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ORIGINAL ARTICLE

Genome editing in almond using hairy root transformation system

Veronika Jedličková[1] · Marie Štefková[1] · Juan Francisco Sánchez López[1,2] · Jérôme Grimplet[3,4] · María José Rubio Cabetas[3,4] · Hélène S. Robert[1]

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Abstract

Woody plant species are often challenging to transform, which complicates gene function studies using molecular biology techniques. To develop an effective gene editing tool for almond (*Prunus dulcis*, syn. *Prunus amygdalus*), we established a new method for inducing hairy roots in almond seedlings based on the injection of agrobacterial culture. We generated composite plants comprising wild-type shoots and transgenic hairy roots. This approach was also successful in almond x peach hybrids. The hairy root transformation system was used alongside Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 gene editing to target two transcription factor genes (*ETHYLENE RESPONSE FACTOR 74* and *GIBBERELLIC ACID INSENSITIVE*). We successfully generated hairy roots with knockouts of the target genes and evaluated the efficiency of guide RNAs for gene editing in almond. Our transgenic root method could be a valuable tool for routine gene function studies in almond.

Key message

Hairy root induction and composite plant generation were proved to be a suitable system for gene editing using CRISPR/Cas9 in almond, a species highly recalcitrant to transformation.

Keywords *Prunus dulcis* · Hairy root culture · Composite plants · CRISPR/Cas9 · ERF74 · GAI

Abbreviations

CP	Composite plant
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
ERF74	ETHYLENE RESPONSE FACTOR 74
GAI	GIBBERELLIC ACID INSENSITIVE
gRNA	Guide RNA
MS + B5	Murashige and Skoog medium with Gamborg B5 vitamins
NLS	Nuclear localization signal
pcoCas9	Plant codon optimized Cas9
Ri	Root-inducing
rol	Root oncogenic loci
SBH	Smith, Bailey and Hough (medium)
T-DNA	Transfer DNA
WPM	McCown Woody Plant medium
Wt	Wild-type

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✉ Hélène S. Robert
helene.robert.boisivon@ceitec.muni.cz

[1] Hormonal Crosstalk in Plant Development, Mendel Center for Plant Genomics and Proteomics, CEITEC MU—Central European Institute of Technology, Masaryk University, Brno 625 00, Czech Republic

[2] National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno 625 00, Czech Republic

[3] Departamento de Ciencia Vegetal, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Zaragoza 500 59, Spain

[4] Instituto Agroalimentario de Aragón-IA2, CITA-Universidad de Zaragoza, Zaragoza 500 13, Spain

Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) system has been adapted from prokaryotes and used by researchers to conduct targeted gene editing across diverse organisms. For over a decade, CRISPR/Cas has been

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employed to modify plant genomes, enabling the study of specific genes or biosynthetic pathways, and accelerating breeding efforts in various plant species, including model and non-model crops (reviewed in Cardi et al. 2023). 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 (Doudna and Charpentier 2014).

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 than the often time-consuming or less effective *A. tumefaciens*-based transformation and subsequent regeneration. Hairy roots are specialized adventitious roots that form in plants due to 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 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 Christey 2001).

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 Kiryushkin et al. 2022). In woody plants, hairy root cultures successfully facilitated the CRISPR/Cas-based editing of target genes in *Citrus sinensis* and *Poncirus trifoliata* (Wang et al. 2023), or hybrid poplar (*Populus tremula* × *alba* (Triozzi et al. 2021). Creating composite plants with wild-type shoots and transgenic hairy roots provides a platform to study a gene of interest in the context of the whole plant. This approach has been successfully employed for studying wood-related genes using CRISPR/Cas-based gene editing in *Eucalyptus grandis* (Dai et al. 2020).

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 regarding their recalcitrance to transformation or in vitro regeneration (Mir and Patel 2018; Ricci et al. 2020). As an alternative method for *A. tumefaciens*-mediated transformation, a hairy root transformation protocol has been successfully established for peach (Xu et al. 2020), as well as for Myrobalan plum and interspecific hybrids of Myrobalan plum x almond-peach (Bosselut et al. 2011).

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% (Miguel and Oliveira 1999; Costa et al. 2006; Ramesh et al. 2006). In search of an alternative transformation method, our study focused on developing a protocol for inducing hairy roots in this species. To evaluate the suitability of the system for studying endogenous gene function, we combined the hairy root transformation with CRISPR/Cas9 gene editing technology. Specifically, we targeted genes encoding the transcription factors ETHYLENE RESPONSE FACTOR 74 (ERF74) and the DELLA protein GIBBERELLIC ACID INSENSITIVE (GAI). ERF74, a member of the ERF-VII family, is involved in the regulation of plant response to osmotic and hypoxic stress (Yao et al. 2017). The DELLA GAI protein is a repressor of the gibberellin signaling pathway (Peng et al. 1997). The sequence of the *GAI* gene and its promoter has been studied in detail in another nut tree, the Persian walnut (*Juglans regia*) (Mohseniazar et al. 2021). Hence, there is significant interest in dissecting the function of these factors and exploring how their disruption affects the expression of other genes. Our transgenic root method represents a significant advancement, facilitating gene function assays through the knockout of various genes of interest. This is particularly relevant for almond, a species highly recalcitrant to transformation.

