Genome editing in almond: A CRISPR-based approach through hairy root transformation

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Abstract

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) technology has revolutionized genome manipulation for crop enhancement, providing a powerful toolkit. However, the tissue culture and plant regeneration steps that are critical to the CRISPR/Cas editing framework are often challenging, especially in some woody plant species that exhibit substantial resistance to these procedures. To address this, we have developed an injection-based protocol for inducing hairy roots in almond (*Prunus dulcis*, syn. *Prunus amygdalus*), a species known for its recalcitrance to conventional transformation methods. Notably, the hairy root induction method also proved effective in almond x peach hybrids. To evaluate its utility for gene functional analysis, we combined the hairy root transformation system with CRISPR/Cas9 gene editing technology, targeting two transcription factor genes (*ERF74* and *GAI*). Our efforts resulted in transformants with target knock-out, suggesting the potential of this genetic transformation technology as a valuable tool for future routine gene function studies in almond.

Keywords

Prunus dulcis, hairy root culture, composite plants, CRISPR/Cas9, ERF74, GAI

Introduction

To meet the increasing food demand of a growing human population while developing more sustainable practices, it is necessary to develop enhanced crop varieties with higher yields, improved nutritional content, or strengthened resistance to both biotic and abiotic stresses.

Traditional breeding methods in plant improvement, however, face limitations in terms of efficiency and precision. To address these challenges, the application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas)-mediated genome editing in plants emerges as a promising solution.

The immune CRISPR/Cas system has been adapted from prokaryotes and used by researchers to conduct targeted gene editing across diverse organisms. The mechanism involves a guide RNA (gRNA) designed to align with a specific genomic DNA sequence. The Cas protein is then guided to the target locus, inducing a cut that can lead to gene silencing, repair, or the insertion of new genetic material[1]. For more than a decade, CRISPR/Cas has been employed to modify plant genomes, enabling the study of specific genes or biosynthetic pathways, and accelerating breeding efforts in various plant species, including both model and non-model crops (reviewed in [2]).

In woody species, challenges such as limited transformation and *in vitro* regeneration capabilities, coupled with their inherently slow growth rate, represent bottlenecks for the broader implementation of CRISPR/Cas technology. An alternative approach involves inducing hairy roots in woody plants, offering a more efficient solution compared to the often time-consuming or less effective *A. tumefaciens*-based transformation and subsequent regeneration. Hairy roots are specialized adventitious roots that form in plants as a result of infection with *Agrobacterium* strains harboring a root-inducing (*Ri*) plasmid. During infection, a specific fragment of the *Ri* plasmid (transfer DNA, T-DNA) is transferred into the plant cells and integrated into the plant genome. The expression of the genes encoded by the T-DNA, mainly the *root oncogenic loci* (*rol*) genes, leads to the development of the characteristic hairy roots (reviewed in [3]).

Hairy root cultures, coupled with the CRISPR/Cas technique, provide a rapid and efficient approach to study gene function. This strategy has been widely used in various scientific studies (reviewed in [4]). In woody plants, hairy root cultures successfully facilitated the CRISPR/Cas-based editing of target genes in *Citrus sinensis* and *Poncirus trifoliata*[5], or hybrid poplar (*Populus tremula* × *alba* [6]). Creating composite plants with wild-type shoots and transgenic hairy roots provides a platform to study the gene of interest in the context of the whole plant. This approach has proven successful in studying wood-related genes using CRISPR/Cas-based gene editing in *Eucalyptus grandis*[7].

The genus *Prunus* (Rosaceae) contains a rich diversity of fruit trees and ornamental species, including cherries, plums, peaches, and almonds. While only a few members of this genus have efficient transformation protocols using *A. tumefaciens*, many species and cultivars pose challenges in terms of their recalcitrance to transformation or *in vitro* regeneration[8,9]. As an alternative method for *A. tumefaciens*-mediated transformation, a hairy root transformation protocol has been successfully established for peach[10], as well as for Myrobalan plum and interspecific hybrids of Myrobalan plum x almond-peach[11]. Interestingly, the hybrids combining the genome of three species exhibit superior transformation efficiency compared to genotypes that are pure Myrobalan plums[11].

Almond (*Prunus dulcis*, syn. *Prunus amygdalus*) is a major nut crop of significant economic importance. However, existing transformation protocols using *A. tumefaciens* have demonstrated modest efficiencies, ranging from 0.1 % to 12.3 %[12–14]. In search of an alternative transformation approach, our study focused on the development of a protocol for

83 inducing hairy roots in this species. The use of a fluorescent protein (VENUS-NLS) as a
84 visual marker allowed us to distinguish the wild-type hairy roots (transformed only with the
85 *Ri* plasmid) from those carrying the T-DNA from both the *Ri* plasmid and a binary vector. To
86 evaluate the suitability of the system for studying endogenous gene function, we combined
87 the hairy root transformation system with CRISPR/Cas9 gene editing technology.
88 Specifically, we targeted genes encoding the transcription factors ETHYLENE RESPONSE
89 FACTOR 74 (ERF74) and the DELLA protein GIBBERELLIC ACID INSENSITIVE (GAI).
90 Our transgenic root method represents a significant advancement, paving the way for gene
91 function assays, particularly through the knockout of various genes of interest. This is
92 especially relevant in almond, a species that is highly recalcitrant to transformation.

