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Agrobacterium rhizogenes-Mediated Genetic Transformation and Establishment of CRISPR/Cas9 Genome-Editing Technology in *Limonium bicolor*

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Abstract: *Limonium bicolor* is a perennial herbaceous plant belonging to the Plumbaginaceae family. It can be used as a dried flower or in cut flower arrangements and serves as a model recretohalophyte. Its genome sequencing has been recently completed. However, the research on *L. bicolor* is limited by the absence of a highly efficient genetic transformation system. In this study, we established a highly efficient *Agrobacterium rhizogenes*-mediated *L. bicolor* genetic transformation method. The transgenic hairy roots were induced from the hypocotyl of *L. bicolor* using *A. rhizogenes* strain K599 harboring pRdGa4Cas9 plasmid (which carries an expression cassette of 35S::*DsRed2*). The transgenic shoots were regenerated from hairy root segments (~0.1 cm diameter), and induction efficiency was achieved at 100%. The transgenic shoots with 4–5 rosette leaves were directly planted into the soil to induce the transgenic roots. Therefore, transgenic plantlets were produced. The *DsRed2* can be used as a reliable reporter gene in screening transgenic plantlets. Furthermore, we also established a CRISPR/Cas9 system in *L. bicolor* employing the *A. rhizogenes*-mediated genetic transformation approach. The highly efficient transformation method and CRIPSP/Cas9 system established will provide a valuable tool for functional genomics investigation and trait improvement in *L. bicolor*.

Keywords: sea lavender (*Limonium bicolor*); *Agrobacterium rhizogenes*-mediated transformation; genetic transformation; K599; CRISPR/Cas9; genome editing

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1. Introduction

Sea lavender (*Limonium bicolor* [Bunge] Kuntze), also known as valentine grass or star flower, is a diploid perennial herbaceous plant that typically reaches a height of 30–170 cm. It is widely distributed in China, Russia, and Mongolia and belongs to the genus Limonium, family Plumbaginaceae. A typical plant has 2–3 determinate inflorescences that unusually consist of thousands of flowers. The flowers originate from the primary inflorescence axis and form compound inflorescences, where secondary (or higher order) inflorescence branches give rise to flowers. In each floret, oblong-ovate bracts, funnel-shaped and persistent calyx with light pink or pale purple limb, and spoon-shaped to oval yellow corolla indicate two different colors in the blooming flowers. Inflorescence branches are characterized by their low water content and high fiber content, resulting in the production of rigid and straight branches. The dried flowers are as bright as fresh ones. Therefore, *L. bicolor* is widely used as both a dried flower and in cut flower arrangements in floriculture. *L. bicolor* is also a pasturage. *L. bicolor* is well known and used in traditional Chinese medicine for the treatment of anemia, hemostasis, emmeniopathy, cancer, and carcinoma uteri due to the high content of polysaccharides, flavonoids, steroids, and sulfated phenolics [1–3].

Much more attention has been paid to *L. bicolor*, which is a pioneer plant in saline soil. It can survive and complete its life cycle in high-saline conditions and produce high-quality seeds because it can secrete excess salt onto the leaf surface via multicellular salt glands

on its leaves and stems; this balances ion homeostasis under salt stress and protects from salt-associated damage [1–4]. It has been reported that the inclusion of 100 mM NaCl in the growth medium could markedly promote the growth of *L. bicolor* and obtain maximal biomass production. Under high NaCl conditions, both the Na^+ excretion rate of a single salt gland and salt gland density were significantly increased and reached their maximum levels at 200 mM [4]. *L. bicolor* is regarded as a model recretohalophyte and has recently fascinated researchers with its mechanisms of salt resistance. Understanding how *L. bicolor* tolerates salinity will help to facilitate the breeding engineering of salt-tolerant crops [1–3].

To elucidate the molecular mechanism of salt resistance in *L. bicolor*, a number of candidate genes possibly related to salt gland development and salt secretion have been predicted based on transcriptomic and proteomic analyses [5–8]. Among these candidate genes, only a few genes potentially involved in salt gland development and salt secretion have been functionally characterized. For example, the overexpression of *SUPER SENSITIVE TO ABA AND DROUGHT2* (*LbSAD2*) or WD40-repeat protein *TRANSPARENT TESTA GLABRA1* (*LbTTG1*) in *Arabidopsis* led to a notable augmentation of trichome development and an enhanced capacity for salt resistance [9,10]. Ectopic expression of *L. bicolor HELIX-LOOP-HELIX* (*LbHLH*) in *Arabidopsis* enhanced salt tolerance by increasing trichome development, decreasing root hair development, and enhancing osmotic resistance under NaCl stress [11]. Overexpression of *Lb1G04202* in *Arabidopsis* resulted in increased salt tolerance by promoting proline biosynthesis [12]. When an uncharacterized gene *Lb1G04794* was overexpressed in *Arabidopsis*, transgenic *Arabidopsis* showed that the number of trichomes was increased, root hairs were decreased, and salt resistance was enhanced [13]. Overexpression of an R1-type MYB transcription factor *LbMYB48* in *Arabidopsis* has been shown to increase salt tolerance through maintaining ion and osmotic balance and might be involved in the abscisic acid signaling pathway [14]. Besides those genes involved in the positive regulation of salt resistance, two negative regulation genes, *Lb2G14763* and *TRIPTYCHON* (*LbTRY*), have also been reported. The constitutive expression of *Lb2G14763* in *Arabidopsis* resulted in a higher accumulation of Na^+ and lower expression levels of salt resistance genes. The transgenic *Arabidopsis* plants overexpressing *LbTRY* showed increased root hair development and salt sensitivity [15].

