



Optimization of Hairy Root Transformation and Application of *RUBY* as a Reporter in *Lotus corniculatus*

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Abstract: *Lotus corniculatus* is an important perennial legume forage species and has been widely used to study the relationships between plants and rhizobia or arbuscular mycorrhizal fungi. Composite plants (wild-type shoots with transgenic hairy roots) can be produced via genetic transformation mediated by *Agrobacterium rhizogenes*. The *A. rhizogenes* puncture method and a method of cutting embryonic roots and coating the hypocotyl incision with *A. rhizogenes* were used to induce hairy roots from seedlings. The identification of transgenic hairy roots from composite plants is also an important task. Here, young branches from adult plants were used as explants to produce composite plants instead of seedlings via the one-step cutting method in the natural environment. The results showed that the explants of young branches had higher transformation frequency than those of seedlings, and the composite plants obtained from the young branches had greater growth and were also more robust than the plants obtained from the seedlings. In this study, a system for the biosynthesis of betalains named *RUBY* was integrated into an expression vector as a reporter gene. It is very easy to distinguish transgenic roots from nontransgenic roots based on the red color of the hairy roots. Young branches were used as explants for hairy root transformation with higher transformation frequency than the seedlings. *RUBY* was used as a reporter gene to distinguish transgenic roots from nontransgenic roots based on the red color of the hairy roots.

Keywords: *Lotus corniculatus*; *Agrobacterium rhizogenes*; hairy root transformation; *RUBY*; betalain



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1. Introduction

Lotus corniculatus is an important perennial legume forage species. *L. corniculatus* has a high nutritional content and contains condensed tannins that prevent ruminant bloating [1]. In addition, *L. corniculatus* is a honey source and ornamental plant. *L. corniculatus* has a well-developed root system with deep soil penetration and strong drought resistance. *L. corniculatus* can symbiotically fix nitrogen with rhizobia in the soil. It has been widely used to study the symbiotic interaction between plants and rhizobia [2]. Genome sequencing of *L. corniculatus* has been completed [3], and efficient transformation methods are a prerequisite for gene functional annotation. However, the transformation method mediated by *Agrobacterium tumefaciens* has a long cycle. When studying the molecular mechanism of the interaction between *L. corniculatus* and rhizobia or mycorrhizal fungi, genetic transformation mediated by *Agrobacterium rhizogenes* is often used to produce transgenic hairy roots for related research [4].

Genetic transformation mediated by *A. rhizogenes* can produce a composite plant with transgenic hairy roots and has the advantages of rapid root formation and easy operation [5]. The transformation of *L. corniculatus* mediated by *A. rhizogenes* was initially carried out using the *A. rhizogenes* puncture method [6]. Subsequently, the transformation method was improved by cutting the embryonic roots, coating *A. rhizogenes* on the hypocotyl, and inducing hairy roots on the culture medium [7,8]. However, this method requires a

sterile environment and tissue culture to induce the formation of hairy roots. Here, the transformation method was improved by using young branches as explants, coating them with *A. rhizogenes*, and then inserting them into vermiculite to produce hairy roots in the natural environment.

The reporter gene in a binary vector can be used to distinguish between transgenic and nontransgenic roots from composite plants. Currently, the visual reporter genes used include *GUS*, a fluorescent protein-encoding gene (*GFP*, *RFP*, etc.), a transcription factor involved in anthocyanin synthesis (such as *AtMYB75*, *MtLAP1*), and *RUBY* [4,9–12]. *GUS* staining is a procedure that is invasive and typically necessitates the destruction of plant specimens. Although fluorescent protein is easy to use, it necessitates the presence of light sources to detect the emitted fluorescence signals. It is difficult to use the transcription factors of anthocyanin biosynthesis as a universal visible reporter gene in plants lacking anthocyanin synthesis genes [4]. Betalains are naturally occurring pigments with a nitrogen-containing heterocyclic structure, predominantly present in plants that belong to the Caryophyllales order [13]. The vivid red color observed in plant tissues and organs is attributed to the presence and accumulation of betalains. *RUBY* is composed of three genes, *CYP76AD1*, *DODA*, and *glucosyltransferase*, and converts tyrosine to red betalain in plant cells. *RUBY* as a reporter is used to detect gene expression and indicate transgenic events in plant genetic transformation [12]. A visual identification technology system for gene-edited soybeans was established by utilizing *RUBY* as a reporter system, and transgene-free genetically edited soybean plants were successfully obtained through seed and cotyledon color [14]. Therefore, *RUBY* can be used as a new visual reporting system [12,15,16]. In this study, *RUBY* was introduced into a binary expression vector as a reporter in *A. rhizogenes*-mediated hairy root genetic transformation in *L. corniculatus*.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Seeds of *Lotus corniculatus* L. (Linn. Li'ao) were kept in our laboratory. The plants were cultivated in a growth chamber (24–26 °C, 16 h/8 h light/dark cycle).

