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1 **Integration of a *FT* expression cassette into CRISPR/Cas9 construct enables fast**

2 **generation and easy identification of transgene-free mutants in Arabidopsis**

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20 **Abstract**

21 The CRISPR/Cas9 genome editing technique has been widely used to generate
22 transgene-free mutants in different plant species. Several different methods including
23 fluorescence marker-assisted visual screen of transgene-free mutants and programmed
24 self-elimination of CRISPR/Cas9 construct have been use to increase the efficiency of
25 genome edited transgene-free mutants isolation, but the overall time length required to
26 obtain transgene-free mutants has remained unchanged in these methods. We report
27 here a method for fast generation and easy identification of transgene-free mutants in
28 Arabidopsis. By generating and using a single *FT* expression cassette-containing
29 CRISPR/Cas9 construct, we targeted two sites of the *AITR1* gene. We obtained many
30 early bolting plants in T1 generation, and found that about two thirds of these plants
31 have detectable mutations. We then analyzed T2 generations of two representative
32 lines of genome edited early bolting T1 plants, and identified plants without early
33 bolting phenotype, i.e., transgene-free plants, for both lines. Further more,
34 homologues *aitr1* mutants were successful obtained for both lines from these
35 transgene-free plants. Taken together, these results suggest that the method described
36 here enables fast generation, and at the mean time, easy identification of
37 transgene-free mutants in plants.

38

39 **Keywords:** CRISPR/Cas9; genome editing; bolting time; FT; AITR1

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40 **Introduction**

41 Shortly after the CRISPR (clustered regularly interspaced short palindromic repeats)
42 RNA-guided Cas9 (CRISPR-associated protein 9) endonuclease being reported to be
43 able to cleave double-stranded DNA, therefore generate mutations in eukaryotic cells
44 [1,2], CRISPR/Cas9 mediated genome-editing has been successful used for gene
45 editing to generate mutations in several different plants including the model plant
46 Arabidopsis and crops such as rice, tobacco and wheat [3-5]. Since then, the
47 CRISPR/Cas9 genome-editing techniques including the double-stranded DNA
48 cleaving based editing and the nucleotide substitution based editing have been widely
49 used to generate mutations in different plant species, in some cases, to improve
50 agronomic traits such as yield, quality and biotic and abiotic stress tolerances [6-12].
51 Thanks to their high efficiency in genome editing and the used of engineered Cas9
52 variants with expanded target space, CRISPR/Cas9 genome-editing systems have
53 brought a bright future for plant breeding [6,12-14].

54 The presence of Cas9 T-DNA in CRISPR/Cas9 genome-edited mutants may
55 affect the phenotypic stability and heritability of the mutation, and transgene-free is
56 likely required for commercial application of CRISPR/Cas9 genome-edited crops [7,
57 10]. Therefore, isolation of transgene-free mutants is one of the most important steps
58 for generation mutants by using CRISPR/Cas9 genome-editing. However, isolation of
59 transgene-free mutants by using the traditional genetic segregation and backcross
60 based genotyping is time consuming and laborious [7,8,10]. To improve the efficiency
61 in transgene-free mutant isolation from CRISPR/Cas9 genome edited plants, a few

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62 different methods have been established [6-8,10]. These methods include the
63 fluorescence maker-assisted selection, which allows to isolation transgene-free
64 mutants based on the observation of the absence of fluorescence in seeds produced by
65 transgenic plants [7]; the active interference element mediated selection, which allows
66 herbicide-dependent isolation of transgene-free plants [8]; and the programmed
67 self-elimination system, which allows only transgene-free male gametophytes to
68 produce seeds [13]. All these methods greatly reduced workload for transgene-free
69 mutant isolation. However, the overall time length required for the whole process of
70 mutant generation, from plant transformation, to mutatant identification, and then
71 transgene-free mutant isolation remained largely unchanged.

72 Appreciate flowering time is critical for successful sexual reproduction in
73 flowering plants [15]. In order to achieve sexual reproductive successful, flowering
74 plants need to sense and respond to environmental stimuli appropriately, and then
75 integrate the environmental information with endogenous signals to make transit from
76 vegetative growth to flowering [16-18]. Accumulated evidence suggest that flowering
77 time in Arabidopsis is controlled by several different regulators, including CO
78 (CONSTANS), SOC1 (SUPRESSOR OF CONSTANS OVEREXPRESSION1), FLM
79 (FLOWERING LOCUS M), FLC, FLK (FLOWERING LOOCUS K HOMOLOGY
80 (KH) DOMAIN), VRN2 (VERNALIZATION 2), MAF2 (MADS AFFECTING
81 FLOWERING 2) and FT (FLOWERING LOCUS T) [15,17,19-24]. Among them,
82 FLC and CO are major regulators involved in vernalization and photoperiod, the two
83 most important environmental stimuli that control the switch from vegetative growth

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84 to flowering, respectively, and they function immediately upstream of FT to regulate
85 the switch [15-17].

86 FT is the key positive regulator of flowering in Arabidopsis, and at least some of
87 the FT homologues in other plants species including medicago, rice, soybean and
88 trees like poplar and pear also function as activator of flowering [25-28]. Due to its
89 important role in flowering promotion, FT has been successfully used to reducing
90 juvenile phase of many plants, therefore accelerated the process for plant breeding
91 [15,27,29].