93

94 **Results**

95 <u>Hairy root induction in *Prunus spp.*</u>

96 To establish an efficient protocol for hairy root transformation in almond, we first evaluated
97 transformation efficiency in seed-grown *P. dulcis* cv. Vairo seedlings. A suspension of
98 *Agrobacterium* C58C1 carrying the virulence *Ri* plasmid was injected into the basal part of
99 the stems of aseptically grown 6-week-old seedlings. Cultivation conditions comprised a
100 temperature of 24 °C and a long-day photoperiod. The initial appearance of calli was observed
101 three to four weeks post-injection. Subsequently, two months after the transformation, hairy
102 roots manifested in 41 ± 7 % of the seedlings (three independent replicates were conducted,
103 each with 12-14 seedlings per replicate).

104 We excised the hairy roots from the stem, established independent hairy root lines, and
105 cultured them on solid media supplemented with cefotaxime (200 mg/L) and ticarcillin
106 (500 mg/L) to inhibit agrobacterial growth. We evaluated three growth media: Murashige and
107 Skoog medium supplemented with B5 vitamins (MS + B5), McCown Woody Plant medium
108 including vitamins (WPM), and Smith, Bailey and Hough medium (SBH). Hairy roots were
109 subcultured every 4 – 5 weeks to a fresh medium. Despite our efforts, the hairy roots
110 exhibited slow growth, and after a few months, they uniformly turned brown across all tested
111 media. Addition of auxin (indole-3-butyric acid [IBA] at concentrations of 0.25 mg/L or
112 0.5 mg/L) did not improve hairy root growth.

113 Consequently, we tested the possibility of growing hairy roots as a part of composite plants.
114 After the appearance of hairy roots, the endogenous roots of the plants were excised, and the
115 plants with emerging hairy roots were transplanted into plant culture boxes with MS + B5
116 medium supplemented with cefotaxime and ticarcillin. The hairy roots in the culture boxes
117 exhibited vigorous growth without any signs of decline. After one month, the plants were
118 transferred to soil and cultivated in phytotrons at 24 °C with a long-day photoperiod. The
119 experimental procedure is shown in Figure 1a.

120 Our study included an evaluation of the co-transformation efficiency using *Agrobacterium*
121 C58C1 carrying the *Ri* plasmid and a binary vector. The optimized procedure for generating
122 composite plants was followed during the experiment. Screening of co-transformed hairy
123 roots was carried out using a fluorescent marker encoded in a binary vector (*p35S:VENUS-*
124 *NLS*). The efficiency of co-transformation varied between 0 % (indicating no positive

125 fluorescent signal in any hairy root of a composite plant) and 75 %. Hairy roots expressing
126 VENUS-NLS are shown in Figure 1b.

127 To evaluate the applicability of the injection-based protocol to other *Prunus* species, we
128 employed the identical procedure to transform an almond x peach hybrid (*P. dulcis* x *P.*
129 *persica*, cv. Monegro). Out of three plants injected with the agrobacterial inoculum, two
130 plants developed hairy roots. The composite almond x peach hybrids were then successfully
131 transplanted into the soil (Figure 1a). Although the replication number was limited in this
132 experiment, the results imply that the methodology may also prove effective in various
133 *Prunus* species or their interspecific hybrids.

134 Construction of CRISPR/Cas9 vectors to mutate two genes coding for transcription factors

135 We used a plant codon optimized Cas9 (pcoCas9) protein derived from *Streptococcus*
136 *pyogenes*. The coding sequence contains the potato IV2 intron for an optimal expression[15] and
137 an SV40 nuclear targeting sequence. The expression was driven by the 35S promoter. The
138 construct also carried a nuclear targeted VENUS fluorescent protein (VENUS-NLS) sequence
139 to monitor the presence of the CRISPR/Cas9 transgene in hairy roots. We aimed to
140 mutagenize two loci encoding *ERF74* and *GAI* in almond (*Prudul26A031706* and
141 *Prudul26A016182* in *P. dulcis* Texas Genome v2.0, respectively).

142 The CRISPR-P 2.0 prediction tool[16] helped to design two guides targeting each of the studied
143 genes. Given the unavailability of the almond genome in this tool, we opted for homologous
144 sequences from the closely related cherry (*P. avium* genome v.1.0;
145 *Pav_sc0000843.1_g200.1.mk* and *Pav_sc0000221.1_g210.1.mk* for *ERF74* and *GAI*,
146 respectively). The high on-score guides, which preferentially target functional domains in
147 ERF74 (AP2 domain) and GAI (DELLA domain and GRAS domain), were selected (Figure
148 2a). The corresponding genomic loci were then amplified and sequenced in the studied
149 almond cultivar to ensure the absence of SNPs. Since the U6 promoter was used for gRNA
150 transcription, we added an extra G at the 5´ end of *ERFguide1* and *GAIguide1*
151 (Supplementary Table S1). This adjustment was made because these guides start with a
152 different nucleotide, thus ensuring accurate transcription[17].