2.2. Construction of the Binary Vector

To produce a *RUBY* (including three genes, *CYP76AD1*, *DODA*, and *glucosyltransferase*, linked with 2A self-cleavage peptides) overexpression vector, a *RUBY* cassette fragment was amplified using pDR5:RUBY as a template with the primer sets RubyS1 (5'-GTGTTGTCGACTAGATGGATCATGCGACCC-3') and RubyS2 (5'-GTGTTGTCGAC TCACTATCACTGGAGGCTTGG-3') (*Sall* restriction site underlined) [12]. The PCR fragment was digested with *Sall* and the pCambia1305 binary vector was digested with *XhoI*, and then those digested DNA fragments were mixed in a one-step digestion–ligation reaction with *XhoI* and T4 ligase [17]. The purpose of adding *XhoI* in the one-step digestion–ligation reaction is to prevent the self-cyclization of the vector digested with *XhoI*. The one-step digestion–ligation reaction was performed as follows: 10 cycles (37 °C, 3 min; 16 °C, 5 min) and then 37 °C for 5 min. The reaction mixture was transformed into chemically prepared competent *E. coli* DH5 cells through thermal stimulation at 42 °C for 45 s.

2.3. *A. rhizogenes*-Mediated Hairy Root Transformation

L. corniculatus seeds were treated with 100% sulfuric acid for 8 min, rinsed 5 times with sterile water, and then placed on wet filter paper in a sterilized culture dish. After approximately 30 h of germination, the radicle grew to approximately 1 cm, and the hypocotyl was cut off, inoculated with *A. rhizogenes* K599 harboring p35RUBY through the incision, and then placed on 1/2 MS culture medium [7].

The young branches of *L. corniculatus* as explants used for hairy root transformation should be the tender branches in the vegetative growth stage, rather than in the flowering stage. Young branches with 1–2 internodes are preferred. The transverse incision sites of young branches were coated with *A. rhizogenes* K599 harboring p35RUBY for hairy root

transformation and then the explants were planted in sterile vermiculite wetted with sterile water and covered with a highly transparent plastic bag in a growth chamber [5]. A total of 30 seedlings and young branches were taken as biological replicates, and 3 replicates were set to evaluate the transformation frequency.

2.4. Genetically Modified Identification with PCR Amplification

To characterize the transgenic and nontransgenic hairy roots, the 35S enhanced promoter and the *CYP76AD1* gene were used for polymerase chain reaction (PCR) analysis. Independent hairy roots from composite *L. corniculatus* were subjected to DNA isolation. Genomic DNA was extracted according to a previously described method [5]. The specific primer 35S-F (5'-GACGCACAATCCCACTATCC-3') located in the 35S enhanced promoter and the primer RUBY2 (5'-CTGTGGGGAGAGCAGATGC-3') located in *CYP76AD1* were used for PCR analysis.

2.5. Statistical Analysis of Hairy Root Transformation Frequency

Thirty plants and thirty young branches were used for one-step ARM hairy root transformation. The composite plants were observed after 14 days. As long as a composite plant contains one transgenic hairy root, it is considered a transgenic composite plant. The data were analyzed using the software GraphPad Prism 8.

2.6. Nodulation Assay

The synthesis of betalain is carried out through enzymatic reactions using tyrosine as a substrate. To evaluate whether betalain in transgenic roots interferes with legume nodulation and whether *RUBY* can serve as a reporter for nitrogen-fixing nodule development, *Mesorhizobium loti* MAFF303099 was inoculated with composite *L. corniculatus*. The nodulation phenotype was evaluated at 30 days postinoculation. The data were analyzed using the software GraphPad Prism 8.

