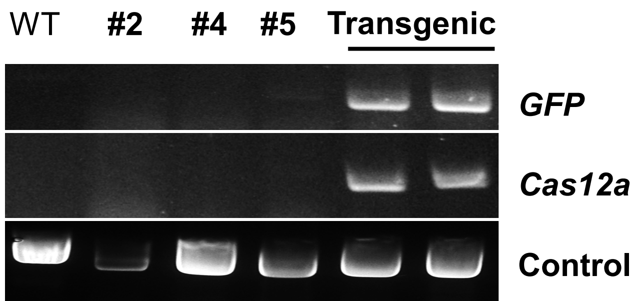
a



b

```
AGCTTTGGTTCTGTGGTGTCTGATGATGGTGAGTAGAGTAGAGTAG SlER WT

AGCTTTGGTTCTGTGGTGT-----------GAGTAGAGTAGAGTAG allele 1, -11 bp   Line 2   biallelic
AGCTTTGGTTCTGTGGT-------------GAGTAGAGTAGAGTAG allele 2, -13 bp

AGCTTTGGTTCTGTGGTGTCTG-------TGAGTAGAGTAGAGTAG allele 1, -7 bp    Line 4   biallelic
AGCTTTGGTTCTGTGGTGTCTGATGA-----------GTAGAGTAG allele 2, -11 bp

AGCTTTGGTTCTGTGGTGTCTG--------------AGTAGAGTAG allele 1, -14 bp   Line 5   biallelic
AGCTTTGGTTCTGTGGTGTCTG-----------------AGAGTAG allele 2, -17 bp

AGCTTTGGTTCTGTGGTGTCTGATGATGGTGAGTAGAGTAGAGTAG allele 1, WT       Line 9   heterozygous
AGCTTTGGTTCTGTGGTGTCTGATGA------GTAGAGTAGAGTAG allele 2, -6 bp

AGCTTTGGTTCTGTGGTGTCTGATGATGGTGAGTAGAGTAGAGTAG allele 1, WT       Line 10  heterozygous
AGCTTTGGTTCTGTGGTGTCTGATGATGG---GTAGAGTAGAGTAG allele 2, -3 bp
```