153 Gene editing in almond hairy roots

154 To induce targeted mutations in almond hairy roots, we used *Agrobacterium* carrying both the
155 *Ri* plasmid and the CRISPR vector. Using an optimized protocol, we successfully generated
156 composite plants that were then cultured *in vitro* for one month. The initial step of the *ERF74*
157 analysis involved excision of a hairy root tip (approximately 1 cm in length), which was
158 immediately frozen for subsequent RNA extraction. Another 1 cm fragment of the root was
159 screened for the fluorescent signal and then frozen for genomic DNA isolation (Figure 1c). To
160 analyze *GAI* editing, root tip freezing was omitted, and the analysis focused solely on the
161 fluorescent signal detection and subsequent genomic DNA isolation. When the hairy roots
162 branched, the analysis was performed on both the primary root and the lateral roots. Genomic
163 DNA was extracted from the roots with a fluorescent signal, and the DNA loci containing the
164 target sites were PCR amplified and sequenced. As a control, 2-3 roots without any
165 fluorescent signal were analyzed for selected composite plants.

166 The gene encoding the ERF74 transcription factor was subjected to editing at two loci using
167 *ERFguide1* (exon 1) and *ERFguide2* (exon 2, containing the AP2 functional domain) (Figure

2a). Of the six composite plants screened, three showed a positive fluorescent signal in their hairy roots (Table 1). In the case of the ERF-targeted composite plant number one (E-CP1), we identified one hairy root out of three roots with a fluorescent signal, resulting in a co-transformation efficiency of 33 %. Due to the branching of the root with a positive signal (R1), we analyzed the genomic DNA of the main root and five lateral roots. All six roots exhibited a heterozygous mutation at the locus targeted by *ERFguide2*, with an insertion of 1 bp (+1 bp) in one allele, while the other allele remained unaltered. Thus, this mutation shared by main and lateral roots probably occurred in the early stages of root growth. The locus targeted by *ERFguide1* was not mutated (Figure 2b, Table 1). In E-CP3, no mutations in any of the targeted loci were detected in the three roots positive for the fluorescent signal. In E-CP6, two independent roots showed a positive signal. In one root, we identified a biallelic mutation with an insertion of 1 bp (+1 bp) in exon 2, with A or C inserted in each allele. In the second root, a homozygous deletion of 1 bp (-1 bp) was detected. The sequence targeted by *ERFguide1* was not mutated in either root (Figure 2b, Table 1). As expected, hairy roots R1 and R2 from E-CP2, R2 from E-CP1, and R3 and R4 from E-CP6, where no signal was observed, had no mutations.

To analyze *GAI* locus editing, we examined six composite plants, four of which displayed a fluorescent signal in their hairy roots (Table 1). In composite plants G-CP1 and G-CP6, we did not find any mutations in either of the target loci, the DELLA domain sequence targeted by *GAIguide1* or the GRAS domain sequence targeted by *GAIguide2*. In G-CP4, one hairy root (out of three) had a VENUS signal and a heterozygous mutation with a 2 bp deletion (- 2 bp) in one allele, while the other allele remained wild-type. In G-CP5, we identified one root with a biallelic mutation in the GRAS domain – one allele with a 1 bp insertion (+ 1bp) and the other allele with a 12 bp deletion (-12 bp). Roots lacking a VENUS signal and selected for genomic analysis were all wild-type for the two *GAI* loci.

ERF74 gene transcript analysis in mutant hairy roots

When base insertions or deletions cause a frame shift in the reading frame, premature stop codons may appear, resulting in reduced transcript levels. Thus, we assessed the transcript levels of the *ERF74* gene in hairy roots carrying heterozygous mutations (plant E-CP1, root R1, main root tip and one lateral root tip were analyzed) and biallelic/homozygous mutations (plant E-CP6, roots R1 and R2). As a control, we used hairy roots from the same composite plants without any fluorescent signal and confirmed mutation-free by sequencing (R2 in E-CP1, and R3 in E-CP6). We observed a reduction in *ERF74* transcript levels in both E-CP1 and E-CP6. In roots of E-CP6 carrying a biallelic/homozygous mutation, *ERF74* expression decreased to less than 10 % of the control level (Figure 2c).

Off-target editing analysis

Off-target editing, where the CRISPR/Cas system induces non-specific and unintended mutations in the genome, was examined in almond hairy roots transformed with CRISPR plasmids. Using the Cas-OFFinder tool, we predicted potential off-target sites in the almond (*P. dulcis* cv. Texas) genome for each gRNA. All predicted sites had at least 3 mismatches (MMs) compared to the original on-target sequence (Table 2, Supplementary Table S2).

For analysis, we focused on hairy roots mutated at target sites by CRISPR. Specifically, for the guides targeting *ERF74*, we amplified and sequenced potential off-target sites from one

211 mutant root of E-CP1 (comprising the main and five lateral roots) and from two independent
212 mutant roots of E-CP6. For the guides targeting *GAI*, we examined mutant roots of G-CP4
213 and G-CP5. We evaluated all predicted off-target sites for all guides, except *ERFguide1*. For
214 this guide, we analyzed all loci with 3 MMs out of 26 predicted loci.

215 The amplified off-target sites for *GAIguide1* on chromosome Pd07 and for *GAIguide2* on
216 chromosomes Pd01 and Pd02 differed from the predicted off-target sequence. This difference
217 is due to SNPs between the genome of the transformed cultivar (Vairo) and the reference
218 genome (cv. Texas) used for the *in silico* analysis, as confirmed by the sequencing of wild-
219 type Vairo roots. A total of 10 loci were screened and no mutations were detected in any of the
220 predicted off-target sites (Table 2).