92 Considering that early flowering phenotype caused by overexpression of *FT* in
93 plants is easy visible, and the resulted short life cycle will accelerate mutants
94 generation, integration of a FT expression cassette into CRISPR/Cas9 may enable fast
95 generation and easy identification of transgene-free mutants in plants. In this study,
96 we introduced a *GmFT2a* expression cassette into the *pHEE* CRISPR/Cas9 vector,
97 and inserted two *sgRNA* expression cassettes to target the *AITR1* (*ABA induced*
98 *transcription repressor1*) gene, which encodes a novel ABA signaling and abiotic
99 stress tolerance regulating transcription factor in Arabidopsis [30]. We successfully
100 obtained detectable mutations in *AITR1* in early bolting T1 Arabidopsis transgenic
101 plants, and obtained homologues transgene-free *aitr1* mutants from T2 plants with
102 normal bolting phenotypes.

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104 **Results**

105 **Generation of a *FT* expression cassette-containing CRISPR/Cas9 construct**

106 Overexpression of *FT* in plants promoted flowering, therefore shorten the juvenile
107 phase of the transgenic plants even in trees [27]. If used in CRISPR/Cas9 mediated
108 gene editing, the easy visible early bolting phenotype caused by overexpression of *FT*
109 may serve as an assistant selection marker for easy identification of transgene-free
110 mutants, whereas shorten in juvenile phase resulted by early flowering may reduce the
111 length of the overall time required for generating genome edited mutants, thereby
112 providing a method for fast generation and easy identification of genome edited
113 transgene-free mutants.

114 Considering that overexpression of *GmFT2a* in both Arabidopsis and soybean
115 promoted flowering in transgenic plants [31,32], we decided to integrate a *GmFT2a*
116 expression cassette into CRISPR/Cas9 vector for gene editing. The full-length coding
117 sequence of *GmFT2a* was synthesized, and cloned into to the *pUC19* vector to
118 generate the *35S:GmFT2a* construct. The whole *35S:GmFT2a-nos* expression cassette
119 was then PCR amplified and cloned into the *pHEE* vector [33], at the *pme1* site to
120 generate the *FT* expression cassette-containing CRISPR/Cas9 vector *pHEE-FT*
121 (Figure 1a). In this vector, the *FT* expression cassette was inserted within the T-DNA
122 fragment of the *pHEE-FT* vector as an independent expression cassette, and the
123 expression of *GmFT2a* was controlled by the strong double *35S* promoter, which
124 enables that *GmFT2a* can be expressed in a high level in transgenic plants generated
125 by using the *pHEE-FT* vector, therefore may led to an easy visible early flowering

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126 phenotype in the transgenic plants.

127

128 **T1 transgenic plants generated using the *FT* expression cassette-containing**
129 **CRISPR/Cas9 construct showed an early bolting phenotype**

130 To examine if the genome editing using the *pHEE-FT* vector may enable fast
131 generation and easy identification of transgene-free mutants, we made a *pHEE-FT*
132 CRISPR/Cas9 genome editing construct to target *AITR1* (Figure 1b), a novel
133 transcription factor gene that has been shown to regulate ABA signaling and abiotic
134 tolerance in Arabidopsis [30], at two different target sites (Figure 1c).

135 After selected on antibiotic-containing plates, more than 70 T1 independent
136 transgenic plants were obtained. As expected, early bolting phenotype was observed
137 in the transgenic plants (Figure 2a). Bolting was observed in transgenic plants as early
138 as 8 days after the seedlings were transferred from the antibiotic-containing plates into
139 soil pots, and the bolting time for these T1 transgenic plants ranged from 8-19 days
140 after the transfer, whereas that in the Col wild type plants ranged from 17-20 days
141 after the transfer (Figure 2b).

142

143 **Homozygous genome edited mutants were obtained in the T1 transgenic plants**

144 The *pHEE-FT-AITR1* CRISPR/Cas9 construct was made to target two sites in *AITR1*,
145 therefore fragment deletion should be expected in the transgenic plants if both sites
146 can be edited. Because we intended to use early bolting phenotype as an assistant
147 marker for transgene-free mutant isolation, transgenic plants with an early bolting

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148 phenotype, i.e., bolted 8 or 9 days after transferred, were chosen for fragment deletion
149 examination by PCR. Yet we included a few plants with medium or normal bolting
150 time in this experiment to examine if this is a co-relationship between early bolting
151 phenotype and genome editing status.

152 Indeed, smaller PCR product band was obtained (Figure 3a). However, among
153 about 50 T1 plants examined, only two plants, i.e., lines 14 and 59 with early and
154 medium bolting phenotype, respectively yield two PCR bands, but none of them
155 produced only one smaller band (Figure 3b). All other plants produced only one larger
156 PCR band with expected site for the full-length coding sequence of *AITR1*(Figure 3b).
157 These results suggest that both target sites in *AITR1* can be edited, but homozygous
158 fragment deletion mutants were not obtained in the plants examined.

159 We therefore sequenced the full-length *AITR1* PCR products obtained from some
160 of the plants that only produced one PCR product band, to see if we may get
161 homozygous mutants with only one *AITR1* target site edited. Indeed, two of the early
162 bolting plants, lines 11 and 15 were identified as homozygous mutants with a single
163 nucleotide insertion at the second target site of *AITR1* (Figure 3b). One of the T1
164 plants with normal bolting phenotype was edited at the second site with a single
165 nucleotide insertion, but was a biallelic mutant (Figure 3b). We also found that
166 another 9 early bolting plants sequenced was edited in one of the target sites but were
167 not homozygous (Figure 3b). It should note that among the single site edited mutant,
168 only one was edited at the first target site (Figure 3b).

169

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170 **Homozygous genome edited transgene-free mutants were obtained in the T2**
171 **plants**

172 Having shown that homozygous mutants can be obtained in early bolting T1
173 transgenic plants (Figure 3), we decided to further examine whether transgene-free
174 mutants can be easily obtained in T2 generation base on phenotypic observation. Two
175 represent early mutant lines, i.e., line 14 and 15 were chosen, and T2 seeds were
176 germinated directly in soil pots. Line 14 was chosen because PCR results indicated
177 that both target sites of *AITR1* were edited in this line, where as line 15 was a
178 homozygous mutant identified in T1 generation.