c



d

e

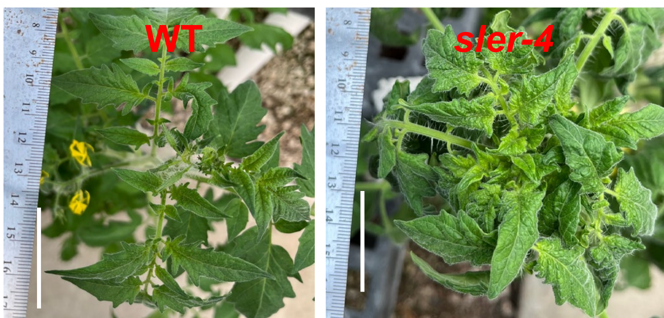


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Fig. 3

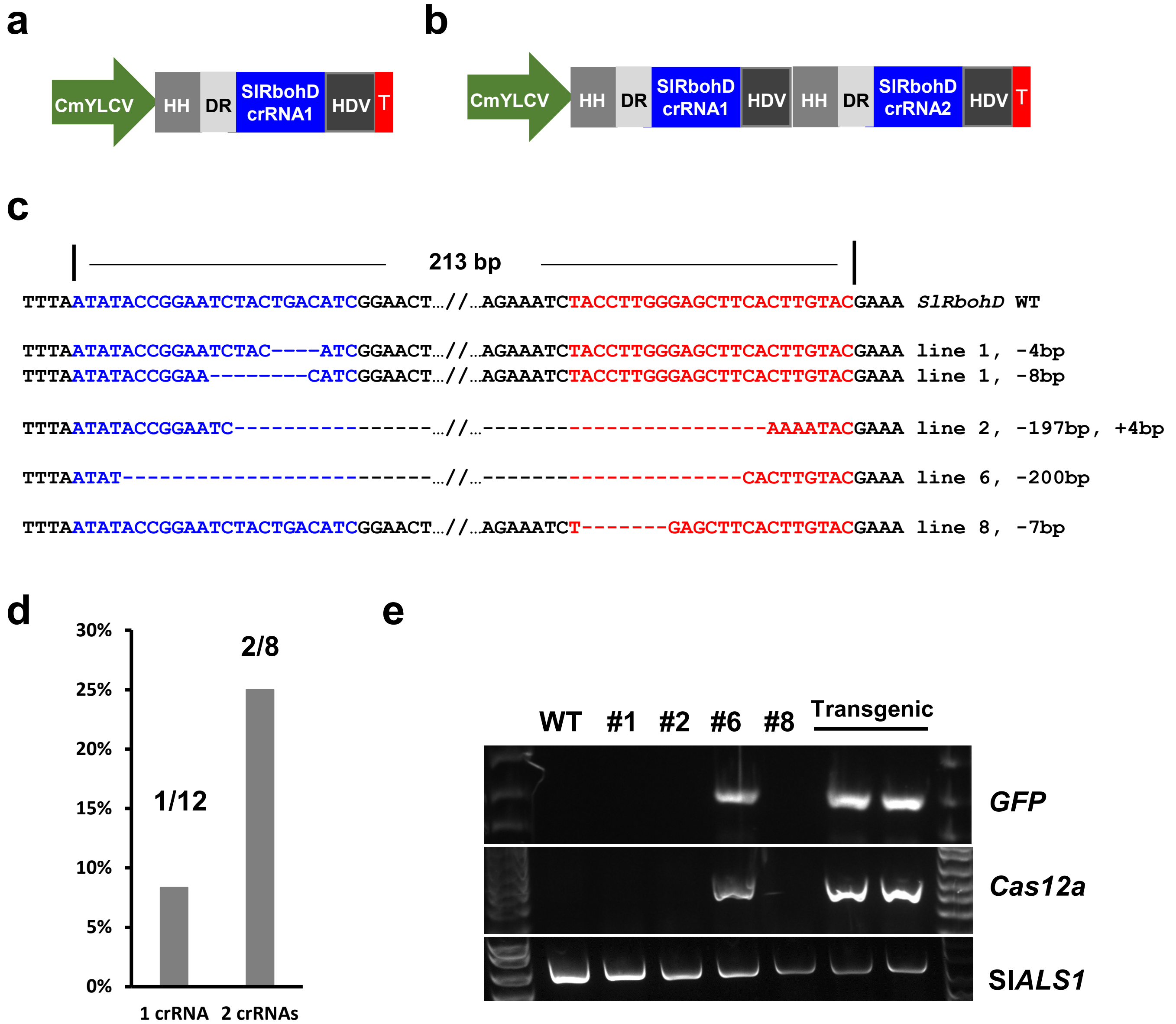

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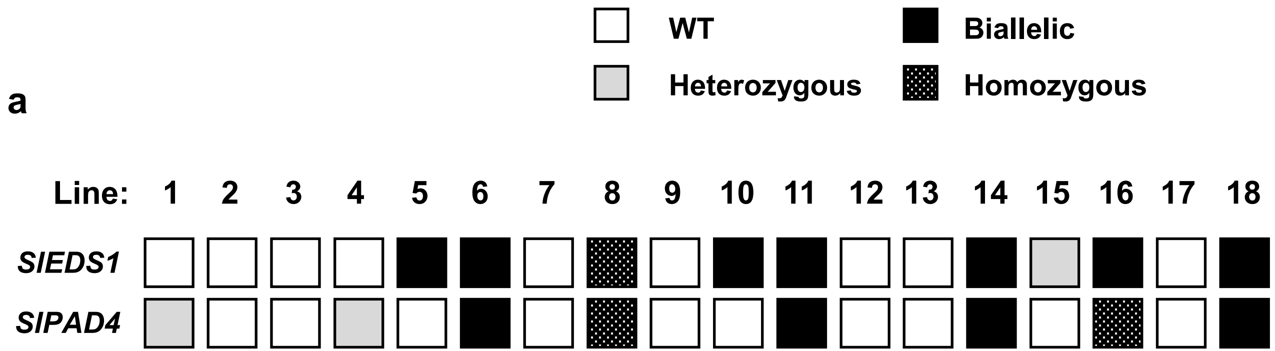
Fig.4