221

Discussion

223 The CRISPR/Cas system is a revolutionary gene-editing tool derived from the bacterial
224 immune system. It enables the precise modification of the genome, such as the insertion,
225 deletion, or exchange of specific DNA sequences. This capability allows researchers to create
226 specific genetic changes in target genes, helping to elucidate their functions.

227 To fully exploit the vast potential of CRISPR/Cas technology in woody plants, the
228 development of robust transformation protocols tailored to the studied species is essential. In
229 almond, conventional transformation methods employing *A. tumefaciens* have achieved a
230 maximum efficiency of 12.3 % [14]. As an alternative approach, we have developed a hairy root
231 induction protocol with high transformation efficiencies, averaging 41 % in the almond
232 cultivar Vairo. Considering the genotype-dependent nature of transformation efficiency, it is
233 plausible that other cultivars may exhibit even higher performance. Our protocol covers the
234 generation of composite plants with vigorously growing hairy roots enabling the study of
235 (trans)genes in the context of the whole plant. Such a composite plant system has been
236 effectively used for gene function analysis in the related species *Prunus persica*[10] and has
237 been shown to be suitable for plant-nematode interaction studies in *Prunus* spp.[11].

238 The efficiency of CRISPR/Cas-based editing can vary significantly, influenced by the choice
239 of the Cas9 protein or guide RNAs. Therefore, for our study, we chose the potent vector for
240 inducing mutations, based on research conducted in *Brassica napus*[18]. This vector carries the
241 nuclear-targeted pcoCas9 derived from *Streptococcus pyogenes* Cas9 (SpCas9), with the
242 potato IV2 intron within the Cas9 sequence[15]. Additionally, the construct includes a
243 fluorescent reporter (VENUS-NLS) to monitor the presence of the T-DNA from the binary
244 vector in hairy roots. To further facilitate the identification of roots containing the transgene, a
245 non-invasive gene expression reporter, such as RUBY[19], which converts tyrosine to vivid red
246 betalain, can also be utilized. This visual indicator is easily detected without the need for
247 special equipment or chemical treatments.

248 In our design, we used two gRNAs selected by the CRISPR-P v.2 tool[16] to enhance
249 mutagenesis efficiency. For both the *ERF74* and *GAI* genes, *guide1* had a slightly higher on-
250 target score (representing the predicted cleavage efficiency of Cas9) compared to *guide2* (see
251 Methods). The higher efficiency scores for *guide1* compared to *guide2* were confirmed by two
252 other gRNA prediction tools[20,21]. Interestingly, *ERF74guide1* and *GAIguide1* failed to induce
253 mutations in almond roots, while *ERFguide2* and *GAIguide2* were successful in targeted

6

254 mutagenesis. This result might be caused by the addition of an extra G at the 5´ end of the
255 *ERFguide1* and *GAIguide1* sequences, done to facilitate efficient transcription from the U6
256 promoter. Previous study in rice and Arabidopsis has demonstrated that such addition has no
257 significant impact on editing efficiencies[17]. Therefore, it is essential to validate the selected
258 guides on the specific genome, and for this purpose, a simple and rapid transformation system
259 such as the hairy root protocol proves invaluable.

260 The use of CRISPR/Cas technology to target transcription factors is a powerful approach for
261 unraveling gene regulation mechanisms, given the central role these proteins play in
262 modulating gene expression. A recent study by Yang et al. (2022) showcases the application
263 of CRISPR/Cas to target *GmNAC12* that encodes a transcription factor of the
264 NAM/ATAF1/2/CUC2 (NAC) superfamily, to elucidate its function in soybean[22]. In the non-
265 model species *Fagopyrum tataricum* and *Scutellaria baicalensis*, CRISPR/Cas was employed
266 to mutate the genes encoding two MYB transcription factors *FtMYB45* and *SbMYB3*,
267 respectively. The studies investigated the involvement of these proteins in flavonoid
268 biosynthesis using a hairy root transformation system[23,24]. In our proof-of-concept study, we
269 aimed to mutate two transcription factors in almond. ERF74, a member of the ERF-VII
270 family, is involved in the regulation of plant response to osmotic and hypoxic stress[25]. The
271 DELLA GAI protein is a repressor of the gibberellin signaling pathway[26]. Hence, there is
272 significant interest in dissecting the function of these transcription factors and exploring how
273 their disruption affects the expression of other genes. The prospect of integrating CRISPR/Cas
274 editing in hairy roots with omics analyses, encompassing transcriptomics and proteomics,
275 represents a promising avenue for future research. This integrated approach offers the
276 potential for rapid, accurate, and cost-effective functional analysis of genes and their
277 associated cellular pathways.

278 In summary, our experiments with composite plants validated the efficiency of CRISPR/Cas-
279 based genome editing in almond, demonstrated a reduction in transcript levels of the selected
280 gene in mutant hairy roots, and revealed the absence of off-target mutations. Despite the
281 primary drawback of CRISPR/Cas-edited transgenic hairy roots, which is the inability to
282 transmit the mutation to progeny, they provide valuable resources for dissecting the functions
283 of target genes without the need for extensive investments in generation of transgenic plants.
284 The composite plant system may also simplify the study of complex root traits, which are
285 typically challenging to breed for. It has the potential to reveal important genes and pathways
286 that enhance root water use, stress tolerance, or fertilizer absorption.