Although a previous *Agrobacterium*-mediated genetic transformation of *L. bicolor* method has been reported [16], among those genes mentioned above, their gain-of-function phenotype(s) were obtained by heterologous overexpression in *Arabidopsis*, except for *Lb1G04794*. As for *Lb1G04794*, it was only the phenotype associated with salt glands that was analyzed in the 35S::*Lb1G04794 L. bicolor*. The further function of *Lb1G04794* in salt stress was analyzed in detail in *Arabidopsis* [13]. These results indicate that functional analyses of genes of interest have still been carried out via heterologous expression in *Arabidopsis*. This is greatly possible due to low efficiency and recalcitrant to genetic transformation mediated by *A. tumefaciens* in *L. bicolor*. Heterologous expression in other model plants (lacking the salt gland structure) cannot well validate the gene function in native species, and it also cannot aid in obtaining the loss-of-function mutant, such as the use of CRISPR/Cas9 genome-editing technology to induce the mutant in *L. bicolor*. With the conduction of multi-omics carried out in *L. bicolor* [5–8], assembly, and annotation of the *L. bicolor* genome [1], further parsing and unraveling of the molecular mechanism of the numerous genes potentially associated with the development of the salt gland structure and the salt secretion mechanism need to be carried out. Therefore, establishing a highly efficient genetic transformation approach and genome-editing system is urgently required. In this study, we established a highly efficient genetic transformation method in *L. bicolor* using *A. rhizogenes*-mediated transformation and genome editing mediated by a CRISPR/Cas9 system in *L. bicolor*.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Sea lavender (*Limonium bicolor*) seeds used in this study were kindly provided by Prof. Min Chen (the College of Life Science, Shandong Normal University, Jinan, China). The *L. bicolor* was grown in the greenhouse under the following conditions: 16 h photoperiod (80 μM photons m^{-2} s^{-1}) and 8 h in the dark at 22 ± 2 °C.

2.2. Binary Vector Construction and A. rhizogenes Strains

To assess whether the DsRed2 fluorescent proteins can be used for the screening of transgenic hairy roots and transgenic plantlets and to optimize the transformation protocol, we used a previously constructed plant binary expression vector pRdGa4Cas9 [17], which carries an expression cassette of 35S::*DsRed2*. The pRdGa4Cas9 plasmid was constructed based on the backbone of pHSE401 [18]. The expression cassette of 35S::*DsRed2* cloned from an intermediate recombined vector pRed1305 [19] was recombined into the pHSE401, and then the promoter $AtGCSpro_{1178}$ [17] replaced the double CaMV35S promoter and therefore produced the vector pRdGa4Cas9.

To construct a genome-editing vector and target-editing *L. bicolor PDS* gene (encoding a phytoene desaturase enzyme), the genomic sequences of *LbPDS* were amplified via PCR and confirmed by Sanger sequencing before gRNA design. The CRISPR/Cas9-mediated genome-editing vector pRdUbiCas9 [17] was used as a backbone. Since there is no genomic database of *L. bicolor* available for a web tool for synthetic gRNA design, we downloaded the genome sequence of *L. bicolor* from NCBI (BioProject number PRJNA753199). Local BLAST of SeqHunter software [20] was used for designing the gRNA for specifically targeting the extron regions of *L. bicolor PDS* (*LbPDS*). Two target sites of *LbPDS* were designed as CGAGATGTTTTGGGTGGAAAGG and TAATGATCGACTACAGTGGAAGG (PAM sequences were underlined). Oligos KtLbPD1 (5′-GTGTGGTCTCAGTCACGAGATGTTTTGGGTGGAAGTTTTAGAGCTAGAAATAG-3′) and ktLbPD2 (5′-CTCTGGTCTCGAAACTCCACTGTAGTCGATCATTCAATCTCTTAG TCGA CTCTAC-3′) were synthesized for gene editing vector construction. A CRISPR/Cas9-mediated gene mutation vector, designated as pCas9LjPDS, was developed according to the previously published protocol [18]. Specifically, to construct the two-gRNA-expressing vectors for *LbPDS* targeting, the fragments were amplified via PCR with oligos KtLbPD1 and ktLbPD2 using the vector pCBC-DT1T2 [18] as a template and then cloned into the *Bsa*I site of pRdUbiCas9 via the Golden Gate cloning method.

The vectors pRdGa4Cas9 and pCas9LjPDS were transformed into *A. rhizogenes* via electroporation. *A. rhizogenes* strains K599 (NCPPB2659; carrying pRi2659 Ri plasmid; GenBank accession no. EU186381), ArA4 (GenBank accession no. CP073112.1), ARqua1 (GenBank accession no. PRJNA976066) [21], and MSU440 [22] were used to test the hairy root transformation efficiency. The strain K599 was kindly provided by Prof. Tianfu Han and Wensheng Hou (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China) and strains ArA4, ARqua1, and MSU440 were bought from Shanghai WEIDI Biotech Co. (China). The fresh *A. rhizogenes* cells were used for infection of the seedlings. The *A. rhizogenes* strain carrying the desired vector was streaked onto the agar-solidified LB medium and cultured for 2 days at 28 °C. A single bacterial colony was inoculated into 1 mL of liquid LB medium on a shaker agitated at 200 rpm overnight at 28 °C. Then, 200 μL of the overnight fresh culture of *A. rhizogenes* was scattered onto the agar-solidified LB medium and incubated at 28 °C overnight for infection of the seedlings. In these *A. rhizogenes* culture steps, antibiotics (depending on the plant binary expression vector used) were always added to the LB medium.