3. Results

3.1. Binary Vector with RUBY as a Reporter System

A *RUBY* cassette, harboring *CYP76AD1*, *DODA*, and *glucosyltransferase* linked with 2A self-cleavage peptides, was cloned between the *XhoI* sites of pCambia1305 to replace *HptII* (Hygromycin Phosphotransferase II). The *RUBY* cassette was driven by the enhanced 35S promoter, and the resulting *RUBY* overexpression vector was termed p35RUBY (Figure 1).

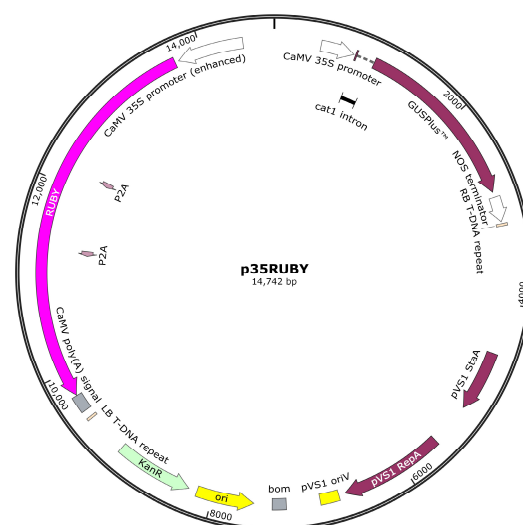


Figure 1. Binary p35SRUBY vector map.

3.2. Vividly Red Hairy Roots Were Produced from Hypocotyl Incisions

At 14 days postinfection with K599 harboring p35SRUBY, composite plants of *L. corniculatus* were obtained from two different explants. Red hairy roots were produced from the hypocotyl incision of *L. corniculatus* (Figure 2a).

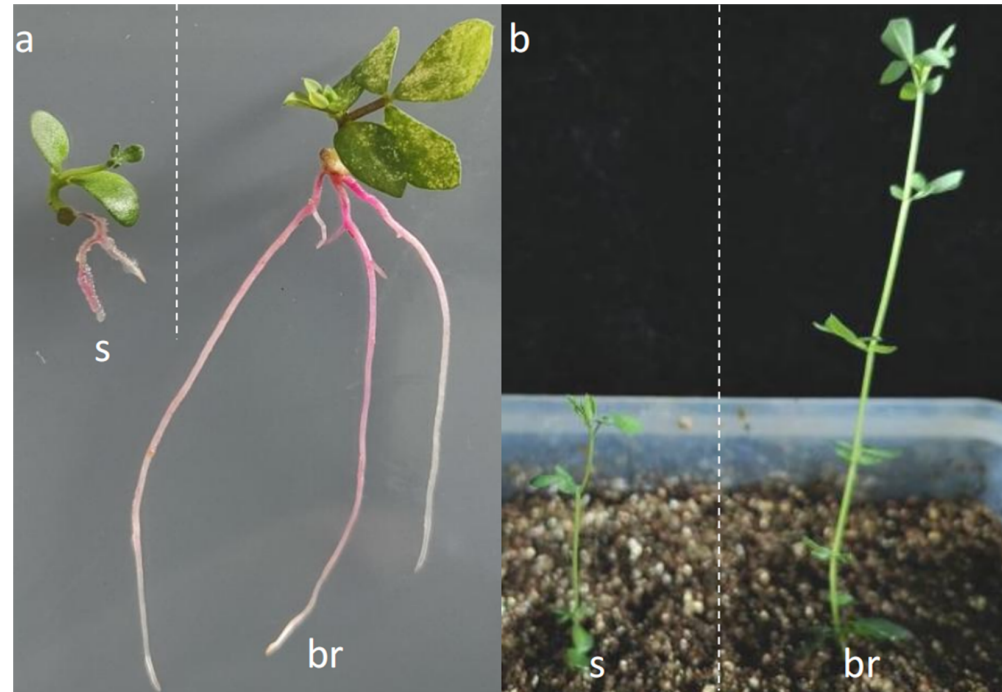


Figure 2. Composite plants of *L. corniculatus* transformed with K599. (a): The roots showed red color in composite plants after 14 days postinfection with K599 harboring p35SRUBY; (b): composite plants transplanted into vermiculite after 7 days. s: composite plant from seedlings; br: composite plant from branches.