179 Segregation on bolting phenotype was observed in T2 plants (Figure 4a). Multiple
180 plants without early bolting phenotype were obtained for both lines, and the
181 segregation ratio for early and normal bolting plants were about 3:1 (Figure 4b).

182 We then examined if the plants without early bolting phenotype were
183 transgene-free plants by amplification of *Cas9* gene. As shown in Figure 5, no PCR
184 products were obtained in all the normal bolting plants examined, whereas PCR
185 products were obtained in early bolting plants. These results suggest that
186 transgene-free plants can be easily identified by observation of bolting phenotype.

187 By using PCR amplification, we found that two of the normal flowering plants in
188 line 14 produced only a small band (Figure 6a), indicating that they were homozygous
189 mutants. Sequence result showed that a 428bp fragment in *AITR1* was deleted in this
190 mutant, leading to a few amino acid substitutions and premature stop after the 89th
191 amino acid residue (Figure 6a).

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192 We also sequenced AITR PCR product from one of the transgene-free plants
193 obtained from line 15 to confirm the genome editing status. We found that it was
194 indeed a homozygous mutant with a single nucleotide insertion occurred in the
195 sequence of the second target site. In this mutant, the single nucleotide insertion
196 *AITR1* also led to a few amino acid substitutions, and premature stop occurred after
197 the 232nd amino acid residue (Figure 6b).
198

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200 **Discussion**

201 CRISPR/Cas9 genome-editing has been used to generate mutants in different plant
202 species [6-12], and may have a bright future in using for plant breeding [6,12-14].
203 However, remove of the Cas9 T-DNA from the transgenic plants is likely necessary to
204 get stable and heritable mutants, expecially for commercial use of the genome-edited
205 crops [7,10].

206 FT promotes flowering in many plant species including the model plants
207 Arabidopsis, crops such as rice and soybean, and fruit trees such as apple and pear
208 [24-27,33], and has been used to accelerate plant breeding process by reducing
209 juvenile phase of many plants [15,27,29].

210 By inserting the *GmFT2a* expression cassette into the *pHEE* CRISPR/Cas9 vector
211 (Figure 1), we established a method for fast generation and easy identification of
212 genome-edited transgene-free mutants in Arabidopsis (Figure 7).

213 In this method, the easy visible early flowering phenotype can be used as an
214 indicator of plants with Cas9 T-DNA. In the T1 generation, only plants with early
215 bolting phenotypes should be selected and genome editing status should be examined
216 to identify mutations. In the T2 generation of the genome-edited T1 plants, only plants
217 without early flowering phenotypes, i.e., transgene-free plants should be selected, and
218 genome editing status should be examined to ensure that genome-edited
219 transgene-free homozygous mutants will be obtained (Figure 7).

220 By using the *pHEE-FT* vector to generate a CRISPR/Cas9 genome editing
221 construct for simultaneously targeting two sites in the ABA signaling and abiotic

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222 stress tolerance regulator gene *AITR1* [30], we successfully obtained genome-edited
223 transgene-free *aitr* homozygous mutants (Figure 6). In the T1 generation, about two
224 thirds of the early bolting plants examined have at least one target site edited (Figure
225 3), indicating that insertion of the *GmFT2a* expression cassette into the CRISPR/Cas9
226 vector did not affect the editing efficiency of Cas9. However, we noted that including
227 the two plants with fragment deletion, only three plants had mutations at the first
228 target site, suggesting that the two targets selected have different editing efficiency.
229 Had both target sites have high editing efficiency, we should able to obtained
230 homozygous fragments deletion mutants in T1 generation. Nevertheless, we obtained
231 homozygous mutants with mutations occurred at only one target site from the T1
232 plants (Figure 3), and we obtained homozygous fragments deletion mutants in T2
233 generations (Figure 6).

234 Even though only the double-stranded DNA cleave based CRISPR/Cas9
235 genome-editing system was examined in this study, however, the concept used in this
236 study may also applied to the nucleotide substitution based CRISPR/Cas9
237 genome-editing system to facility transgene-free mutant isolation.

238 It should note that target site editing were also observed in T1 transgenic plants
239 with medium or normal bolting time (Figure 3), suggesting that editing efficiency may
240 not positively correlated with early bolting phenotypes. This is likely because *FT* and
241 *Cas9* in the vector were in two different expression cassettes, and were driven by
242 different promoters (Figure 1), thus their expression levels may not always positively
243 related in the transgenic plants. Consider that mutants can obtained from early bolting

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244 T1 plants based on PCR results only or combined with sequencing (Figure 2), and
245 transgene-free plants can be easily obtained from offsprings of the early bolting T1
246 plants based solely on bolting phenotype segregation (Figure 5), only T1 plants with
247 early bolting phenotypes should be selected for next step analysis when using this
248 method to generate for genome edited transgene-free mutants.

249 In addition to enable easy identification of transgene-free mutants base on
250 phenotypic observation, the early bolting phenotype also reduce the length of the
251 juvenile phase, which led to reduced overall time length required for generating
252 genome edited transgene-free mutants. In our case, the different bolting time between
253 early bolting transgenic plants and Col wild type plants were more than 10 days
254 (Figure 2). Because early bolting plants were selected in T1, but plants with normal
255 bolting time were selected in T2, the overall time length required for generating
256 genome edited transgene-free mutants in Arabidopsis will be reduced at least 10 days.
257 In some cases, transgene-free mutants may not be able to be identified in T2
258 generations, and had to be identified on T3 generations. In that case, identify mutant
259 from offsprings of the gene-edited early bolting T2 plants may save more time than
260 from that of the transgene-free heterozygous T2 mutants.