2.3. Induction of Transgenic Hairy Roots and Regeneration of Plantlets

To induce the transgenic hairy roots, *L. bicolor* seeds were sterilized with 75% ethanol for 1 min and 50% bleach (2.5% sodium hypochlorite, final volume) for 10 min, followed by rinsing five times with sterile water. The sterilized seeds were laid out in a Petri dish

containing 1/2 × MS medium (More Better, Hangzhou, China) and 0.8% agar (Solarbio, Beijing, China) and grown for 7 days in a growth greenhouse. Transgenic hairy roots were induced according to the previously published transformation protocol [19,23] with the following minor modifications. The 7-day-old seedlings were used for the induction of transgenic hairy roots. The primary roots were cut off, and a slant cut was produced on the hypocotyl using a sterile scalpel in the *A. rhizogenes* suspension solution transformed with the pRdGa4Cas9 or pCas9LjPDS vector (the *A. rhizogenes* grown on agar-solidified LB medium were diluted to OD600 ≈ 0.6 at 600 nm using sterile water). The fresh *A. rhizogenes* grown on the LB solid plate were collected using a sterile surgical blade and used to inoculate the oblique wound site of the hypocotyl of the seedling. The infected seedlings were placed on the 1/2 × MS solid medium (supplemented with 0.8% agar) and kept in a greenhouse. The hairy roots were induced and generated 2 to 3 weeks post inoculation.

To obtain whole transgenic plantlets, transgenic shoots were induced from the transgenic hairy roots-induced. When the induced hairy roots reached up to ~10 cm in length, they were excised from the non-transgenic shoots, cut into ~1 cm segments, and placed on the induction medium (1 L containing phytotech M519 Murashige and Skoog basal medium with vitamins (Solarbio, Kansas, USA) 4.43 g, sucrose 30 g, 6-BA 1 mg, agar 8 g, zeatin 0.5 g, cefotaxime 200 mg, and carbenicillin 200 mg) to induce the generation of transgenic shoots. The hairy root segments or calli were changed to the same fresh medium at 3-week intervals. After ~2 months of culture, the transgenic shoots were induced from the hairy roots. The transgenic shoots with 4–5 leaves were transplanted in a pot with soil mixed with vermiculite (nutrient soil:vermiculite = 1:2), covered with a plastic bag, and grown in the greenhouse for 5~7 days. To acclimate the environment, the humidity was gradually decreased by puncturing holes in the plastic bag, then the bag was removed, and the transgenic shoots formed transgenic roots. The whole transgenic plantlets were further grown in the greenhouse. The diagram of the *A. rhizogenes*-mediated genetic transformation of *L. bicolor* is presented in Figure 1.

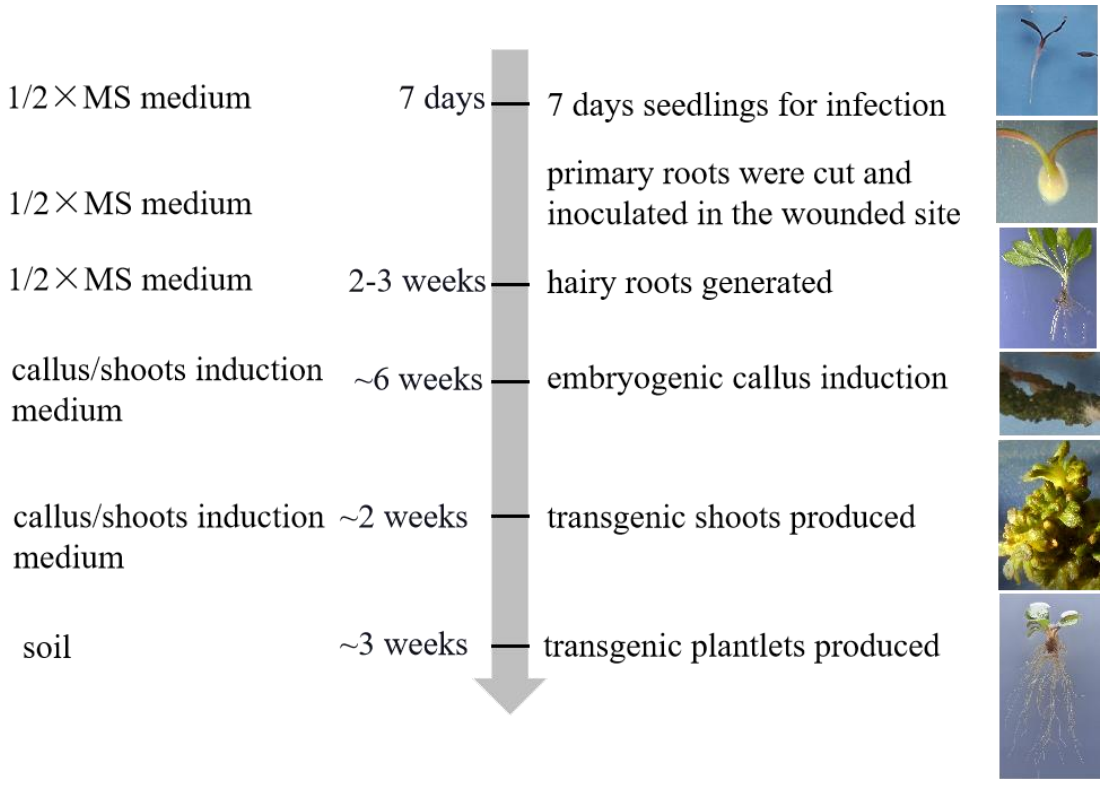


Figure 1. Diagram of *A. rhizogenes*-mediated genetic transformation of *L. bicolor*. The schedule and medium are indicated on the left, and the corresponding figures are shown on the right.