287

Methods

Plant material

290 Almond pits were obtained from cultivar Vairo grown in a commercial plot in Yecla Region of
291 Murcia, Spain. Pits from almond x peach hybrid (cv. Monegro) were obtained from plants
292 grown in a Nursery mother plant plot in Caspe Region of Zaragoza, Spain. The almond and
293 hybrid pits were mechanically removed, and the seeds were placed in a wet tissue at 4°C
294 overnight to soften the testa for easier removal. They were then surface sterilized using
295 chlorine gas for seven hours followed by one hour of ventilation. Sterile seeds were placed in
296 plant cultivation boxes containing Smith, Bailey and Hough (SBH) medium[27] with 1 % Plant

297 Preserve Mixture (PPM, Plant Cell Technology). Cultivation boxes were stored at 4 °C for 45
298 days to fulfill the chilling requirement for germination. The boxes were transferred to the
299 phytotron (24 °C, long-day photoperiod). Six-week-old seedlings were used for the
300 transformation process.

301 **CRISPR/Cas9 vector construction**

302 The two guide RNAs (gRNA) targeting the coding sequences of the *ERF74* and *GAI* genes
303 were designed using the CRISPR-P v2.0 prediction tool[16]. Since the almond genome sequence
304 was not available in this tool, we used the genome sequence of the closely related *P. avium*
305 species (*P. avium* genome v.1.0; *Pav_sc0000843.1_g200.1.mk* and
306 *Pav_sc0000221.1_g210.1.mk* for *ERF74* and *GAI*, respectively). The predicted efficiency
307 scores for the guides were as follows: 0.5387 for *ERFguide1*, 0.4083 for *ERFguide2*, 0.7625
308 for *GAIguide1*, and 0.6448 for *GAIguide2*. Predicted gRNA efficiency scores were also
309 calculated using the SSC and CHOPCHOP tools[20,21]. The genomic loci corresponding to these
310 sequences were amplified and sequenced in the studied almond cultivar to confirm the
311 absence of SNPs. The sequences have been deposited at the Zenodo repository
312 (https://zenodo.org/doi/10.5281/zenodo.10945763). The guides were synthesized as
313 oligonucleotides with added Esp3I sites (Supplementary Table S1) and annealed by boiling
314 and slow cooling. They were used for the standard assembly protocol with the prepared
315 universal plasmids. Plasmid construction was performed by modular cloning using the MoClo
316 Tool Kit and the MoClo Plant Parts Kit (Addgene, [28–30]). A detailed MoClo protocol and the
317 list of vectors used can be found in [18].

318 **Hairy root transformation**

319 Hairy roots were obtained by transforming almond seedlings with the transconjugant *Ti*-less
320 *A. tumefaciens* C58C1 carrying a hairy-root-inducing plasmid *pRiA4b*[31]. The agrobacterial
321 suspension was grown in Luria-Broth medium at 28 °C until the OD_{600} reached a value
322 between 0.9 and 1. The suspension was injected with an insulin syringe into the basal part of
323 the seedling stem. After 6-8 cultivation weeks, hairy roots emerging from the inoculation sites
324 were excised and placed on one of the solid media: 1) Murashige and Skoog medium
325 including Gamborg B5 vitamins (MS + B5, Duchefa; 4.4 g/L), 2) McCown Woody Plant
326 medium including vitamins (WPM, Duchefa, 2.46 g/L), or 3) SBH medium[27]. All media were
327 supplemented with 0.3 % phytagel, 30 g/L sucrose, 500 mg/L ticarcillin, and 200 mg/L
328 cefotaxime. Hairy roots were grown on Petri dishes at 24 °C in the dark and transferred to
329 fresh media after 5-6 weeks of cultivation.

330 To generate composite plants, the endogenous roots were removed after emergence of hairy
331 roots, and the composite plants were transferred to the cultivation boxes with fresh MS + B5
332 medium supplemented with cefotaxime (200 mg/L) and ticarcillin (500 mg/L) and grown at
333 24 °C with a long-day photoperiod. After a month, the plants were transferred to the soil and
334 cultivated at the same conditions.

335 **Detection of the VENUS signal**

336 A Zeiss Axio Imager Z2 microscope was used to detect VENUS fluorescence signals in the
337 hairy roots. The roots remained unfixed during screening to ensure their availability for
338 subsequent genomic DNA extraction. For confocal microscopy (Figure 1b), hairy roots were
339 fixed in 4 % paraformaldehyde (PFA) in PBS-T (pH 7.4) for one hour under vacuum

340   conditions at 4 °C. Subsequently, the samples underwent three one-hour washes in PBS-T (pH

341   7.4) and were cleared with fresh ClearSee alpha solution for 5 days. The counterstain SCRI

342   Renaissance 2200 (Renaissance Chemicals Ltd) labeled the cell walls. Visualization was

343   conducted using an upright microscope Zeiss Axio Imager Z2 with a confocal unit LSM 700.