Materials and methods

Plant material

Almond pits were obtained from cultivar Vairo grown in a commercial plot in Yecla Region of Murcia, Spain. Pits from almond x peach hybrid (cv. Monegro) were obtained from plants grown in a Nursery mother plant plot in Caspe Region of Zaragoza, Spain. The almond and hybrid pits were mechanically removed, and the seeds were placed in a wet tissue at 4 °C overnight to soften the testa for easier removal. They were then surface sterilized using chlorine gas for seven hours, followed by one hour of ventilation. Sterile seeds were placed in plant cultivation boxes containing Smith, Bailey and Hough (SBH) medium (Smith et al. 1969) with 1% Plant Preserve Mxture (PPM, Plant Cell Technology). Cultivation boxes were stored at 4 °C for 45 days to fulfill the chilling requirement for germination. The boxes were transferred to the phytotron (24 °C, long-day photoperiod). Six-week-old seedlings were used for the transformation process.

CRISPR/Cas9 vector construction

The two guide RNAs (gRNA) targeting the coding sequences of the *ERF74* and *GAI* genes were designed using the CRISPR-P v2.0 prediction tool (Liu et al. 2017). Since the almond genome sequence was not available in this tool, we used the genome sequence of the closely related *P. avium* species (*P. avium* genome v.1.0; *Pav_sc0000843.1_g200.1.mk* and *Pav_sc0000221.1_g210.1.mk*). The predicted efficiency scores for *ERFguide1*, *ERFguide2*, *GAIguide1*, *GAIguide2* were 1.2503, -0.1317, 0.2878, -0.1053 by the SSC tool (Xu et al. 2015), and 68.49, 62.27, 67.99, 59.47 by the CHOPCHOP tool (Montague et al. 2014). The genomic loci corresponding to the target sites were amplified with specific primers (Supplementary Table S1) and PrimeSTAR GXL DNA Polymerase (Takara) from DNA of *Prunus dulcis* seedlings extracted by a cetyltrimethylammonium bromide (CTAB) method (Allen et al. 2006). The PCR fragments were sequenced to confirm the absence of SNPs in the studied almond cultivar. The sequences have been deposited at the Zenodo repository [https://zenodo.org/doi/10.5281/zenodo.10945763]. Plasmid construction was performed by modular cloning using the MoClo Tool Kit and the MoClo Plant Parts Kit (Addgene, (Weber et al. 2011; Werner et al. 2012; Engler et al. 2014). For the Cas9 cassette cloning in L1 (p35S: Cas9-NLS: t35S), we used Addgene plasmids pICH47811, pICH41373, pAGM533, pICH41414, and plasmid containing the pco-Cas9 cds (Li et al. 2013). For the VENUS cassette cloning in L1 (p35S: VENUS-NLS: t35S), we used Addgene plasmids pICH47751, pICH41388, pAGM5331, pJOG171, and pICH41414. For guide RNA cassette cloning (pAtU6:guide RNA1/2:RNAscaffold), we used our previously constructed plasmids (pL1M-F4-pAtU6-LacZ-pcoCas9-RNA-scaffold and pL1M-F5-pAtU6-LacZ-pcoCas9-RNA-scaffold) containing an AtU6-26 promoter, followed by two Esp3I sites for guide RNA insertion instead of a counter-selective LacZ gene, and a scaffold gRNA sequence (Jedličková et al. 2022). The guides were synthesized as oligonucleotides with added Esp3I sites (Supplementary Table S1), annealed by boiling and slow cooling and inserted into the constructs by restriction reaction with Esp3I. For the L2 cloning, we combined all the cassettes and the backbone (Addgene plasmid pAGM4673). The final plasmids were sequenced before use.

Hairy root transformation

Hairy roots were obtained by transforming almond seedlings with the transconjugant *Ti*-less *A. tumefaciens* C58C1 carrying a hairy-root-inducing plasmid *pRiA4b* (Petit et al. 1983). The agrobacterial suspension was grown in Luria-Broth medium at 28 °C until the OD_{600} reached a value between 0.9 and 1. The suspension was injected with an insulin syringe into the basal part of the seedling stem. After 6–8 cultivation weeks, hairy roots emerging from the inoculation sites were excised and placed on one of the solid media: (1) Murashige and Skoog medium including Gamborg B5 vitamins (MS + B5, Duchefa; 4.4 g/L), (2) McCown Woody Plant medium including vitamins (WPM, Duchefa, 2.46 g/L), or (3) SBH medium (Smith et al. 1969). All media were supplemented with 0.3% phytagel, 30 g/L sucrose, 500 mg/L icarcillin, and 200 mg/L cefotaxime. Hairy roots were grown on Petri dishes at 24 °C in the dark and transferred to fresh media after 4–5 weeks of cultivation.