PCR amplification was performed with the primer set 35S-F and RUBY2, with genomic DNA isolated from the red hairy roots and white hairy roots serving as templates. The 535-bp amplified fragments were obtained from red hairy roots, while no amplified fragments were detected from white hairy roots (Figure 3). PCR analysis indicated that the red hairy roots were transgenic hairy roots and that the white hairy roots were nontransgenic roots.

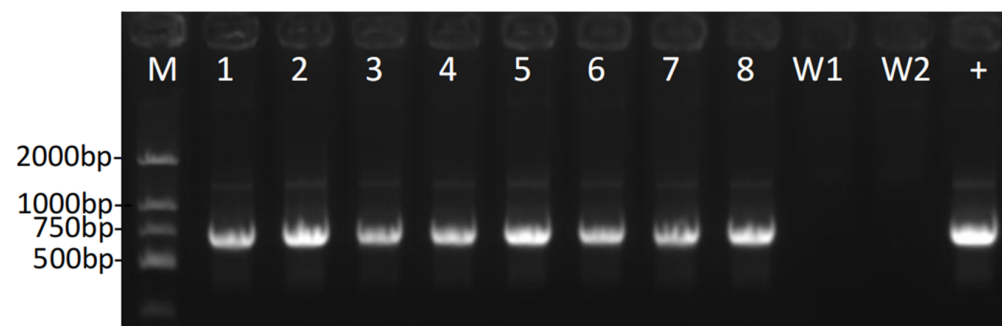


Figure 3. PCR analysis of hairy roots expressing RUBY. M, DL2000 DNA marker; lanes 1–8, independent red roots; lanes W1–2, independent white roots; +, p35SRUBY.

3.3. Young Branches as Explants Are Better Than Those as Seedlings

Provided that a composite plant harbors one red-colored root, it is considered a transgenic composite plant. In total, 20, 17, and 18, and 26, 24, and 23 transgenic composite

plants were obtained from explants of the seedlings and young branches, respectively (Table 1). Therefore, the transgenic hairy root transformation frequencies were 62.2% and 81.1% for the seedlings and young branches, respectively. The average numbers of total hairy roots and positive transgenic hairy roots in the transgenic composite plants were 2.70 and 1.60, respectively, for the seedlings, and 3.98 and 2.19, respectively, for the young branches (Table 1). There was a significant difference in the average number of transgenic hairy roots between the transgenic composite plants obtained from the seedlings and those obtained from the young branches.

Table 1. Hairy root transformation frequency of the *A. rhizogenes*-mediated method with two different explants.

Explants	Numbers of Explants	Numbers of Composite Plants	Transformation Frequency	Average of Total Roots in Transgenic Composite Plant	Average of Transgenic Hairy Roots in Transgenic Composite Plant
seedling	30	20	62.2%	3.00 ± 0.56^B	1.65 ± 0.49^B
	30	17		2.53 ± 0.72^B	1.59 ± 0.51^B
	30	18		2.56 ± 0.74^B	1.56 ± 0.62^B
young branch	30	26	81.1%	4.15 ± 0.88^A	2.27 ± 0.72^A
	30	24		3.96 ± 1.08^A	2.17 ± 0.82^A
	30	23		3.82 ± 0.89^A	2.13 ± 0.76^A

Letters A and B are significantly different at $p = 0.01$ according to two-tailed unpaired t tests. The values are presented as the means $\pm$ standard deviations of 30 independent composite plants.

The composite plants were transplanted into vermiculite, and 7 days later, the composite plants obtained from young branches were stronger and taller than the composite plants obtained from seedlings (Figure 2b).