344   Two laser lines were used: a 405 nm wavelength for imaging SCRI Renaissance 2200, and

345   488 nm for VENUS. The images were processed with ZEN black software.

Analysis of gene editing in composite plants

347   The fragment of the hairy root (1 cm) screened for the fluorescence signal was frozen at -

348   80 °C. Tissues were ground in liquid nitrogen, and genomic DNA was isolated using a

349   cetyltrimethylammonium bromide (CTAB) method[32]. The target loci in *ERF74* and *GAI* genes

350   were PCR amplified using Phusion High-Fidelity DNA polymerase (New England Biolabs)

351   and gene-specific primers (Supplementary Table S1), and sequenced. The chromatograms

352   were decoded manually or using the tool TIDE[33]. For G-CP5 root R1, amplified fragments

353   were subcloned into the pGEM-T-Easy Vector (Promega), and 6 clones were sequenced. The

354   chromatograms have been deposited at the Zenodo repository

355   (https://zenodo.org/doi/10.5281/zenodo.10945763).

Off-target analysis

357   Using the Cas-OFFinder tool[34], we predicted potential off-target sites in the almond genome

358   (*P. dulcis* cv. Texas v2.0, Genome Database for Rosaceae[35]) for each gRNA, allowing up to 4

359   mismatches (MMs) in the sequences. All predicted sites are listed in Supplementary Table S2.

360   The sequence analysis conducted on the loci Pd06:+3018280 and Pd06:+3042881 revealed

361   that the off-target sites of *ERFguide2*, along with the neighboring regions (250 bp up- and

362   down-stream to the target site), displayed 100% homology. Thus, only one primer pair was

363   designed to amplify both regions (Supplementary Table S1). The genomic DNA sequences

364   surrounding the potential off-target sites selected for further analysis (Table 2) were amplified

365   by PCR using specific primers (Supplementary Table S1) and PrimeSTAR GXL DNA

366   Polymerase (Takara) from CRISPR/Cas-edited hairy roots (R1 of E-CP1 [the main root and

367   five lateral roots], R1 and R2 of E-CP6, R1 of G-CP4, and R1 of G-CP5). PCR products were

368   analyzed by sequencing. The amplified off-target sites for *GAIguide1* (Pd07: +4850919) and

369   for *GAIguide2* (Pd01: +8709365 and Pd02: -14236738) in Vairo hairy roots were divergent

370   from the *in silico* predicted off-target sequence derived from the cultivar Texas. Thus, the

371   corresponding genomic regions were amplified from Vairo wild-type roots and sequenced.

RNA analysis

373   We extracted total RNA from hairy root tips using TRIzol reagent (Invitrogen) according to

374   the manufacturer's protocol. To eliminate any contaminant DNA, the RNA isolates were

375   treated with TURBO DNase (Invitrogen). Subsequently, cDNA was synthesized using

376   SuperScript III Reverse Transcriptase (Invitrogen) with 0.3 µg of RNA from the hairy roots.

377   PCR amplification was carried out using the FastStart Essential DNA Green Master (Roche)

378   on the QuantStudio 12K Flex (Applied Biosystems). Actin (*LOC117630898*) served as the

379   internal reference gene. The corresponding sequence was amplified and sequenced in the

380   Vairo cultivar to confirm the absence of any SNPs. We assessed the efficiency of each primer

381   pair (Supplementary Table S1) by generating a standard curve with five serial dilutions. Each

382   sample was analyzed in technical triplicate. Relative gene expression levels were determined
383   following the method outlined by [36].

384

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462

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Author contributions

VJ, JG, MJRC, and HSR conceived the project. VJ performed the hairy root culture experiments and CRISPR-mutagenesis screen, analyzed and interpreted the data. MŠ performed the RT-qPCR expression analysis, cared for the plants, and documented plant growth. JFSL performed the clearing and imaging of roots. VJ and HSR drafted the manuscript. All authors read and approved the manuscript.

482

Data availability statement

The data generated during the current study are included in this published article (and its Supplementary Information file), and are available in the Zenodo repository [https://zenodo.org/doi/10.5281/zenodo.10945763].

487

Competing interests

The authors declare no competing interests.

490

Figure legends

492 **Figure 1.** Composite plants generation and screening of hairy roots. (**a**) Transformation of
493 almond or almond x peach hybrid with *Agrobacterium* carrying the *Ri* plasmid and generation
494 of composite plants (scale bars represent 3 cm). (**b**) Detection of VENUS fluorescence signal
495 in almond hairy roots co-transformed with the *Ri* plasmid and a binary vector encoding the
496 nuclear-targeted fluorescent protein VENUS-NLS. Scale bars represent 100 µm (left) and 20
497 µm (right). (**c**) Composite plants generated by transforming almonds using *Agrobacterium*
498 carrying the *Ri* plasmid and a CRISPR vector. For the analysis of *ERF74* editing in hairy
499 roots, the root tip was frozen for RNA isolation, while the remaining portion of the root was
500 screened for VENUS fluorescence signals and subsequently used for genomic DNA
501 extraction. To analyze *GAI* editing, the freezing of root tips was omitted, and the analysis
502 focused solely on detecting the VENUS fluorescence signal and subsequent genomic DNA
503 isolation.