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

Detection of the VENUS signal

A Zeiss Axio Imager Z2 microscope was used to detect VENUS fluorescence signals in the hairy roots. The roots remained unfixed during screening to ensure their availability for subsequent genomic DNA extraction. For confocal microscopy (Fig. 1b and c), wild-type hairy roots and hairy roots co-transformed with the binary vector were fixed in 4% paraformaldehyde (PFA) in PBS-T (pH 7.4) for one hour under vacuum at 4 °C. Subsequently, the samples underwent three one-hour washes in PBS-T (pH 7.4) and were cleared with fresh ClearSee alpha solution for 5 days. The counterstain SCRI Renaissance 2200 (Renaissance Chemicals Ltd) labeled the cell walls. Visualization was conducted using an upright microscope Zeiss Axio Imager Z2 with a confocal unit LSM 700. Two laser lines were used: a 405 nm excitation wavelength for imaging SCRI Renaissance 2200, and 488 nm for VENUS. The images were processed with ZEN black software.

Analysis of gene editing in composite plants

The fragment of the hairy root (1 cm) screened for the fluorescence signal was frozen at -80 °C. Tissues were ground in liquid nitrogen, and genomic DNA was isolated using a CTAB method (Allen et al. 2006). The target loci in *ERF74* and *GAI* genes were PCR amplified using Phusion High-Fidelity DNA polymerase (New England Biolabs) and gene-specific primers (Supplementary Table S1), and sequenced. The chromatograms were decoded manually or using the TIDE web tool (Brinkman et al. 2014). For G-CP5 Root1,