3.4. Evaluating the Reliability of RUBY as a Reporter in the Study of Rhizobia–Legume Symbiosis

After 30 days of transplanting the composite plants back into the soil, mature nitrogen-fixing nodules formed normally with a red color in the red transgenic hairy roots (Figure 4a–c). We found that the redness of the transgenic roots gradually decreased, and even disappeared, but the surface of the nodules that formed in the transgenic roots remained clearly red (Figure 4a–c).

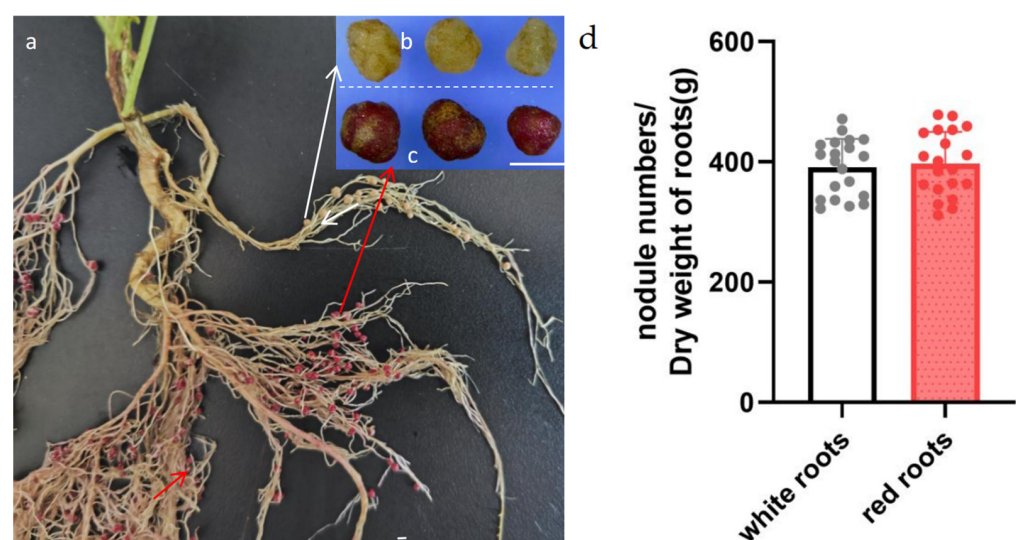


Figure 4. (a): Composite plants of *L. corniculatus* with transgenic and nontransgenic roots; (b): nodules from nontransgenic roots; (c): nodules from transgenic roots; (d): numbers of nodules formed per dry weight of *L. corniculatus* hairy roots. White arrows: white nodules; Red arrows: red color nodules; Bars: 2 mm.

Mature nitrogen-fixing nodules from red transgenic roots and nontransgenic roots were counted. The nodule numbers in red transgenic roots were not different from those in nontransgenic roots for the same weight of dry roots (Figure 4d). These results indicated that the production of betalain in the roots does not affect the formation of root nodules.

4. Discussion

Seedlings were used as explants in previous hairy root transformations of *L. corniculatus* [7]. The seeds were surface-sterilized and germinated, after which the plants were used as explants. After approximately two days, the hairy roots were induced in the culture medium and operated on an ultraclean workbench. In this study, young branches were used as explants for hairy root transformation. There are several advantages to using young branches as explants for transformation. First, hairy root transformation can be undertaken at any time if plants are growing in the laboratory. There is no need to germinate seeds. Given that *L. corniculatus* is a perennial herb, new branches will grow soon after mowing. It is therefore easy and saves time to use young branches as explants for hairy root transformation. Second, more transgenic hairy roots are obtained from young branches as explants than from seedlings. Finally, the composite plants obtained from young branches grow quickly and robustly.