504 **Figure 2.** Design of CRISPR constructs and gene editing analysis in hairy roots. (**a**) Structure
505 of the *ERF74* and *GAI* genes. The arrowheads indicate the positions of the two gRNAs for
506 each gene. The PAM sequences in the target sites are bolded. (**b**) Targeted mutagenesis in
507 almond hairy roots compared to the wild-type sequence. The gRNA target sites are
508 underlined, and the PAM sequences are bolded. Indels are indicated in red. (**c**) RT-qPCR
509 analysis of the *ERF74* gene in mutant hairy roots. The transcript levels of the *ERF74* gene
510 were analyzed in hairy roots carrying heterozygous mutations in composite plant E-CP1
511 (main root tip R1a and a lateral root tip R1b) and biallelic/homozygous mutations in plant E-
512 CP6 (roots R1 and R2). Hairy roots from the same composite plants lacking any VENUS
513 fluorescence signal and confirmed to be mutation-free by sequencing were used as controls
514 (R2 in E-CP1, and R3 in E-CP6).

515

516 **Tables**

517 **Table 1.** Gene editing in almond hairy roots. Hairy roots of composite plants (CP) were
518 screened for VENUS fluorescence signal (the number of roots with detected signals out of all
519 screened roots is indicated for each composite plant). The target loci in *ERF74* and *GAI* genes
520 were sequenced in roots positive for VENUS fluorescence signal (+) and in selected roots
521 without any signal (-).

522

Target	Plant	n of VENUS positive roots (n of all roots)	Root (positive or negative for VENUS signal)	Genomic analysis locus 1	Genomic analysis locus 2
ERF74	E-CP1	1 (3)	R1 (+)	wt	wt / +1 bp
			R2 (-)	wt	wt
	E-CP2	0 (3)	R1 (-)	wt	wt
			R2 (-)	wt	wt
	E-CP3	3 (7)	R1 (+)	wt	wt
			R2 (+)	wt	wt
			R3 (+)	wt	wt
	E-CP4	0 (6)	-	-	-
	E-CP5	0 (2)	-	-	-

	E-CP6	2 (4)	R1 (+)	wt	+ 1 bp / +1 bp
			R2 (+)	wt	- 1 bp / -1 bp
			R3 (-)	wt	wt
			R4 (-)	wt	wt
GAI	G-CP1	2 (6)	R1 (+)	wt	wt
			R2 (+)	wt	wt
	G-CP2	0 (3)	R1 (-)	wt	wt
	G-CP3	0 (1)	-	-	-
	G-CP4	1 (3)	R1 (+)	wt	wt / -2 bp
			R2 (-)	wt	wt
	G-CP5	1 (3)	R1 (+)	wt	+1 bp / -12 bp
			R2 (-)	wt	wt
	G-CP6	1 (3)	R1 (+)	wt	wt

Table 2. Off-target editing analysis. Potential off-target sites were predicted using the Cas-OFFinder tool in the almond genome (*P. dulcis* cv. Texas). The PAM motif is highlighted in bold, and the mismatched bases to the original target sites are shown in red lower-case letters. MMs indicate the number of mismatches. Mutation rate represents the number of hairy roots with mutations divided by the total number of tested hairy roots. * indicates the amplified off-target site in the studied cultivar that differed from the *in silico* predicted off-target in cultivar Texas. Consequently, the corresponding genomic regions were sequenced in wild-type roots of the studied cultivar and compared to those from hairy roots.

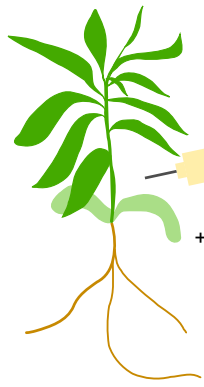| gRNA	Putative off-target locus	Sequence	MMs	Mutation rate
ERFguide1	Pd01: -16219319	aTTTCCAGCAGAaAAAaCAG**TGG**	3	0/8
	Pd03: -9068856	gTTTCCAGgAGAGAAtGCAG**TGG**	3	0/8
	Pd04: -9549693	agTTCCAGCAGAGAAgGCAG**GGG**	3	0/8
ERFguide2	Pd04: -22567141	GGAgTcCGCaAGCGgCCATG**GGG**	4	0/8
	Pd06: +3018280	GGAgTcCGCCgGCGaCCATG**GGG**	4	0/8
	Pd06: +3042881	GGAgTcCGCCgGCGaCCATG**GGG**	4	0/8
GAIguide1	Pd07: +4850919	TACaGTTCgCTATAcaCCGT**TGG**	4	0/2*
GAIguide2	Pd01: +8709365	aaAACtgATCGGAGAATGAG**TGG**	4	0/2*
	Pd02: -14236738	GCAAaATATaaGAaAATGAG**AGG**	4	0/2*
	Pd08: -6198750	GCAtgATATCGaAGAATGAa**AGG**	4	0/2

Supplementary Table S1. List of oligonucleotides.