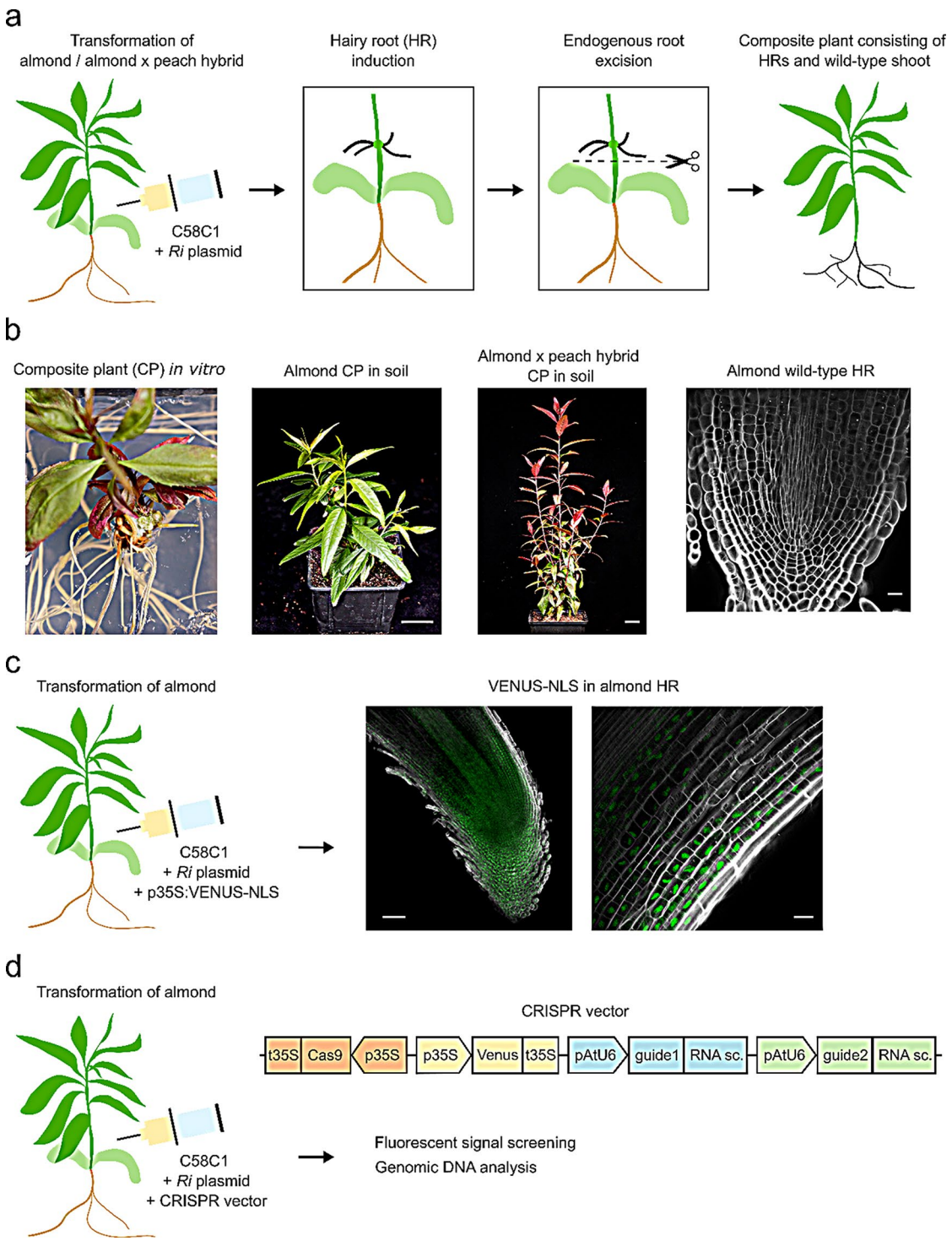

Fig. 1 Composite plant generation and screening of transformed hairy roots. (**a**). Workflow scheme for the transformation of almond or almond x peach hybrid with *Agrobacterium* carrying the *Ri* plasmid and generation of composite plants. (**b**) Photo documentation of composite plants, both in vitro and in soil (scale bars represent 3 cm), and confocal microscopy image of wild-type hairy root (i.e., transformed only with the *Ri* plasmid; scale bar represents 20 µm). (**c**) Detection of VENUS fluorescence signal in almond hairy roots transformed with *Agrobacterium* containing the *Ri* plasmid and a binary vector encoding the nuclear-targeted fluorescent protein VENUS-NLS. Scale bars represent 100 µm (left) and 20 µm (right). (**d**) Generation of almond composite plants using *Agrobacterium* carrying the *Ri* plasmid and a CRISPR vector consisting of the Cas9 cassette (p35S: Cas9-NLS: t35S), the VENUS cassette (p35S: VENUS-NLS: t35S), and two guide RNA cassettes (AtU6 promoter, guide1 or guide2, RNA scaffold). Hairy roots were screened for VENUS fluorescence signals and subsequently used for analysis of the genomic DNA for mutations

amplified fragments were subcloned into the pGEM-T-Easy Vector (Promega), and 6 clones were sequenced. The chromatograms have been deposited at the Zenodo repository [https://zenodo.org/doi/10.5281/zenodo.10945763].

RNA analysis

We extracted total RNA from individual hairy root tips using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To eliminate any contaminant DNA, the RNA isolates were treated with TURBO DNase (Invitrogen). Subsequently, cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) with 0.3 µg of RNA from the hairy roots and anchored oligo (dT)20 primer. PCR amplification was carried out using the FastStart Essential DNA Green Master (Roche) on the QuantStudio 12 K Flex (Applied Biosystems) with 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 25 s. PCR reactions (10 µL) contained 2.5 µL of cDNA (8x diluted), 5 µL SYBR Green mix, 0.5 µL of each primer (10 µM) and 1.5 µL of H2O. Actin (*LOC117630898*) served as the internal reference gene (the corresponding sequence of actin was amplified and sequenced in the Vairo cultivar to confirm the absence of any SNPs in the primer binding sites). We assessed the efficiency of each primer pair (Supplementary Table S1) by generating a standard curve with five serial dilutions. Each sample was analyzed in technical triplicate. Relative gene expression levels were determined following the method outlined by Pfaffl (2001).

Off-target analysis

Using the Cas-OFFinder tool (Bae et al. 2014), we predicted potential off-target sites in the almond genome (*P. dulcis* cv. Texas v2.0, Genome Database for Rosaceae (Jung et al. 2019) for each gRNA, allowing up to 4 mismatches (MMs) in the sequences. All predicted sites are listed in Supplementary Table S2. The sequence analysis conducted on the loci Pd06:+3018280 and Pd06:+3042881 revealed that the off-target sites of *ERFguide2*, along with the neighboring regions (250 bp up- and down-stream to the target site), displayed 100% homology. Thus, only one primer pair was designed to amplify both regions (Supplementary Table S1). The genomic DNA sequences surrounding the potential off-target sites selected for further analysis (Table 1) were amplified by PCR using specific primers (Supplementary Table S1) and PrimeSTAR GXL DNA Polymerase (Takara) from CRISPR/Cas-edited hairy roots (Root1 of E-CP1 [the main root and five lateral roots], Root1 and Root2 of E-CP6, Root1 of G-CP4, and Root1 of G-CP5). PCR products were analyzed by sequencing.

Results

An improved protocol for hairy root induction in *Prunus* spp.

To establish an efficient protocol for hairy root transformation in almond, we first evaluated transformation efficiency in seed-grown *P. dulcis* cv. Vairo seedlings. A suspension of *Agrobacterium* C58C1 carrying the virulence *Ri* plasmid was injected into the basal part of the stems of aseptically grown 6-week-old seedlings. Cultivation conditions comprised a temperature of 24 °C and a long-day photoperiod. The initial appearance of calli was observed three to four weeks post-injection. Subsequently, two months after the transformation, hairy roots manifested in 41 ± 7% of the

Table 1 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 Vairo 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

seedlings (three independent replicates were conducted, each with 12–14 seedlings per replicate). In one seedling, we usually observed 2–4 hairy roots.