261 In this study, the effects of integration of *FT* expression cassette into
262 CRISPR/Cas9 vector to accelerate transgene-free mutant isolation was tasted only in
263 Arabidopsis, however, considering that the juvenile phase for most of the crops such
264 as rice and soybean lasts for moths, and that for most of the trees including fruit trees
265 apple and pear lasts for years, whereas overexpression of *FT* greatly reduced the

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266 length of juvenile phase in most of the plants examined, including all the plants
267 mentioned above [25-28,34-36], the method described here may benefit even more for
268 genome editing based breeding for the plants with a long juvenile phase.

269 On the other hand, transgenic plants of different plant species may need to be
270 selected in different antibiotics, and multiple genes may need to be edited
271 simultaneously, by replacing the antibiotic gene and/or the sgRNA clone cassette, the
272 pHEE-FT vector reported here may be used for genome editing for a single or
273 multiple genes in different plant species.

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275 **Materials and methods**

276 **Plant materials and growth conditions**

277 The Columbia ecotype 'Col-0' (Col) Arabidopsis (*Arabidopsis thaliana*) was used for
278 plant transformation, and as controls for bolting time assays.

279 Seeds of Col wild type were germinated in soil pots, and grown in a growth room.
280 T1 transgenic plants were selected by plating T1 seeds on antibiotic-containing 1/2
281 MS plates. Transgenic seedlings were transferred into soil pots, and grown in a
282 growth room. As a control, seeds of Col wild type were germinated on 1/2MS plates,
283 seedlings were transferred into soil pots and grown in a growth room. The growth
284 conditions in the growth room have been described previously [37,38].

285

286 **Constructs**

287 The *pHEE* CRISPR/Cas9 vector has been described previously [33]. To insert the FT
288 expression cassette into *pHEE* to generate the *pHEE-FT* vector, the full-length open
289 reading frame (ORF) sequence of *GmFT2a* was synthesized and cloned into *pUC19*
290 under the control of the double *35S* promoter, and terminated by *nos* [39]. The
291 sequence of the *35S:GmFT2a-nos* cassette was then amplified by PCR, and cloned
292 into the *pHEE* vector at the *Pme*1 site by using Gibson assembly. The primers used to
293 amplify *35S:GmFT2a-nos* from the *pUC19* construct were:
294 5'-CCTGTCAAACACTGATAGTTTGTCGACTCTAGAGGATCC-3' and
295 5'-GTCGTTTCCCGCCTTCAGTTTACGACGGCCAGTGAATTC -3'.

296 To generate CRISPR/Cas9 construct for genome editing of *AITR1*, appropriate

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297 target sequences were first identified by scanning coding sequence of *AITR1* on
298 CRISPRscan (http://www.crisprscan.org/?page=sequence), and then evaluated on
299 Cas-OFFinder (http://www.rgenome.net/cas-offinder/). Two target sequences were
300 selected and used for editing *of AITR1*, 5'-GATCTGACTTGTCTGATGAT(CGG)-3',
301 and 5'-GGTGGCGGAGGAGGCAACGG(CGG)-3'. The sgRNA expression cassettes
302 targeting *AITR1* were cloned into the *pHEE-FT* vector to generate *pHEE-FT-AITR1*
303 construct by following the procedures described by Wang et al [39]. The primers used
304 to insert the target sequences into sgRNA expression cassettes were, *DT1-BsF*,
305 5'-ATATATGGTCTCGATTGATCTGACTTGTCTGATGATGTT-3', *DTI-F0*, 5'-T
306 GATCTGACTTGTCTGATGATGTTTTAGAGCTAGAAATAGC-3', *DT2-R0*,
307 5-AACCCGTTGCCTCCTCCGCCACCAATCTCTTAGTCGACTCTAC-3', and
308 *DT2-BsR*, 5'-ATTATTGGTCTCGAAACCCGTTGCCTCCTCCGCCACC-3'. The
309 primers used for colony PCR and to sequence the sgRNA expression cassettes in the
310 generated *pHEE-FT-AITR1* construct were, *U6-26-IDF*,
311 5'-TGTCCCAGGATTAGAATGATTAGGC-3' and *U6-26-IDR*,
312 5'-AGCCCTCTTCTTTCGATCCATCAAC-3'.
313
314 **Plant transformation and transgenic plant selection**
315 Col wild type plants have several mature flowers on the main inflorescence
316 (~5-week-old) were used for transformation. The plants were transformed via
317 Agrobacterium *GV3101* mediated floral dipping [40]. T1 seeds collected were
318 germinated on 1/2 MS plates containing 30 μg/ml hygromycin and 100 μg/ml

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319 carbenicillin to select transgenic plants.

320 Bolting time of the T1 plants was observed, and gene editing status was examined

321 by amplifying the coding sequence of *AITR1* and sequencing the PCR products

322 obtained.

323

324 **Isolation of transgene-free homozygous mutants**

325 T2 seeds were collected from selected plants that bolting early and with *AITR1* gene

326 edited. The seeds were then sown directly into soil pots. T2 plants with normal bolting

327 time were selected, the absence of *Cas9* T-DNA insertion was confirmed by PCR

328 amplification of *Cas9* fragment, and gene editing status was further

329 examined/confirmed amplifying the coding sequence of *AITR1* and sequencing the

330 PCR products obtained.

331

332 **DNA isolation and PCR**

333 To examine gene editing status of *AITR1*, DNA was isolated from leaves of the

334 transgenic plants, and the coding sequence of *AITR1* was amplified by PCR. PCR

335 products was recovered from gel and sequenced. The sequencing results obtained

336 were then examined and aligned with coding sequence of *AITR1* obtained from

337 Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html). The primers used for PCR

338 amplification of *ACT2* and *AITR1* coding sequence and have been described

339 previously [30,41].