In previous reports, transcription factors involved in anthocyanin synthesis, such as *AtMYB75* and *MtLAP1*, were shown to initiate anthocyanin synthesis in roots; thus, *AtMYB75* or *MtLAP1* can serve as a reporter gene in hairy root transformation [4,18]. Compared with those of plants transformed with *AtMYB75* or *MtLAP1*, the roots of composite plants transformed with the reporter gene *RUBY* exhibited a more vivid red color in the early stages of root production. The red color of the roots gradually weakens as the roots grow, while the color of the nodules still shows as a very obvious red. The color of transgenic roots overexpressing *AtMYB75* or *MtLAP1* does not weaken or disappear as the roots grow [4]. Other studies have shown that the overexpression of *Aomelos*, *BvCYP76AD1S*, and *BvDODA1S* in carrots leads to the accumulation of betalain [19]. However, during the process from differentiation to seedling formation in carrots, the betalain content gradually decreases or even disappears. When supplemented with exogenous tyrosine, the roots of transgenic plants can synthesize betalain again [19]. The disappearance or reduction of betanin content in transgenic roots may be related to the glycosylation of intermediate metabolites. Glycosylation enhances the half-life of betanidin and isobetanidin when exposed to reactive oxygen species, resulting in a 17-fold increase [20]. Glycosylation serves as a crucial modification process that significantly impacts solubility, physicochemical stability, biological longevity, and permeability through biological membranes of plant-derived secondary metabolites [21]. So, in this study, the difference in color between roots and nodules tissues may be related to the degree of glycosylation of intermediate metabolites of betalain synthesis in different organs or developmental stages.

Betalains constitute a group of plant natural compounds and encompass the red-violet betacyanins and the yellow-orange betaxanthins, which can be utilized as additives for coloring food [22]. Betalains are renowned for their potent antioxidant capabilities, and it is believed that their intake can offer numerous advantages for human health [23]. Metabolic engineering of betalains may be an effective method to enhance the anti-inflammatory properties of common foods such as tomatoes [24]. Betanin biosynthetic pathways have been reconstructed with the *BvADH α* gene under the control of fruit-specific promoters in tomato. The findings demonstrate that the genetically modified tomatoes exclusively yield betacyanins, bestowing upon the tomato juice a vibrant purple-fuschia hue [25]. People love a colorful world and hope to create plants with a variety of colors. *RUBY* was introduced into three succulent species, *Kalanchoe blossfeldiana*, *Crassula arborescens*, and *Sansevieria trifasciata*, and all transgenic-positive succulent species show a red color [26]. So there may be more and more studies on the genetic improvement of plants using metabolic engineering of betalains. This could involve boosting their concentration, employing them as a reporter, or altering the aesthetic appeal of plants.

5. Conclusions

The young branches were used as explants for hairy root transformation with more transformation frequency than the seedlings. *RUBY*, as a reporter gene, can distinguish transgenic roots from non-transgenic roots easily, based on the color of hairy roots.