2.4. PCR (Polymerase Chain Reaction) Analysis and Observation of DsRed2 Fluorescent Protein

The genomic DNA of leaves was extracted, and PCR analysis was carried out according to Fan et al. [19]. The specific primers DsRed1 and DsRed2 designed for the amplification of *DsRed2* were used [19]. The DsRed2 fluorescent protein was observed in hairy root-induced, embryogenic calli-induced, and whole transgenic plantlet stages using a handheld fluorescent lamp (LUYOR-3415RG, Co. Luyang, Shanghai, China). Digital image processing was performed using Adobe Photoshop 2020.

2.5. Targeted Mutagenesis of LbPDS by CRISPR/Cas9 and Validation of Mutation Genotype(s) of Transgenic Plants

To analyze the edited targeted mutagenesis of *LbPDS* via CRISPR/Cas9, genomic DNA was extracted, and sequences containing target sites were amplified by PCR using primers LbPDS1 (5′-TTGGCTGGTTTGTCTACTGC-3′) and LbPDS2 (5′-TTCTCCAGGCTTGTTTGGC-3′) covering the target sites and further sequenced to identify mutations.

3. Results

3.1. A. rhizogenes-Mediated Hairy Root Transformation

To evaluate whether *A. rhizogenes* can efficiently infect *L. bicolor* and induce the generation of hairy roots, K599, ArA4, ArQual, and MSU440 strains of *A. rhizogenes* were used. The DsRed2 fluorescence observed in each independent co-transformed root was in accordance with that from PCR analysis. The DsRed2 fluorescence was observed in the roots produced, PCR detection indicated positive roots, and vice versa (Figures 2 and 3). Therefore, we used the *DsRed2* gene driven by the CaMV35S promoter harbored on the pRdGa4Cas9 vector as a molecular marker to screen co-transformed hairy roots and to facilitate quantification of transformation efficiency. The results showed that ARqual and MSU440 strains were insufficient to infect *L. bicolor*. Transformation efficiency was 0%. Compared with this, K599 and ArA4 strains could sufficiently infect *L. bicolor* and yielded 80% and 9.1% transformation efficiency, respectively. The transformation efficiency of K599 was the highest among the strains tested (Table 1). Hence, we used the K599 strain to induce the transgenic hairy roots in the subsequent experiments.

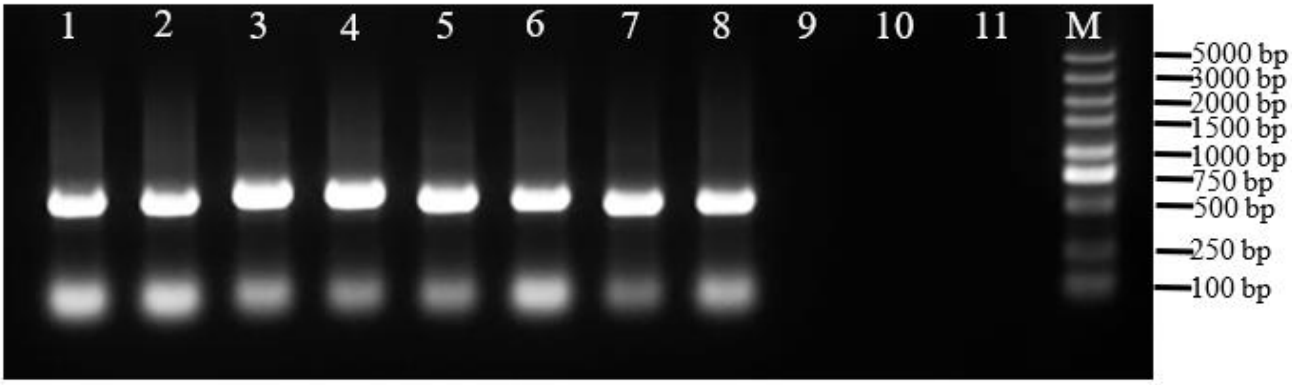


Figure 2. Validation of transgenic hairy roots and non-transgenic via PCR by amplification of *DsRed2* gene. Lane 1, PCR product amplified from K599-harboring pRdGa4Cas9 as a template and used as a positive control. Lanes 2–8, different independent transgenic roots from DsRed2-positive root. Lane 9, DsRed2-negative root infected with K599 carrying pRdGa4Cas9. Lane 10, non-transgenic root infected with K599 as a negative control. Lane 11, ddH_2O used as a template as a blank control. M, DL5000 Plus DNA Marker (Tsingke Biotechnology Co., Qingdao, China).