340 To confirm the transgene-free status of the mutants obtained, DNA was isolated

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341 from leaves of T2 transgenic plants that have a normal bolting time, and *Cas9* gene
342 fragment was amplified by PCR. The primers used for PCR amplification of *Cas9*
343 were, *Cas9-F*, 5'-GGACAACGAGGAGAATGAGG-3', and *Cas9-R*,
344 5'-TGTCTCGACCAGCTGCCTCTT-3'
345
346 **Bolting time assays**
347 For the T1 plants, the date of bolting was recorded, and days after the transgenic
348 seedlings were transferred into soil pots were calculated and used as bolting time.
349 Transferred Col wild type plants were used as controls.
350 For T2 plants, the date of bolting was recorded, and days to bolting after the seeds
351 were germinated were calculated. Col wild type plants germinated and grown in soil
352 pots were used as controls.

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354 **Author contribution statement**

355 S.W. conceived the study. S.W., Y.C. and N.Z. designed the experiments. Y.C., N.Z.,
356 S.H., S.A., and W.Y. performed the experiments. S.W., Y.C. and N.Z. analyzed the
357 data. S.W. drafted the manuscript. All the authors participated in the revision of the
358 manuscript.

359

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364

365 **Conflict of interest statement**

366 The authors declare no conflict of interest.

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498 **Figure legends**

499 **Figure 1. Generation of a *FT* expression cassette containing *pHEE* CRISPR/Cas9**
500 **construct for *AITR1* editing.**

501 (**a**) *pHEE* vector with a *FT* expression cassette. The full-length ORF sequence of
502 *GmFT2a* was synthesized, and cloned into *pUC19* vector under the control of the *35S*
503 promoter and terminated by the *nos* sequence. The *35S:GmFT2a-nos* cassette was
504 amplified by PCR and then inserted into the *pHEE* vector at the *Pme*1 site by using
505 Gibson assembly to generate *pHEE-FT* vector. (**b**) The *sgRNA* expression cassettes in
506 the *pHEE-FT-AITR1* construct. The *sgRNA* sequences corresponding to the target
507 sequences of *AITR1* were introduced into the *sgRNA* expression cassettes by PCR
508 amplification, followed by Golden Gate reaction with the *pHEE-FT* vector. (**c**) Target
509 sequences in *AITR1*. Numbers indicated the nucleotide position relative to the first
510 nucleotide in the coding sequence of *AITR1*, PAM sites after the target sequences
511 were indicated in the brackets.

512

513 **Figure 2. Early bolting phenotypes observed in the T1 transgenic plants.**

514 (**a**) Early bolting phenotype in some T1 transgenic plants. Transgenic plants were
515 selected on antibiotic-containing 1/2 MS plates, and ~5-day-old transgenic seedlings
516 were transferred into soil pots and grown in a growth room. As a control, seeds of Col
517 wild type were germinated on 1/2 MS plates, and seedlings ~5-day-old were
518 transferred into soil pots. Pictures were taken 10 days after the transfer. (**b**) Bolting
519 time of the T1 transgenic plants. The date of bolting for the plants was recorded, and

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520 days after the transfer were calculated. For Col wild type plants, n=11. For transgenic
521 plants, n=53.
522
523 **Figure 3. *AITR1* editing status in T1 transgenic plants.**
524 (**a**) PCR amplification of *AITR1* coding sequence in the T1 transgenic plants. DNA
525 was isolated from leaves collected from individual T1 transgenic plants, and PCR was
526 used to amplify coding sequence of *AITR1*. Picture is representative image of PCR
527 results, showing the 1 or 2 PCR product bands obtained in different plants. (**b**) *AITR1*
528 editing status in 21 individual T1 transgenic plants. PCR products were recovered
529 from gel and sequenced. Sequencing results were examined and aligned with coding
530 sequence of *AITR1* to check the editing status in the T1 transgenic plants. -, not edited,
531 -/+, edited but heterozygous, ins, homozygous or biallelic editing with nucleotide
532 insertions as indicated, N/A, not sequenced.
533
534 **Figure 4. Phenotype segregation in the T2 generation of selected transgenic lines.**
535 (**a**) Bolting phenotype of the T2 plants from a single T1 transgenic line. T2 seeds were
536 collected from selected T1 transgenic lines and sown directly into soil pots and grown
537 in a growth room. Col wild type plants were generated and grown side by side with
538 the T2 plants as a control. Pictures were taken 17 days after germination. Arrows
539 indicate plants that were not bolting early. (**b**) Bolting phenotype segregation of the
540 T2 plants from selected T1 transgenic lines. Chi square analysis was performed on
541 omni calculator (https://www.omnicalculator.com/statistics/chi-square).

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542

543 **Figure 5. T2 plants with normal bolting time are transgene-free plants.**

544 DNA was isolated from leaves collected from six individual T2 plants with normal
545 bolting time, and PCR was used to amplify *Cas9* fragment. For each line, DNA was
546 also isolated from two early bolting plants, and used a positive control for *Cas9*
547 amplification. PCR amplification of *ACT2* was used as a control.

548

549 **Figure 6. Isolation of genome edited transgene-free *aitr1* mutants.**

550 Transgene-free *aitr1* mutants isolated from line 14 (**a**) and line 15 (**b**). DNA was
551 isolated from leaves collected from individual T2 plants with normal bolting time, and
552 used as template to amplify the coding sequence of *AITR1*. The PCR products were
553 recovered from gel and sequenced. Sequencing results were compared with coding
554 sequence of *AITR1* to check the editing status. ORF of the *AITR1* sequences in the
555 *aitr1* mutants were identified on ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/),
556 and corresponding amino acid sequences were used for alignment with AITR1 amino
557 acid sequences. Triangle indicates fragment deletion, arrow indicates nucleotide
558 insertion, underlines in the sequence results indicate the PAM sites. Numbers in the
559 alignment indicate the position of amino acid relative to the first Met of AITR1.