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

Consequently, we tested the possibility of growing hairy roots as a part of composite plants. After the appearance of hairy roots, the endogenous roots of the plants were excised, and the plants with emerging hairy roots were transplanted into plant culture boxes with MS + B5 medium supplemented with cefotaxime and ticarcillin (Fig. 1a). The hairy roots in the culture boxes exhibited vigorous growth without any signs of decline (Fig. 1b). After one month, the plants were transferred to soil and cultivated in phytotrons at 24 °C with a long-day photoperiod (Fig. 1b).

Our study included an evaluation of the co-transformation efficiency using *Agrobacterium* C58C1 carrying the *Ri* plasmid and a binary vector. The optimized procedure for generating composite plants was followed during the experiment. Screening of co-transformed hairy roots was carried out using a fluorescent marker encoded in a binary vector (p35S: VENUS-NLS). The efficiency of co-transformation varied between 0% (indicating no positive fluorescent signal in any hairy root of a composite plant) and 75%. The VENUS fluorescent signal was visible in the nuclei of transformed hairy root cells, demonstrating the efficiency of co-transformation and the suitability of hairy root transformation to map gene expression (Fig. 1c).

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

Constructing CRISPR/Cas9 vectors to mutate *ERF74* and *GAI* genes

We used a plant codon optimized Cas9 (pcoCas9) protein derived from *Streptococcus pyogenes*. The coding sequence contains the potato IV2 intron for an optimal expression (Li et al. 2013) and an SV40 nuclear targeting sequence. The expression was driven by the 35S promoter. The construct also carried a nuclear targeted VENUS fluorescent protein (VENUS-NLS) sequence to monitor the presence of the CRISPR/Cas9 transgene in hairy roots (Fig. 1d).

We aimed to mutagenize two loci encoding *ERF74* and *GAI* in almond (*Prudul26A031706* and *Prudul26A016182* in *P. dulcis* Texas Genome v2.0, respectively). The CRISPR-P 2.0 prediction tool (Liu et al. 2017) helped to design two guides targeting each of the studied genes. Given the unavailability of the almond genome in this tool, we opted for homologous sequences from the closely related cherry (*P. avium* genome v.1.0). *Pav_sc0000843.1_g200.1.mk* shares 97% nucleotide seqence identity with *Prudul26A031706* in the coding region, and *Pav_sc0000221.1_g210.1.mk* shares 98% nucleotide idetity with *Prudul26A016182* in the coding region. The high on-score guides, which preferentially target functional domains in ERF74 (AP2 domain) and GAI (DELLA domain and GRAS domain), were selected (Fig. 2a). The predicted efficiency scores from CRISPR-P 2.0 tool were as follows: 0.5387 for *ERFguide1*, 0.4083 for *ERFguide2*, 0.7625 for *GAIguide1*, and 0.6448 for *GAIguide2*. The corresponding genomic loci were then amplified and sequenced in the studied almond cultivar to ensure the absence of SNPs. Since the U6 promoter was used for gRNA transcription, we added an extra G at the 5′ end of *ERFguide1* and *GAIguide1* (Supplementary Table S1) to ensure accurate transcription (Ma et al. 2015).

Editing *ERF74* and *GAI* loci in almond hairy roots showcased the effectiveness of the transformation protocol

To induce targeted mutations in almond hairy roots, we used *Agrobacterium* carrying both the *Ri* plasmid and the CRISPR vector. Using an optimized protocol, we successfully generated composite plants that were then cultured in vitro for one month. Subsequently, we screened approximately 1 cm fragments of each hairy root for the fluorescent signal. The root fragment was then frozen for genomic DNA isolation. When the hairy roots branched, the analysis was performed on both the primary root and the lateral roots. Roots positive for the fluorescence signal were evaluated for the presence of indels at the targeted loci by sequencing the PCR-amplified genomic DNA fragments containing the