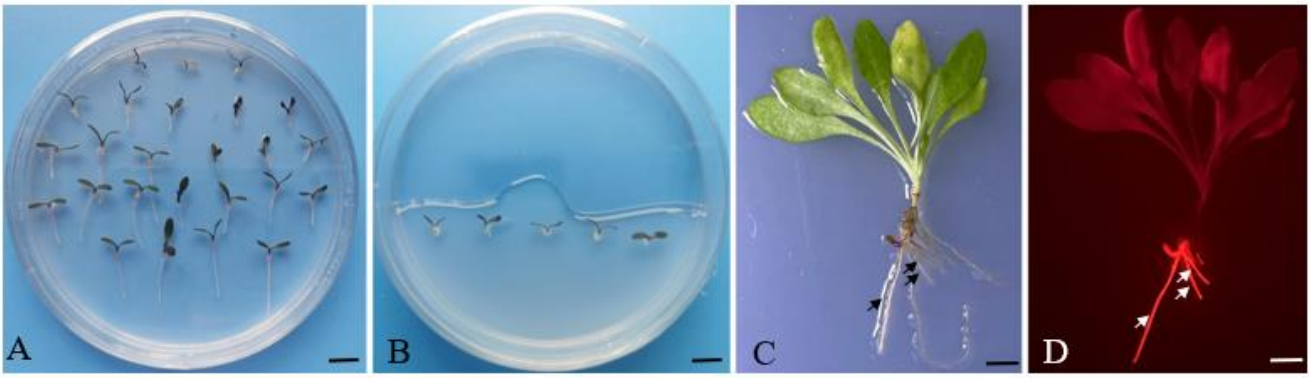


Figure 3. Induction of transgenic hairy roots. Seven-day-old seedlings used for infection (**A**); the primary roots of seedlings were removed and inoculated with the *A. rhizogenes* transformed with the desirable vector (**B**); generation of hairy roots from wounded hypocotyl post 2~4 weeks inoculation (**C**); the DsRed2 fluorescent protein expression on induced transgenic hairy roots (**D**). The arrows indicate the transgenic hairy roots. Scale bars = 1 cm.

Table 1. A comparison of hairy root transformation efficiency among different *A. rhizogenes* strains with pRdGa4Cas9.

A. rhizogenes Strain	Number of Plants Infected	Number of Shoots with at Least One Transgenic Root	Number of DsRed-Positive Roots (per Seedling with Positive Root(s))	Transformation Efficiency
K599	20	16	2.19 ± 0.75	80.0%
ArA4	22	2	1.50 ± 0.71	9.1%
ArQual	20	0	0	0%
MSU440	26	0	0	0%

Note: Transformation efficiency was defined according to Fan et al. [23]: number of shoots with at least one transgenic root/number of infected plants × 100%. The infection experiment was repeated three times. The data were measured 4 weeks after infection.

3.2. Regeneration of Plantlets from Transformed Hairy Roots

The hairy roots (representing 20 independent lines) induced via *A. rhizogenes*-mediated transformation were cut into ~1 cm segments and used to induce the generation of shoots on the induction medium (Figure 4A). The embryonic callus was formed on the whole segments of hairy roots after culturing for ~2 months (Figure 4B). 100% segments of hairy roots can give rise to embryonic callus tissue, which continued to develop into numbers of shoots in the subsequent about 2 weeks (Figure 4C). We observed that the number of shoots induced from the explants is positively related to the diameter of the hairy roots. The transgenic shoots that developed with 4–5 leaves (Figure 4D) were cut from the base of shoots and transplanted into a pot with soil mixed with vermiculite (nutrient soil:vermiculite = 1:2). Fifty transgenic shoots from 20 different independent transgenic roots were transplanted. A plastic bag was used to cover the pot planted with the transgenic shoots to maintain a high-humidity environment. They were cultivated in a greenhouse, and the plastic bag was gradually removed by making some ventilation holes for 5~7 days. The 100% transgenic shoots produced roots within 3 weeks (Figure 4E,F). The DsRed2 fluorescence can be observed from the whole transgenic plantlets, compared with no DsRed2 fluorescence signal in the non-transformed plant (Figure 4F). Therefore, a whole plantlet was generated from the transformed hairy root (Figure 4E,F).