560

561 **Figure 7. Simplified procedure for generating genome edited tansgene-free**
562 **mutants by using *FT* expression cassette-containing CRISPR/Cas9 construct.**

563 Plants can be transformed and transgenic plants can selected in a way similar to that

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564 for other constructs. Within the T1 transgenic plants, select plants with early-bolting
565 phenotype, and sequence to examine genome editing status. Keep only genome edited
566 plants. In the T2 plants germinated from seeds of the individual T1 plants, keep only
567 these bolting normally, and sequence to identify genome edited plants. These plants
568 are genome edited transgene-free mutants. If necessary, confirm the transgene-free
569 status by PCR amplification of *Cas9* fragment.

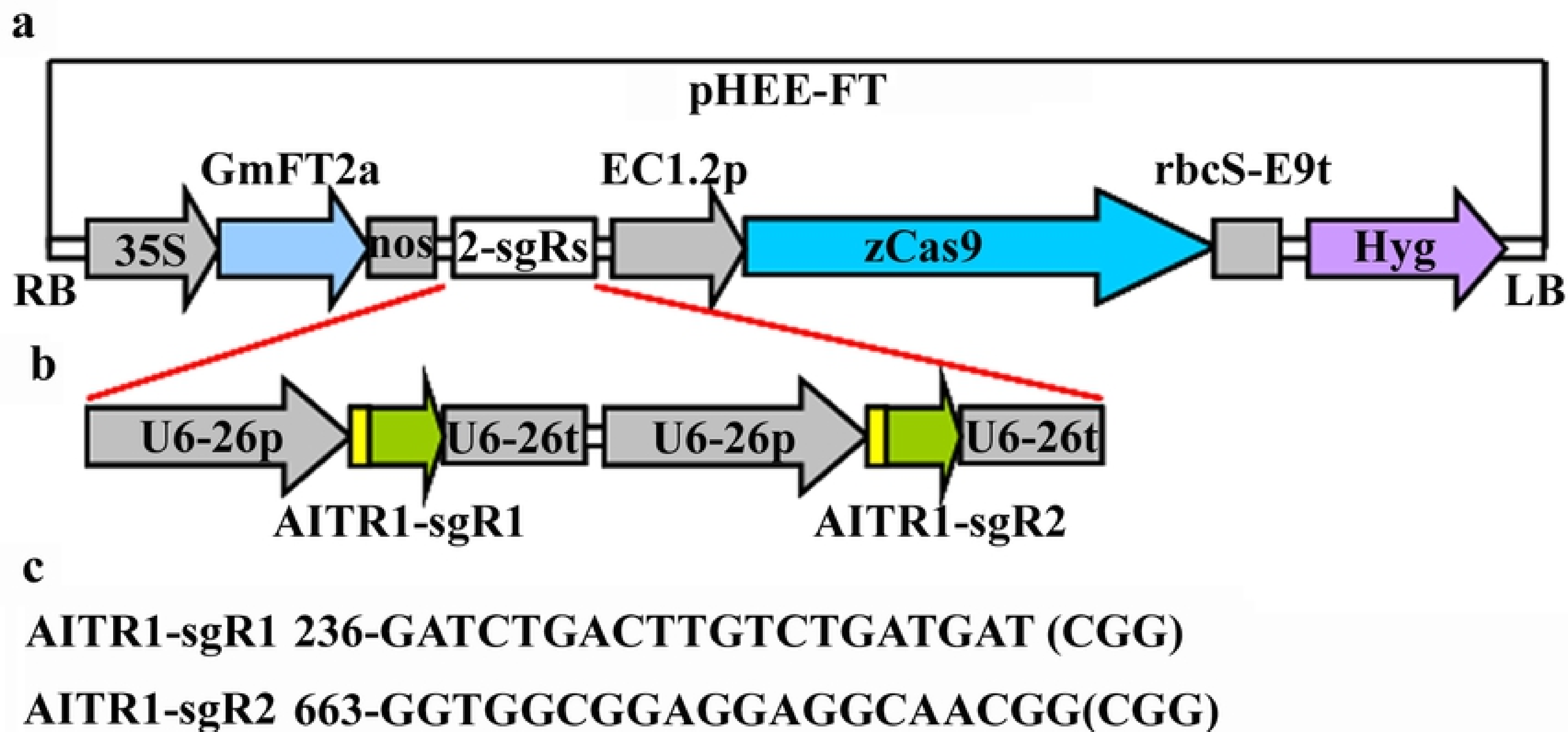

Figure 1

a

Col T1

b

Bolting time (Days after transfer)

25
20
15
10
5
0

Col T1

Figure 2

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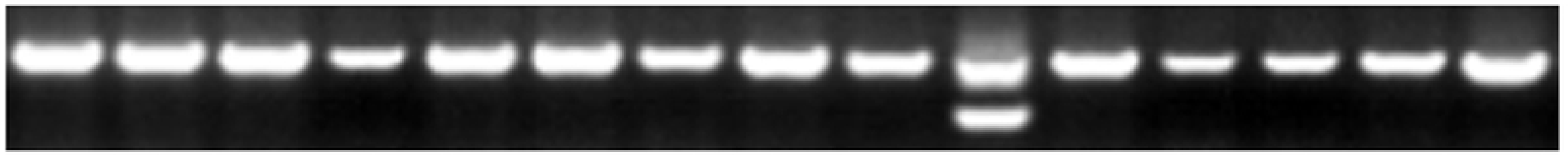
a



b

***AITR1* editing status in T1 plants**

Lines	PCR bands	sgR1	sgR2	Bolting
1	1	-	-/+	early
2	1	-/+	-	early
3	1	-	-	early
4	1	-	-	early
5	1	-	-/+	early
6	1	-	-/+	early
9	1	-	-/+	early
10	1	-	-	early
11	1	-	A ins	early
12	1	-	-/+	early
13	1	-	-	early
14	2	N/A	N/A	early
15	1	-	A ins	early
57	1	-	-/+	early
58	1	-	-/+	early
59	2	N/A	N/A	medium
60	1	-	-/+	early
I7	1	-	A/T ins	nomal
J7	1	-	-	nomal