a



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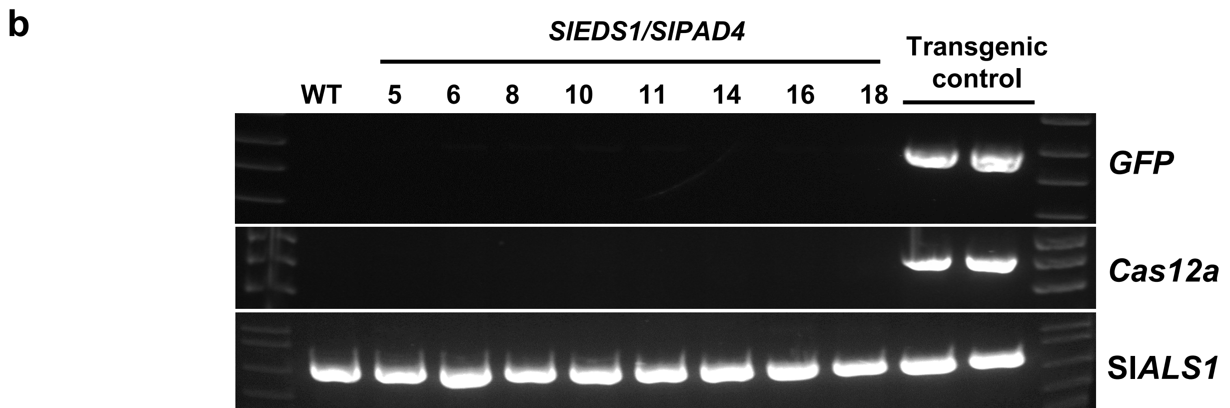

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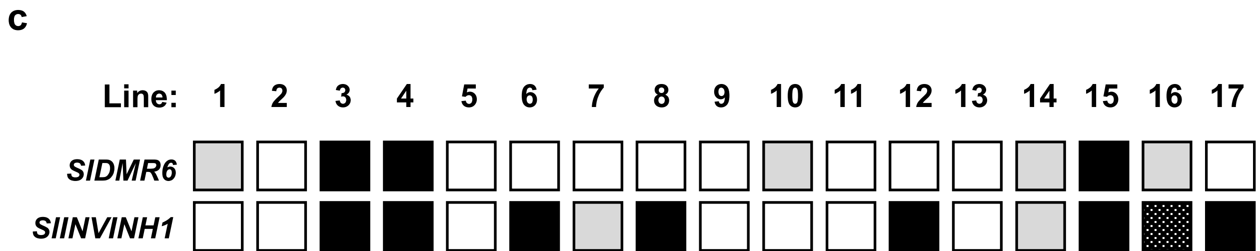