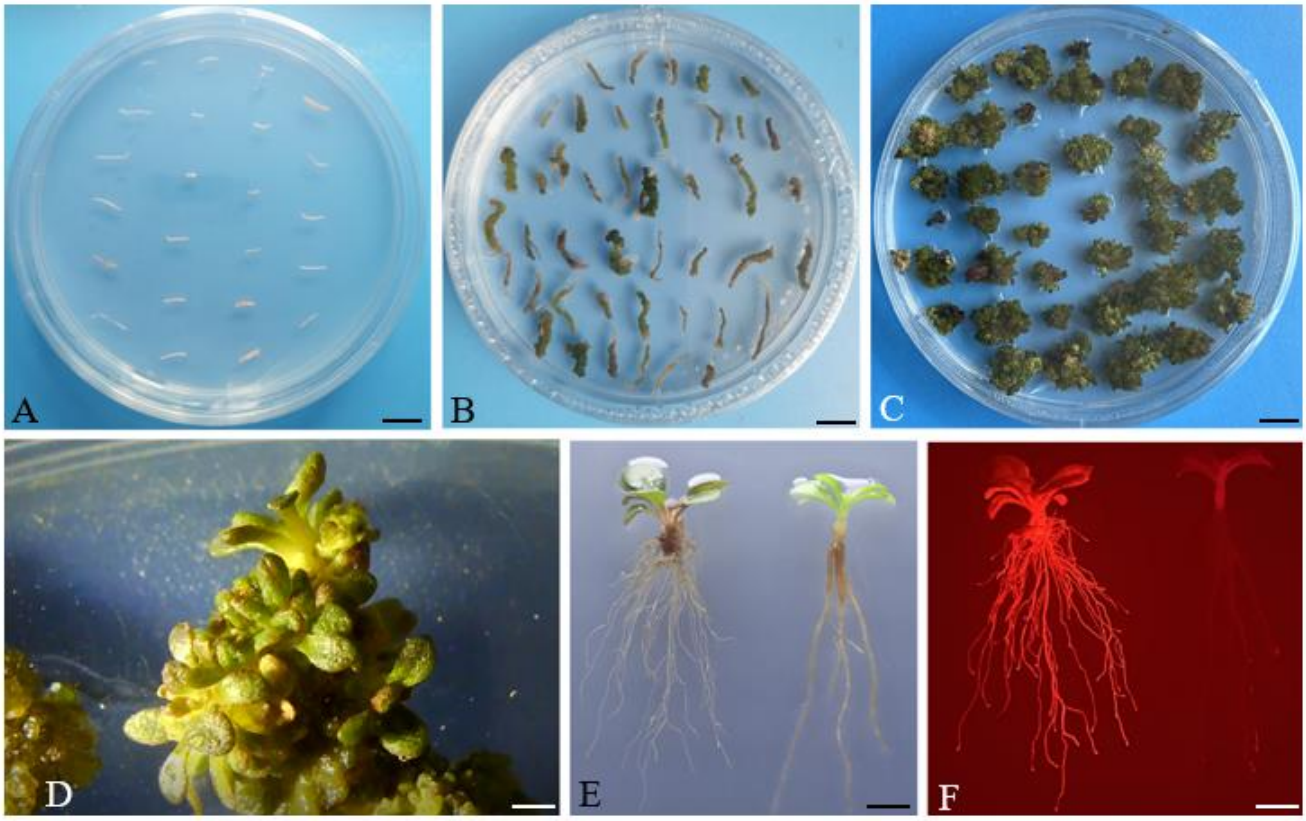


Figure 4. Transgenic plantlet regeneration from transgenic hairy roots. Transgenic hairy roots were cut into ~1 cm segments and used for induction of embryogenic callus on the induction medium (**A**); induction of the embryogenic callus after 6 weeks of culture on the induction medium (**B**); appearance of transgenic buds after 8 weeks of culture on the induction medium (**C**); closed-up transgenic buds regenerated from the transgenic hairy roots after 8 weeks of culture on the induction medium (**D**); a whole transgenic plantlet was generated (left), compared with the untransformed plantlet (right). Pictured under the condition of natural light (**E**); DsRed2 expression on the transgenic plantlets (left) compared with the untransformed plantlet (right) without the DsRed2 fluorescent protein expression (**F**). Scale bars = 1 cm.

3.3. Establishment of a CRISPR/Cas9 System for Genome Editing in L. bicolor

CRISPR/Cas9 is one of the most powerful and extensively employed genome-editing tools that enables the precise introduction of InDels (Insertions and deletions) at specific target sequences. This system has been effectively utilized in many plants, including various crop species, to genetically modify plants and introduce desired traits. However, to date, there have been no reports of the establishment of CRISPR/Cas9 in *L. bicolor*. We established an *A. rhizogenes*-mediated hairy root transformation method in *L. bicolor*, and the transgenic hairy root can be induced, developed, and regenerated into a whole transgenic plant, suggesting that it would be possible to use this system to develop mutants mediated by CRISPR/Cas9. To test this hypothesis, we initiated knockout of the *L. bicolor PDS* gene (encoding a phytoene desaturase enzyme), *LbPDS*. *LbPDS* was selected as the target gene because the *LbPDS* mutant possibly showed a conveniently observable "albino" phenotype caused by disruption of chlorophyll biosynthesis, as observed in some plant species [24,25]. Two gRNAs targeting the conserved regions of the *PDS* exon were designed and cloned in the CRISPR/Cas9 vector pRdUbiCas9 [17]. Targeted mutations of *PDS* were successfully induced in hairy roots using the K599 strain, which carried the CRISPR/Cas9 vector pCas9LjPDS. Each transgenic hairy root induced with a visual DsRed2 fluorescence is considered an independent line. The independent DsRed2-positive transgenic hairy root lines were used for the induction of callus and, subsequently, further

generation of transgenic shoots (Figure 5). All the hairy root explants from 12 independent hairy roots could form calli, and among those, some calli showed a pink/red color (different from the green-colored callus induced from the non-*pds* knockout hairy roots) and could barely further develop into shoots (Figure 5A–E). In some cases, some buds showed a green color, and others had white- or pink/red-colored calli, even from a segment of hairy root explant (Figure 5B). In these developed transgenic buds, broad phenotypic variations were observed (Figure 5). To conveniently describe them, the phenotypes were classified into three groups, as follows: (i) leaves of some transgenic callus/buds showed a uniform pink/red color (Figure 5C–F), (ii) leaves of transgenic shoots with chimeric phenotype showed green, white, and pink/red colorations simultaneously (Figure 5G,H), and (iii) leaves of transgenic shoots revealed a green color, as observed phenotypes in Figure 4D. We further accurately determine the genome-editing type(s) mediated by CRISPR/Cas9 in the three phenotypic groups. The DNA sequences covering the CRISPR target sites were amplified by PCR and Sanger sequencing analysis from each of the independent DsRed2-positive transgenic buds representing the three phenotypic groups induced from different independent transgenic hairy roots. Two examples are given in Figure S1A,B. Figure S1A shows a uniform pink/red color transgenic bud caused by the knockout of *LbPDS* at site 1, and sequence analysis showed two mutant alleles, one with a 1-bp insertion and the other with a 1-bp deletion (Figure S1A). Figure S1B represents an example showing that knockout of *LbPDS* at site 2 also resulted in the formation of a uniform pink/red color transgenic bud; sequence analysis identified two mutant alleles, one with a 20-bp deletion and the other with a 1-bp insertion when sequences were aligned with the wild-type allele. The results indicated that pink/red color buds were homozygous or biallelic mutants at targeted site 1 and/or targeted site 2 (Figure 5D–F). The buds with chimeric phenotypes were related to the edited types in different cells with a heterozygous mutation, wild-type, or homozygous/biallelic mutations in the same buds (Figure 5G,H). The leaves of transgenic shoots showing a green color had a heterozygous mutation or wild-type at both of the two targeted sites. The gene editing efficiencies mediated by CRISPR/Cas9 among the two gRNAs were also evaluated (Table 2). The homozygous/biallelic and heterozygous mutation rates at targeted site 1 were 16.67% (2/12) and 0% (0/12), while at targeted site 2 they were 41.67% (5/12) and 50.0% (6/12), respectively. The editing efficiency is higher at targeted site 2 than that at targeted site 1 (Table 2). Furthermore, we observed that the pink/red color then became more pronounced with callus age. The pink/red color became stronger and more obvious than that of the early stage of the callus in the homozygous or biallelic mutation developing plantlets (Figure 5C–F). In these homozygous/biallelic *PDS* mutants, the callus and buds showed a pink/red color but not the "albino" phenotype, possibly caused by the expression of *DsRed2* in the "albino" background.