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Figure 3

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a

b

Segregation of bolting phenotype in T2 plants

Lines	Bolting early	Bolting normal	x^2 for 3:1
14	40	14	0.007
15	39	9	0.593

Figure 4

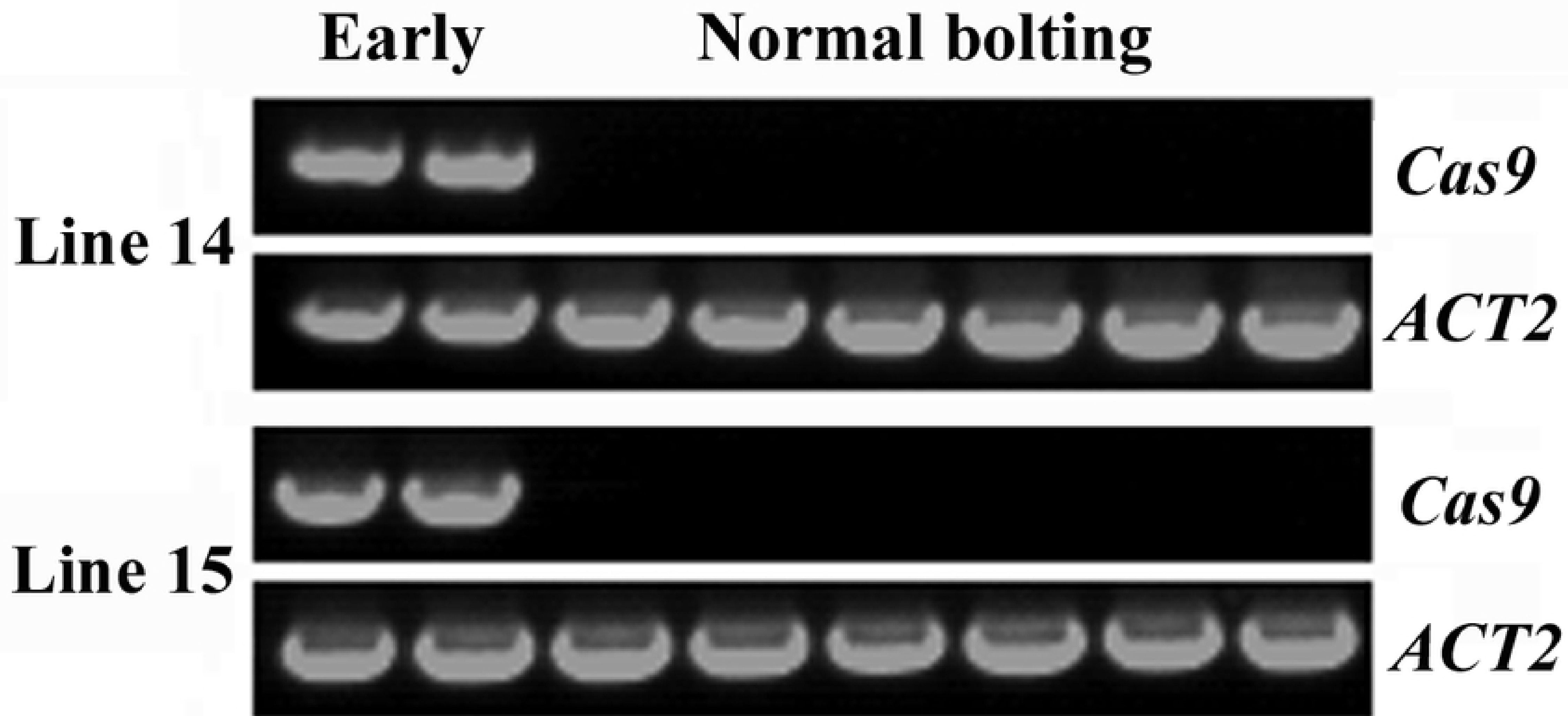
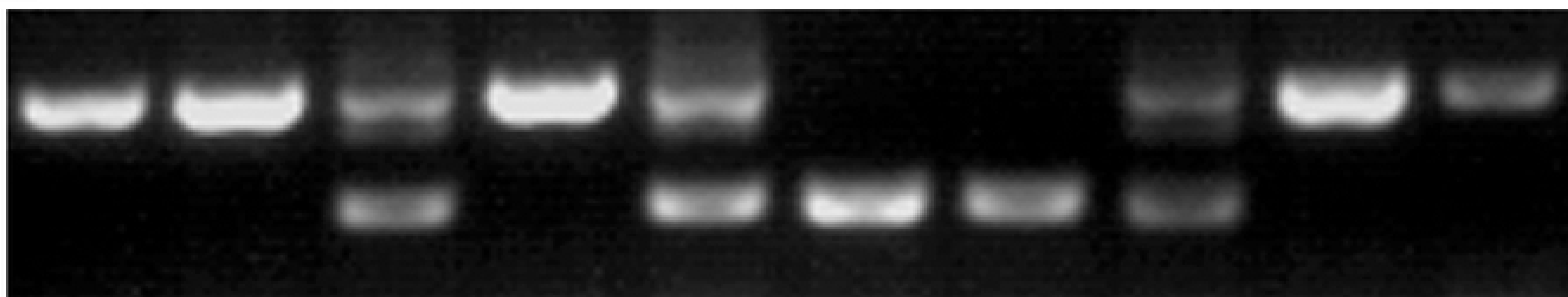