d

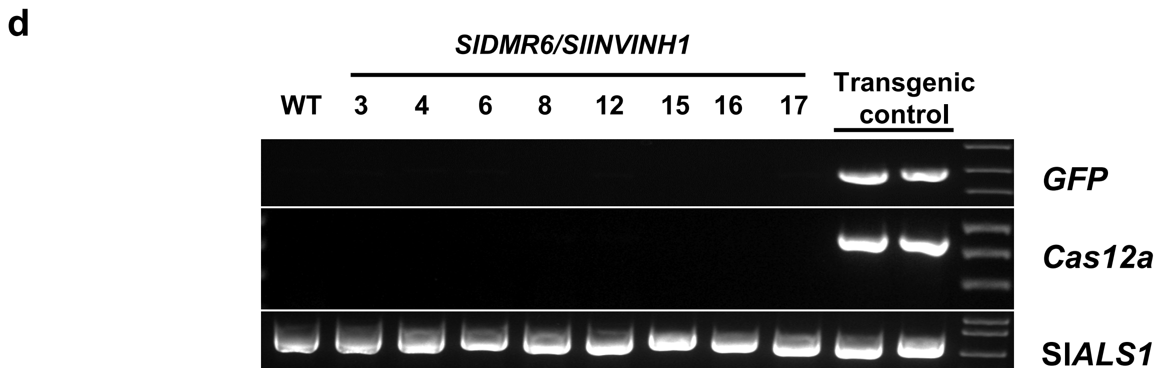

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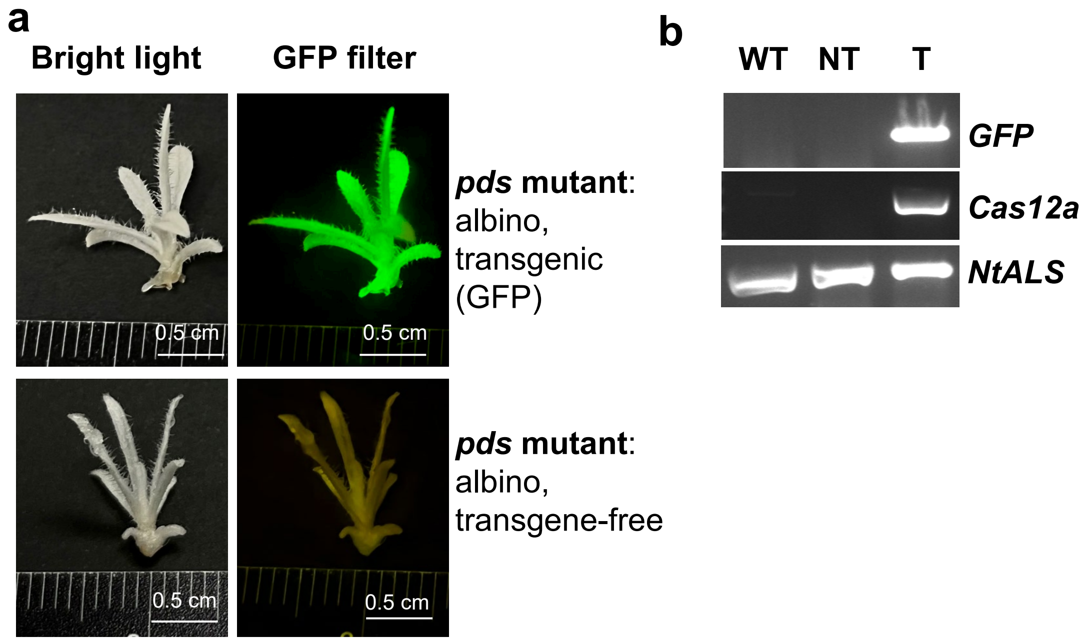
Fig.5



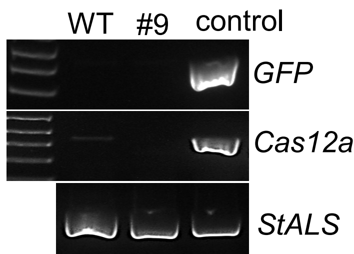
c

```
CAAGTACCACGTAGGATGATTGG NtALS WT
CAAGTATTATGTAGGATGATTGG NtALS line 4

ATGAAATTTCGGCAGATCAGAGCAAAGCAAAAATATTGAAGTATCATG NtPDS1 WT
ATGAAATTTCGGCAGATCAGAGCAA--CAAAAATATTGAAGTATCATG -2bp, line 4
ATGAAATTTCGGCAGATCAGAGCAAAGCA----TATTGAAGTATCATG -4bp, line 4

ACGAAATTTCGGCAGATCAGAGCAAAGCAAAAATATTGAAGTATCACA NtPDS2 WT
ACGAAATTTCGGCAGATCAGAGCAAAGCAAA----TTGAAGTATCACA -4bp, line 4
ACGAAATTTCGGCAGATCAGAGCAAA-------TATTGAAGTATCACA -7bp, line 4
```