Table 2. Genotyping of targeted gene mutations induced by CRISPR/Cas9 in the transformed roots: editing efficiency, homozygous/biallelic mutations, and zygosity (heterozygosity or chimeric).

gRNA	Homozygous/Biallelic (No. Homozygous/Biallelic Hairy Roots/Total Number of Positive Roots)	Zygosity (No. Zygosity Hairy Roots/Positive Roots)	Editing Efficiency (%) (No. Edited Hairy Roots/No. Total Positive Roots)
gRNA1	2/12	0/12	16.67%
gRNA2	5/12	6/12	91.67%

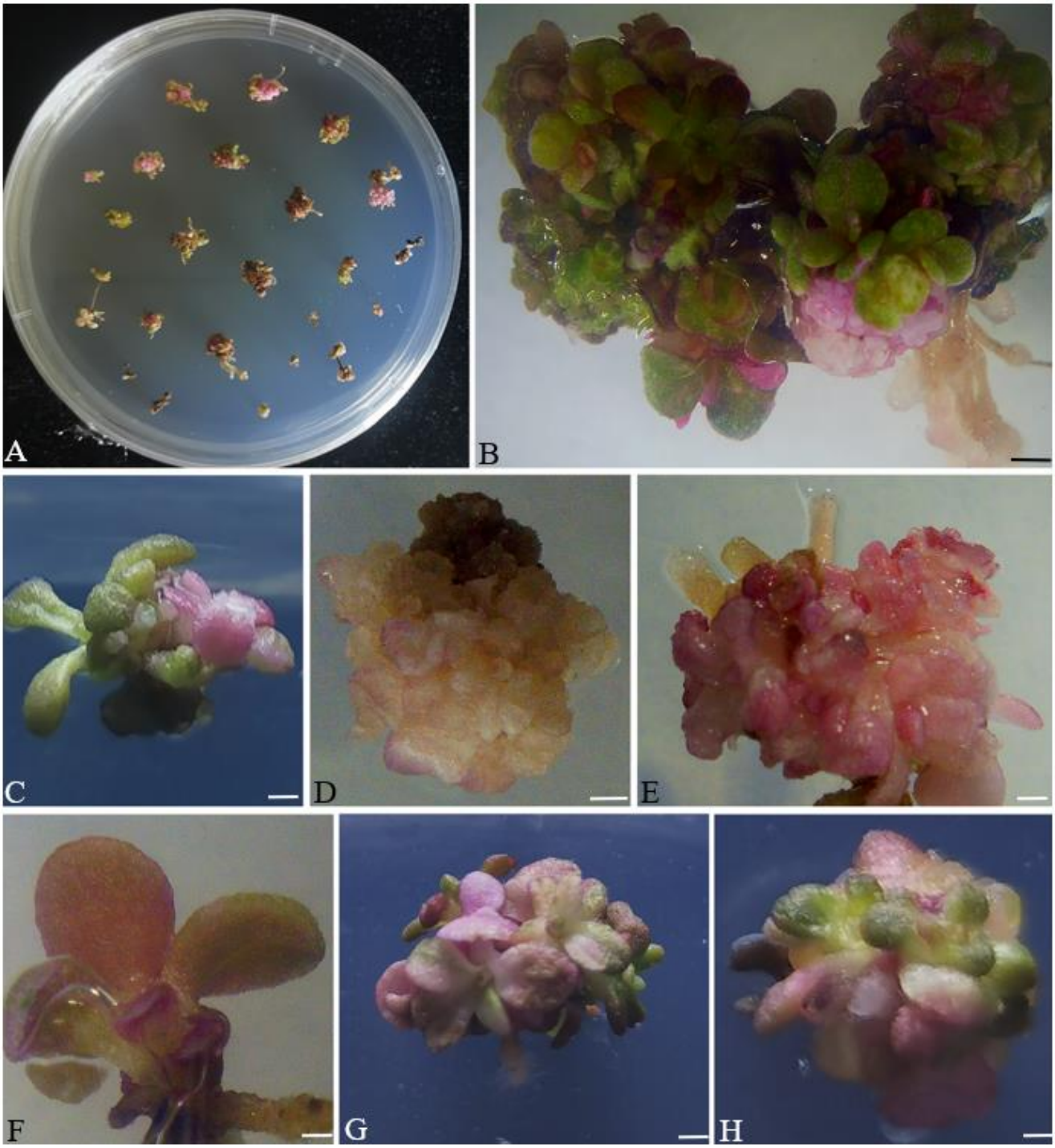


Figure 5. Editing of *L. bicolor* by knockout of *LbPDS* gene with CRISPR/Cas9. Callus induced from *LbPDS* knockout hairy roots by CRISPR/Cas9 (**A**); a closed-up transgenic callus/buds regenerated from an explant of induced independent hairy roots (**B**); chimeric hairy root-regenerated shoots revealed the albino phenotype (**C**); callus and developing buds, characterized by a pink/red color (**D**–**F**); regenerated shoots showing chimeric phenotypes (**G**,**H**). Scale bars = 1 mm.