Figure 5

a



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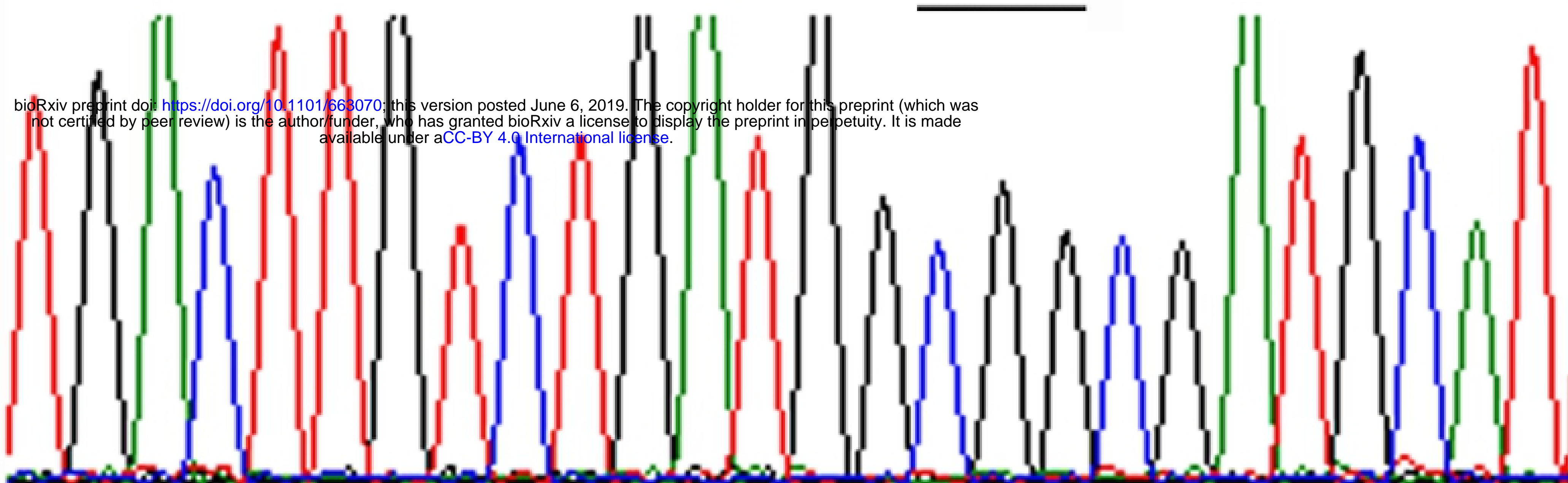

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b

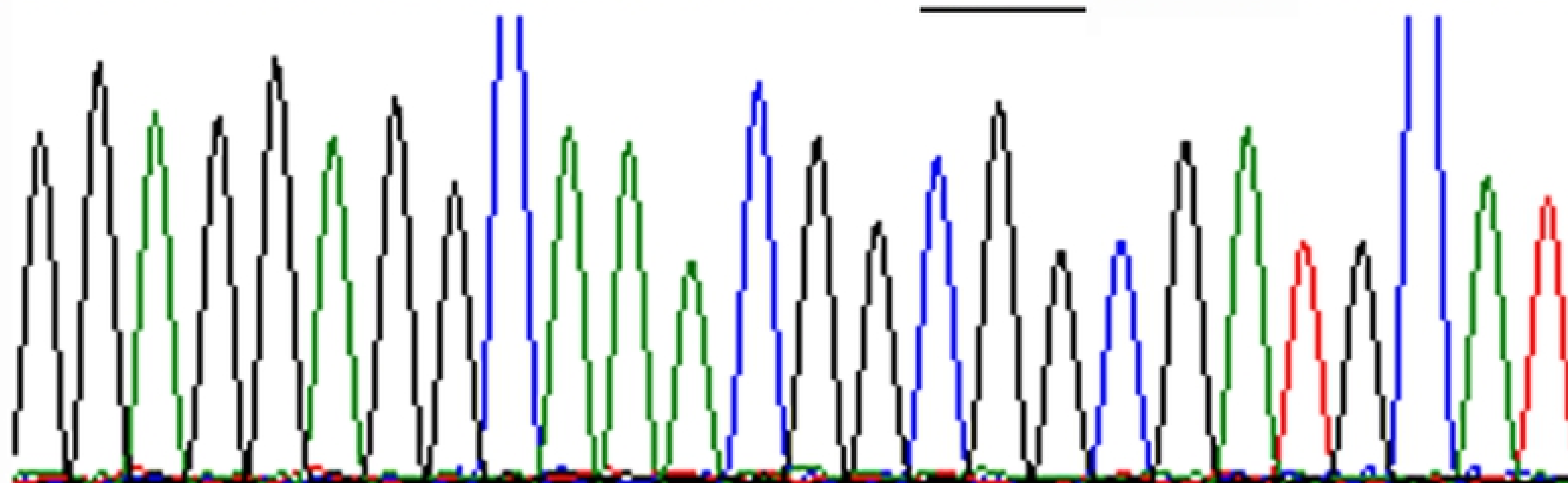
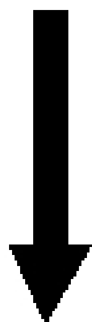


Figure 6

Plant transformation



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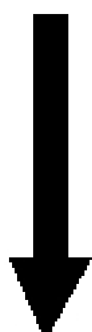
Sequence to identify gene eidted plants from early bolting plants



Sequence to identify homozygous mutants from normal bolting plants

Figure 7