d



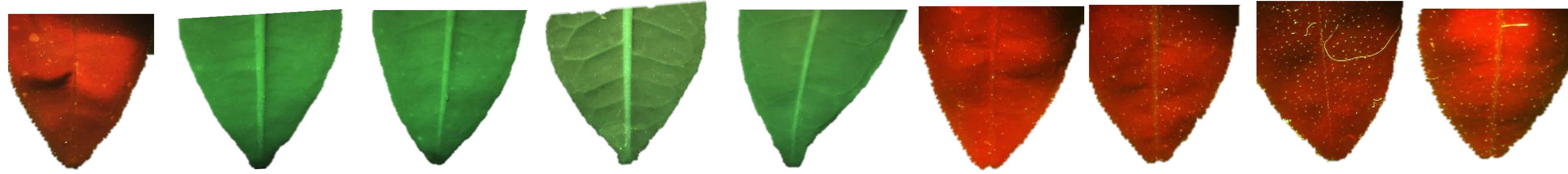
e

```
GAAGCTAAAATTGTATTCAGATGATCCTTCAAA StDMR6 WT

GAAGCTAAAA----ATTCAGATGATCCTTCAAA line 9, allele 1, -4bp
GAAGCTAAAAT--------GATGATCCTTCAAA line 9, allele 2, -8bp
GAAGCTAAAATTGTATTCAGATGATCCTTCAAA line 9, allele 3, WT
GAAGCTAAAATTGTATTCAGATGATCCTTCAAA line 9, allele 4, WT
```

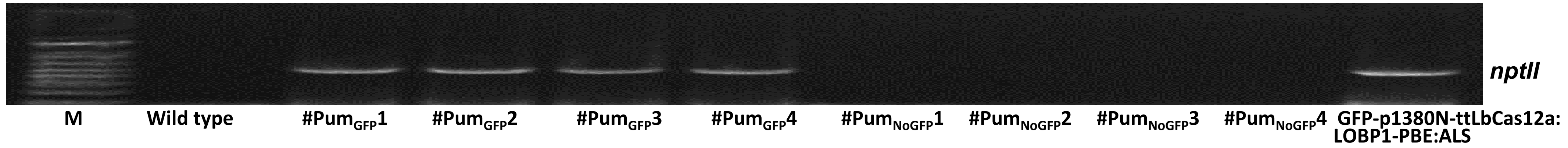
Fig. 6

a



Wild type | #Pum$_{GFP}$1 | #Pum$_{GFP}$2 | #Pum$_{GFP}$3 | #Pum$_{GFP}$4 | #Pum$_{NoGFP}$1 | #Pum$_{NoGFP}$2 | #Pum$_{NoGFP}$3 | #Pum$_{NoGFP}$4

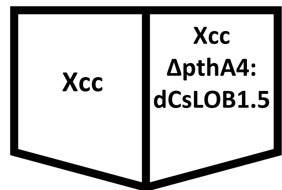
b



c

Edited lines			Genotypes
#Pum$_{NoGFP}$1	Wild type (x0)	TTGCCTTTTCCTTTCTCTATATAAACCCCTTTTGCCTTAACTTT	biallelic
	-CCTTTTGCCT (x6)	TTGCCTTTTCCTTTCTCTATATAAACC-- - - -- - -- - - TAACTTT	
	-ACCCCTTT (X4)	TTGCCTTTTCCTTTCTCTATATAA- - - -- - - -TGCCTTAACTTT	
#Pum$_{NoGFP}$2	Wild type (x5)	TTGCCTTTTCCTTTCTCTATATAAACCCCTTTTGCCTTAACTTT	heterozygous
	-CCCTTTTG (x5)	TTGCCTTTTCCTTTCTCTATATAAAC- - - - - - - -CCTTAACTTT	
#Pum$_{NoGFP}$3	Wild type (x0)	TTGCCTTTTCCTTTCTCTATATAAACCCCTTTTGCCTTAACTTT	homozygous
	-CCTTTTG (X10)	TTGCCTTTTCCTTTCTCTATATAAACC-- -- - - -CCTT AACTTT	
#Pum$_{NoGFP}$4	Wild type (x4)	TTGCCTTTTCCTTTCTCTATATAAACCCCTTTTGCCTTAACTTT	heterozygous
	-CCTTTTGCCT (x6)	TTGCCTTTTCCTTTCTCTATATAAACC-- - - -- - -- - - TAACTTT	

d



Wild type | #Pum$_{GFP}$1 | #Pum$_{GFP}$2 | #Pum$_{GFP}$3 | #Pum$_{GFP}$4 | #Pum$_{NoGFP}$1 | #Pum$_{NoGFP}$2 | #Pum$_{NoGFP}$3 | Pum$_{NoGFP}$4