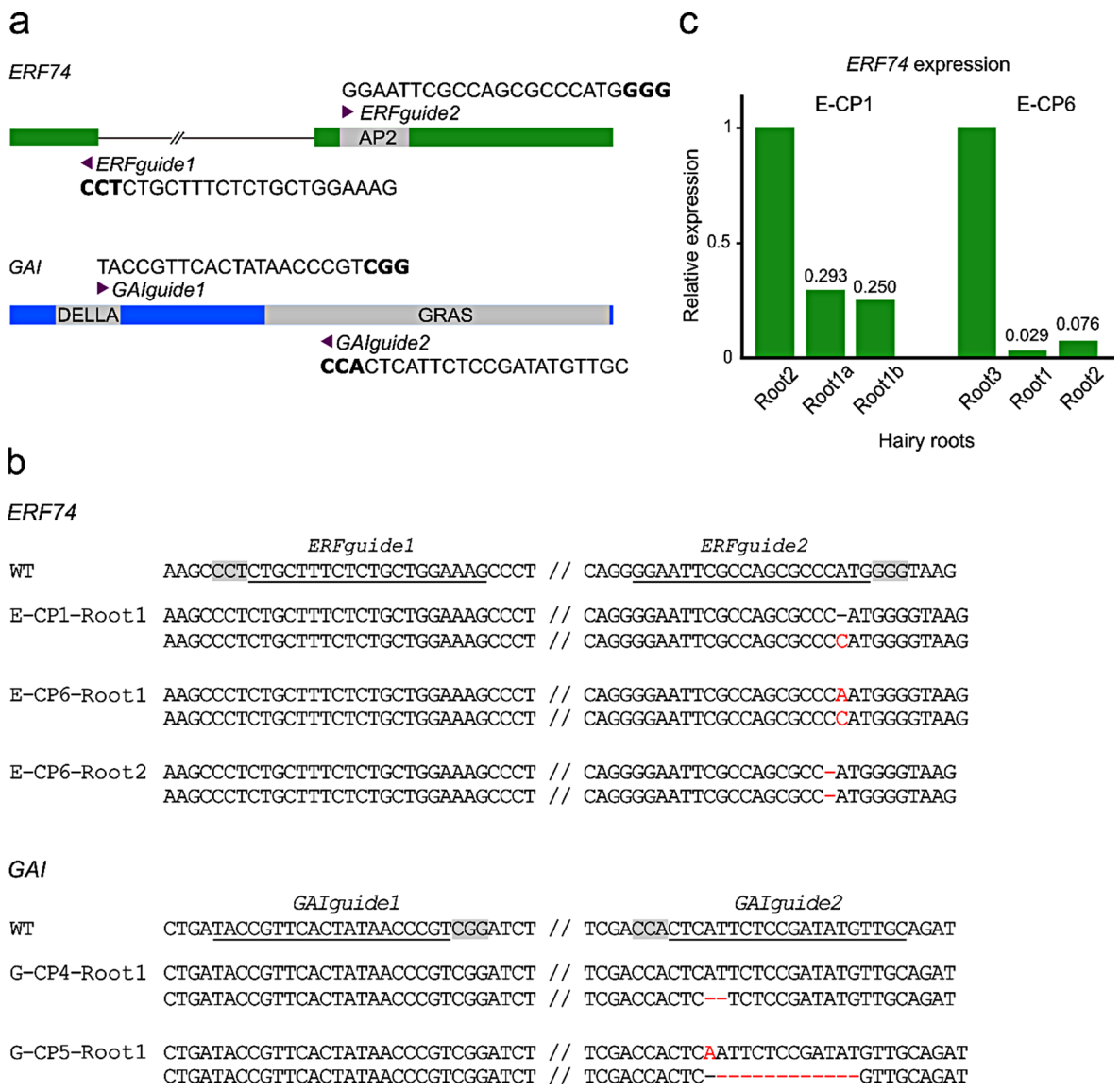

Fig. 2 Localization of the guide RNAs within target genes and gene editing analysis in hairy roots. (**a**) Structure of the *ERF74* and *GAI* genes. The arrowheads indicate the positions of the two gRNAs for each gene. The PAM sequences in the target sites are bolded. (**b**) Targeted mutagenesis in almond hairy roots compared to the wild-type sequence. The gRNA target sites are underlined, and the PAM sequences are highlighted in gray. Indels are indicated in red. (**c**) RT-qPCR analysis of the *ERF74* gene in mutant hairy roots. The transcript levels of the *ERF74* gene were analyzed in individual hairy roots carrying heterozygous mutations in composite plant E-CP1 (main root tip Root1a and a lateral root tip Root1b with wt / +1 bp genotype) and in plant E-CP6 with a biallelic mutation (+1 bp in Root1, with A or C inserted in each allele) or homozygous mutation (− 1 bp in Root2). Hairy roots from the same composite plants lacking any VENUS fluorescence signal and confirmed mutation-free by Sanger sequencing were used as controls (Root2 in E-CP1, and Root3 in E-CP6)

target sites. As a control, 2–3 roots without any fluorescent signal were analyzed for selected composite plants.

The initial step of the *ERF74* analysis involved the excision of a hairy root tip (approximately 1 cm long), which was immediately frozen for subsequent RNA extraction. Another 1 cm root fragment was screened for the fluorescent signal and then frozen for genomic DNA isolation. The *ERF74* gene was subjected to editing at two loci using *ERFguide1* (exon 1) and *ERFguide2* (exon 2, coding for the AP2 functional domain) (Fig. 2a). Of the six composite plants screened, three showed a positive fluorescent signal in their hairy roots (Table 2). In the case of the ERF-targeted composite plant number one (E-CP1), we identified one out of three hairy roots with a fluorescent signal, resulting in a co-transformation efficiency of 33%. Due to the branching of the root with a positive signal (Root1), 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,