Supplementary Table S2. List of all potential off-target sites predicted by the Cas-OFFinder tool in the almond genome (*P. dulcis* cv. Texas).

a

ERF74

GGAATTCGCCAGCGCCCATG**GGG**
▶ ERFguide2

AP2

◀ERFguide1
CCTCTGCTTTCTCTGCTGGAAAG

GAI

TACCGTTCACTATAACCCGT**CGG**
▶GAIguide1

DELLA   GRAS

◀GAIguide2
CCACTCATTCTCCGATATGTTGC

c

ERF74 expression
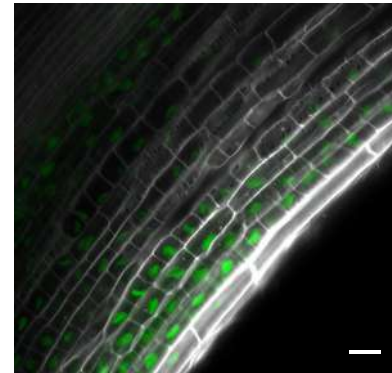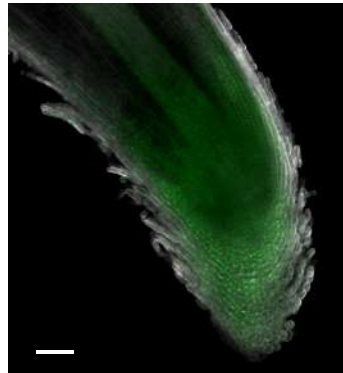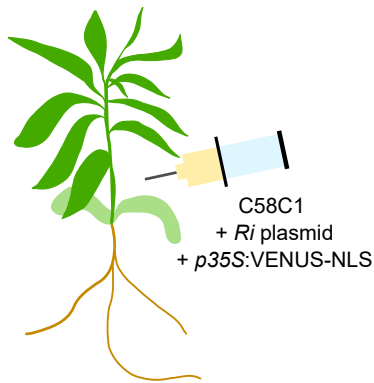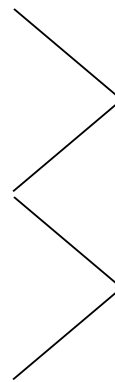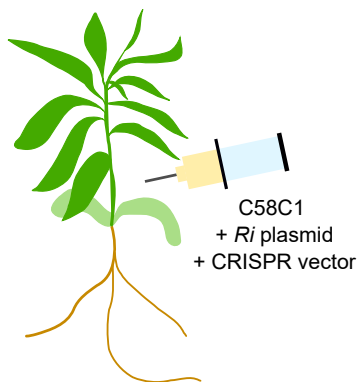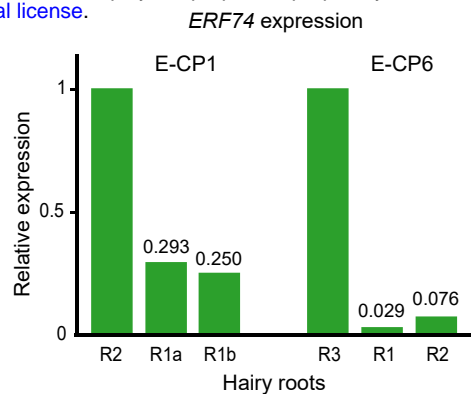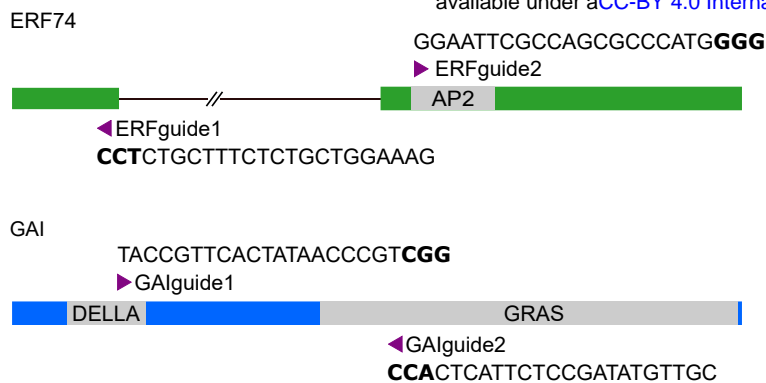
b

ERF74

```
                ERFguide1                              ERFguide2
WT        AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCCCATGGGGTAAG

E-CP1-R1  AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCCC-ATGGGGTAAG
          AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCCCCATGGGGTAAG

E-CP6-R1  AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCCCAATGGGGTAAG
          AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCCCCATGGGGTAAG

E-CP6-R2  AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCC-ATGGGGTAAG
          AAGCCCTCTGCTTTCTCTGCTGGAAAGCCCT // CAGGGGAATTCGCCAGCGCC-ATGGGGTAAG
```

GAI

```
                GAIguide1                              GAIguide2
WT        CTGATACCGTTCACTATAACCCGTCGGATCT // TCGACCACTCATTCTCCGATATGTTGCAGAT

G-CP4-R1  CTGATACCGTTCACTATAACCCGTCGGATCT // TCGACCACTCATTCTCCGATATGTTGCAGAT
          CTGATACCGTTCACTATAACCCGTCGGATCT // TCGACCACTC--TCTCCGATATGTTGCAGAT

G-CP5-R1  CTGATACCGTTCACTATAACCCGTCGGATCT // TCGACCACTCAATTCTCCGATATGTTGCAGAT
          CTGATACCGTTCACTATAACCCGTCGGATCT // TCGACCACTC------------GTTGCAGAT
```