4. Discussion

A. rhizogenes-mediated hairy root transformation has been widely applied in root-related biology studies. For example, interactions between plant and microbe (rhizobia, arbuscular mycorrhizal fungi, pathogens, etc.) [19,26,27], signal communication between root and shoot, interactions between plant and environment (biotic/abiotic stresses), promoter activity analysis in the root [23], genome-editing efficiency analysis at the targeted site mediated by the CRISPR/Cas system [17], and so on. However, so far, there has been no report on a hairy root transformation method in *L. bicolor.* In this research, we established an *A. rhizogenes*-mediated hairy root transformation method in *L. bicolor*. The *A. rhizogenes* K599 strain was highly efficient among the four strains tested and resulted in 80% transformation efficiency (Table 1). This is in accordance with previous reports that different *A. rhizogenes* strains resulted in a remarkable difference in transformation efficiency in *A. rhizogenes*-mediated hairy root transformation [19,28–30].

In our previous study, we observed that some plantlets can be produced from the roots of *L. bicolor* grown on MS culture medium (Figure S2). This inspired us to determine whether the transgenic hairy roots induced via *A. rhizogenes*-mediated transformation can be used as explants to induce the regeneration of transgenic plantlets. This will overcome *A. tumefaciens*-mediated genetic transformation with low transformation efficiency in *L. bicolor* [16]. In this study, our results indicate that transgenic plantlets can be successfully induced from transgenic hairy roots. The *A. rhizogenes*-mediated hairy root transformation efficiency was 80%. Furthermore, the number of positive transgenic hairy roots reached up to 2.19 ± 0.75 (Table 1). The transgenic root was cut into ~1 cm segments, and these were used as explants to induce transgenic shoots, producing 100% induction efficiency. About 25 transgenic shoots could be induced and regenerated from each explant (Figure 4D). Also, 100% of the transgenic shoots could produce roots (Figure 4C). Each independent transgenic root with a length of ~4 cm has the potential to generate about 100 whole transgenic plantlets. Therefore, we established a very highly efficient genetic transformation approach mediated by *A. rhizogenes*.

In a previous study, the hygromycin B-resistance gene was used as a selectable marker in the genetic transformation of *L. bicolor*. It is expensive to purchase the chemical reagent hygromycin. Moreover, the dosage used needs to be balanced in screening transgenic shoots because less dosage used will lead to higher transgenic false-positive plantlets induced, and a higher dosage will result in a high risk of killing the transgenic-positive plantlets [16]. In this study, we developed a convenient and reliable visual reporter gene, DsRed, that can be applied for screening the transgenic-positive callus, shoots, and plantlets in genetic transformation in *L. bicolor*. The DsRed fluorescent protein was used as a selective marker, which overcomes the shortcomings of hygromycin B used as a marker.

L. bicolor is a horticulture plant and recretohalophyte that has a typical salt secretory epidermal structure and salt glands [7]. It is a unique recretohalophyte with a sequenced genome that constitutes an essential genetic resource for improving salt tolerance in crops [1]. So far, it is still a bottleneck for *L. bicolor* to produce transgenic or genome-edited plants. CRISPR-Cas9 represents one of the most powerful genome-editing tools and has great potential for gene functional analysis and trait improvement in plants and agricultural research [17]. Based on the well-established genetic transformation system mediated by *A. rhizogenes* in *L. bicolor*, we established a CRISPR/Cas9 genome-editing system in *L. bicolor*. The establishment of genetic transformation and CRISPR/Cas9 genome-editing systems will lay a foundation for understanding the molecular mechanism of salt resistance and genetic improvement in *L. bicolor*, especially regarding introducing specific new traits to modify or re-edit already existing traits.

For loss-of-function studies or modifying already-existing traits involving the knock-out of a specific gene(s), except for recessive traits, the generation of InDel mutations by CRISPR/Cas9 will result in a mutant phenotype only when there is homozygous/biallelic mutagenesis. In the previously reported transformation method mediated by *A. tumefaciens* [16], from the initiation of transformation to the generation of shoots, it typically

takes approximately two months. Once the transgenic shoots have formed, they can be analyzed to determine the type of mutation(s) present and assess whether homozygous or biallelic mutagenesis has occurred. In contrast, in this study, in the *A. rhizogenes*-mediated genetic transformation protocol, the type of mutation(s) can be determined during the early stages of hairy root development (~4 cm root length). It generally takes about 1 month. Our developed transformation approach offers the advantage of convenience and expedited and earlier detection of the mutation types. Therefore, it is highly efficient to screen the homologous/biallelic mutants. In the future, using a highly efficient promoter to drive the expression of Cas9 can greatly enhance the likelihood of achieving higher rates of homozygous/biallelic mutagenesis in *L. bicolor*. Previous studies have shown that when the *Arabidopsis* gamma-glutamylcysteine synthetase promoter (*AtGCS* promoter) was employed in *A. rhizogenes*-mediated hairy root transformation, it significantly increased the efficiency of inducing homozygous/biallelic mutations in transgenic hairy roots [17]. We speculate that the *AtGCS* promoter has great potential for highly efficient induction of homologous mutagenesis in *L. bicolor*; this needs to be further analyzed.

5. Conclusions

A highly efficient *A. rhizogenes*-mediated genetic transformation method and CRISPR/Cas9-mediated genome-editing system were established in *L. bicolor*. The DsRed fluorescent protein can be used as a reliable selective marker in screening transgenic hairy roots and plantlets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13092244/s1, Figure S1. DNA sequencing peaks showed evidence of successful gene editing in the targeted sites 1 (A) and 2 (B) of *LbPDS*. Figure S2. Plantlets were generated from the roots of *L. bicolor*. The arrows indicate the plantlets. Scale bars = 1 mm.

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