Table 2 Gene editing in almond (*Prunus dulcis*) hairy roots. Almond was transformed with a CRISPR vector with gRNAs targeting either *ERF74* or *GAI*. Hairy roots of composite plants (CP) were screened for VENUS fluorescence signal (the number of roots with detected signals out of all screened roots is indicated for each composite plant). The target loci in *ERF74* and *GAI* genes were sequenced in all roots positive for VENUS fluorescence signal (+) and in selected roots without any signal (-). The type of indels at the targeted locus is indicated

Target	Plant	*n* of roots positive for the VENUS signal (*n* of all roots)	Selected roots for sequencing (positive [+] or negative [-] for VENUS signal)	Genomic analysis locus 1	Genomic analysis locus 2
ERF74	E-CP1	1 (3)	Root1 (+)	wt	wt / +1 bp
			Root2 (-)	wt	wt
	E-CP2	0 (3)	Root1 (-)	wt	wt
			Root2 (-)	wt	wt
	E-CP3	3 (7)	Root1 (+)	wt	wt
			Root2 (+)	wt	wt
			Root3 (+)	wt	wt
	E-CP4	0 (6)	-	-	-
	E-CP5	0 (2)	-	-	-
	E-CP6	2 (4)	Root1 (+)	wt	+ 1 bp / +1 bp
			Root2 (+)	wt	− 1 bp / -1 bp
			Root3 (-)	wt	wt
			Root4 (-)	wt	wt
GAI	G-CP1	2 (6)	Root1 (+)	wt	wt
			Root2 (+)	wt	wt
	G-CP2	0 (3)	Root1 (-)	wt	wt
	G-CP3	0 (1)	-	-	-
	G-CP4	1 (3)	Root1 (+)	wt	wt / -2 bp
			Root2 (-)	wt	wt
	G-CP5	1 (3)	Root1 (+)	wt	+ 1 bp / -12 bp
			Root2 (-)	wt	wt
	G-CP6	1 (3)	Root1 (+)	wt	wt

this mutation shared by main and lateral roots probably occurred in the early stages of root growth. 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 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. In all the analyzed roots, the locus targeted by *ERFguide1* was not mutated (Fig. 2b; Table 2). We also chose several VENUS-negative hairy roots as a control to determine if detecting the fluorescence signal is a reliable method for selecting transformed roots. Indeed, Root1 and Root2 from E-CP2, Root2 from E-CP1, and Root3 and Root4 from E-CP6, where no signal was observed, had no mutations.

To assess the impact of the mutations on *ERF74* transcript levels, we performed RT-qPCR analysis on individual hairy roots carrying heterozygous mutations (plant E-CP1, Root1, main root tip; plant E-CP1, Root1 lateral root tip) and biallelic/homozygous mutations (plant E-CP6, Root1 and Root2). We used hairy roots from the same composite plants to ensure the same genetic material as the control. Those hairy roots did not have any fluorescent signal and were confirmed mutation-free by Sanger sequencing (E-CP1 Root2 and E-CP6 Root3). We observed a reduction in *ERF74* transcript levels in both E-CP1 Root1 and E-CP6 Root1 and Root2. In roots of E-CP6 carrying a biallelic/homozygous mutation, *ERF74* expression decreased to less than 10% of the control level (Fig. 2c). Therefore, since different indels led to a reduction in the transcript levels, pooling the mutated hairy roots could be both feasible and beneficial in future studies. This approach would help to ensure more robust data for omics analyses.

To analyze *GAI* locus editing, we examined six composite plants, four of which displayed a fluorescent signal in their hairy roots (Table 2). In composite plants G-CP1 and G-CP6, we did not find any mutations in either 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 (+ 1 bp) 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.

The accuracy of the CRISPR-based genome editing protocol was validated by an absence of off-target mutagenesis

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 1, Supplementary Table S2).

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

The amplified off-target sites for *GAIguide1* on chromosome Pd07 and *GAIguide2* on chromosomes Pd01 and Pd02 differed from the predicted off-target sequence. This difference is due to SNPs between the genome of the transformed cultivar (Vairo) and the reference genome (cv. Texas) used for the in silico analysis, as confirmed by the sequencing of wild-type Vairo roots. A total of 10 loci were screened, and no mutations were detected in any of the predicted off-target sites (Table 1).

Discussion

The CRISPR/Cas system is a revolutionary gene-editing tool derived from the bacterial immune system. It enables the precise modification of the genome, such as the insertion, deletion, or exchange of specific DNA sequences. This capability allows researchers to create specific genetic changes in target genes, helping to elucidate their functions. To fully exploit the vast potential of CRISPR/Cas technology in woody plants, the development of robust transformation protocols tailored to the studied species is essential. In almond, conventional transformation methods employing *A. tumefaciens* have achieved a maximum efficiency of 12.3% (Costa et al. 2006). As an alternative approach, we have developed a hairy root induction protocol with high transformation efficiencies, averaging 41% in the almond cultivar Vairo. Considering the genotype-dependent nature of transformation efficiency, it is plausible that other cultivars may exhibit even higher performance.

Hairy roots offer a valuable system for studying genes of interest either directly in tissue culture or through regeneration into whole plants. Typically, the regeneration process of hairy roots involves callus induction followed by shoot regeneration from the callus. However, no regeneration protocol for almond hairy roots has been established so far. In the case of peach (*Prunus persica*), a closely related species, only callus induction from hairy roots has been reported (Xu et al. 2020). Additionally, the browning observed in almond hairy roots during tissue culture (likely caused by the oxidation of phenolic compounds, as noted in other woody plant tissue cultures) (Liu et al. 2024) further complicates their regeneration. In light of these challenges, we focused on developing an alternative approach to address these limitations. Our protocol covers the generation of composite plants with vigorously growing hairy roots enabling the study of (trans)genes in the context of the whole plant. Such a composite plant system has been effectively used for gene function analysis in peach (Xu et al. 2020) and has been shown to be suitable for plant-nematode interaction studies in *Prunus* spp. (Bosselut et al. 2011).

The efficiency of CRISPR/Cas-based editing can vary significantly, influenced by the choice of the Cas9 protein or guide RNAs. Therefore, for our study, we chose a potent vector for inducing mutations based on research conducted in *Brassica napus* (Jedličková et al. 2022). This vector carries the nuclear-targeted pcoCas9 derived from *Streptococcus pyogenes* Cas9 (SpCas9), with the potato IV2 intron within the Cas9 sequence (Li et al. 2013). Additionally, the construct includes a fluorescent reporter (VENUS-NLS) to monitor the presence of the T-DNA from the binary vector in hairy roots. To further facilitate the identification of roots containing the transgene, a non-invasive gene expression reporter, such as RUBY (He et al. 2020), which converts tyrosine to vivid red betalain, can also be utilized. This visual indicator is easily detected without the need for special equipment or chemical treatments.

In our design, we used two gRNAs selected by the CRISPR-P v.2 tool (Liu et al. 2017) to enhance mutagenesis efficiency. For both the *ERF74* and *GAI* genes, *guide1* had a slightly higher on-target score (representing the predicted cleavage efficiency of Cas9) compared to *guide2*. The higher efficiency scores for *guide1* compared to *guide2* were confirmed by two other gRNA prediction tools (Montague et al. 2014; Xu et al. 2015). Interestingly, *ERF74guide1* and *GAIguide1* failed to induce mutations in almond roots, while *ERFguide2* and *GAIguide2* were successful in targeted mutagenesis. This result might be caused by the addition of an extra G at the 5′ end of the *ERFguide1* and *GAIguide1* sequences, done to facilitate efficient transcription from the U6 promoter. Previous studies in rice and Arabidopsis have demonstrated that such addition has no significant impact on editing efficiencies (Ma et al. 2015). Therefore, it is essential to validate the selected guides on the specific genome, and for this purpose, a simple and rapid transformation system such as the hairy root protocol proved invaluable.

The use of CRISPR/Cas technology to target transcription factors is a powerful approach for unraveling gene regulation mechanisms, given the central role these proteins play

in modulating gene expression. A recent study by Yang et al. (2022) showcases the application of CRISPR/Cas to target *GmNAC12* that encodes a transcription factor of the NAM/ATAF1/2/CUC2 (NAC) superfamily, to elucidate its function in soybean (Yang et al. 2022). In the non-model species *Fagopyrum tataricum* and *Scutellaria baicalensis*, CRISPR/Cas was employed to mutate the genes encoding two MYB transcription factors *FtMYB45* and *SbMYB3*, respectively. The studies investigated the involvement of these proteins in flavonoid biosynthesis using a hairy root transformation system (Fang et al. 2022; Wen et al. 2022). In our study, we aimed to mutate two transcription factors in almond, ERF74 and GAI. ERF74 regulates plant responses to stress, while the DELLA GAI protein plays a role in the gibberellin signaling pathway (Peng et al. 1997; Yao et al. 2017). In our proof-of-concept study using the almond Vairo cultivar, we achieved a transformation efficiency of approximately 41%, with each composite plant producing around 2–4 hairy roots. Among the hairy roots o-transformed with both the *Ri* plasmid and the CRISPR construct (i.e., those exhibiting a fluorescent signal), gene editing occurred in up to 50% of the cases (3 out of 6 VENUS-positive hairy roots for *ERF74* mutagenesis, and 2 out f 5 for *GAI* editing). Therefore, transforming several dozen seedlings should yield sufficient mutated material for future omics analyses or phenotyping studies to reveal the roles of these factors and explore how their disruption impacts the regulation of other genes. Moreover, to minimize the risk of variability in the starting genetic material, it is advisable to use in vitro propagated almond seedlings derived from a single mother plant, which will ensure genetic uniformity. A protocol for such propagation is available for almond (Miguel et al. 1996).

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

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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.

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

Declarations

Competing interests The authors declare no competing interests.

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