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References

1. Beyaz, R.; MacAdam, J.W. X-Radiation of *Lotus corniculatus* L. Seeds Improves Germination and Initial Seedling Growth. *Int. J. Radiat. Biol.* **2023**, *99*, 1794–1799. [[CrossRef](#)] [[PubMed](#)]
2. Montiel, J.; Reid, D.; Grønbaek, T.H.; Benfeldt, C.M.; James, E.K.; Ott, T.; Ditengou, F.A.; Nadzieja, M.; Kelly, S.; Stougaard, J. Distinct Signaling Routes Mediate Intercellular and Intracellular Rhizobial Infection in *Lotus japonicus*. *Plant Physiol.* **2021**, *185*, 1131–1147. [[CrossRef](#)] [[PubMed](#)]
3. Sato, S.; Nakamura, Y.; Kaneko, T.; Asamizu, E.; Kato, T.; Nakao, M.; Sasamoto, S.; Watanabe, A.; Ono, A.; Kawashima, K.; et al. Genome Structure of the Legume, *Lotus japonicus*. *DNA Res.* **2008**, *15*, 227–239. [[CrossRef](#)] [[PubMed](#)]
4. Fan, Y.; Wang, X.; Li, H.; Liu, S.; Jin, L.; Lyu, Y.; Shi, M.; Liu, S.; Yang, X.; Lyu, S. Anthocyanin, a Novel and User-Friendly Reporter for Convenient, Non-Destructive, Low Cost, Directly Visual Selection of Transgenic Hairy Roots in the Study of Rhizobia-Legume Symbiosis. *Plant Methods* **2020**, *16*, 94. [[CrossRef](#)] [[PubMed](#)]
5. Fan, Y.; Zhang, X.; Zhong, L.; Wang, X.; Jin, L.; Lyu, S. One-Step Generation of Composite Soybean Plants with Transgenic Roots by *Agrobacterium rhizogenes*-Mediated Transformation. *BMC Plant Biol.* **2020**, *20*, 208. [[CrossRef](#)] [[PubMed](#)]
6. Mugnier, J. Establishment of New Axenic Hairy Root Lines by Inoculation with *Agrobacterium rhizogenes*. *Plant Cell Rep.* **1988**, *7*, 9–12. [[CrossRef](#)] [[PubMed](#)]
7. Boisson-Dernier, A.; Chabaud, M.; Garcia, F.; Bécard, G.; Rosenberg, C.; Barker, D.G. *Agrobacterium rhizogenes*-Transformed Roots of *Medicago truncatula* for the Study of Nitrogen-Fixing and Endomycorrhizal Symbiotic Associations. *MPMI* **2001**, *14*, 695–700. [[CrossRef](#)]
8. Stiller, J.; Martirani, L.; Tupale, S.; Chian, R.-J.; Chiurazzi, M.; Gresshoff, P.M. High Frequency Transformation and Regeneration of Transgenic Plants in the Model Legume *Lotus japonicus*. *J. Exp. Bot.* **1997**, *48*, 1357–1365. [[CrossRef](#)]
9. Liu, Y.; Zeng, J.; Yuan, C.; Guo, Y.; Yu, H.; Li, Y.; Huang, C. Cas9-PF, an Early Flowering and Visual Selection Marker System, Enhances the Frequency of Editing Event Occurrence and Expedites the Isolation of Genome-edited and Transgene-free Plants. *Plant Biotechnol. J.* **2019**, *17*, 1191–1193. [[CrossRef](#)]
10. Lin, X.; Jia, Y.; Heal, R.; Prokhorchik, M.; Sindalovskaya, M.; Olave-Achury, A.; Makechemu, M.; Fairhead, S.; Noureen, A.; Heo, J.; et al. Solanum Americanum Genome-Assisted Discovery of Immune Receptors That Detect Potato Late Blight Pathogen Effectors. *Nat. Genet.* **2023**, *55*, 1579–1588. [[CrossRef](#)]
11. Timoneda, A.; Yunusov, T.; Quan, C.; Gavrín, A.; Brockington, S.F.; Schornack, S. MycoRed: Betalain Pigments Enable In Vivo Real-Time Visualisation of Arbuscular Mycorrhizal Colonisation. *PLoS Biol.* **2021**, *19*, e3001326. [[CrossRef](#)]
12. He, Y.; Zhang, T.; Sun, H.; Zhan, H.; Zhao, Y. A Reporter for Noninvasively Monitoring Gene Expression and Plant Transformation. *Hortic. Res.* **2020**, *7*, 152. [[CrossRef](#)] [[PubMed](#)]
13. Polturak, G.; Aharoni, A. Advances and Future Directions in Betalain Metabolic Engineering. *New Phytol.* **2019**, *224*, 1472–1478. [[CrossRef](#)] [[PubMed](#)]
14. Chen, L.; Cai, Y.; Liu, X.; Yao, W.; Wu, S.; Hou, W. The RUBY Reporter for Visual Selection in Soybean Genome Editing. *aBIOTECH* **2024**, 1–5. [[CrossRef](#)]
15. Ajdanian, L.; Niazian, M.; Torkamaneh, D. Optimizing *Ex Vitro* One-step RUBY-equipped Hairy Root Transformation in Drug- and Hemp-type Cannabis. *Plant Biotechnol. J.* **2024**, *22*, 1957–1959. [[CrossRef](#)] [[PubMed](#)]

16. Niazian, M.; Belzile, F.; Curtin, S.J.; De Ronne, M.; Torkamaneh, D. Optimization of *In Vitro* and *Ex Vitro* *Agrobacterium rhizogenes*-Mediated Hairy Root Transformation of Soybean for Visual Screening of Transformants Using RUBY. *Front. Plant Sci.* **2023**, *14*, 1207762. [[CrossRef](#)]
17. Wang, X.; Teng, C.; Wei, H.; Liu, S.; Xuan, H.; Peng, W.; Li, Q.; Hao, H.; Lyu, Q.; Lyu, S.; et al. Development of a Set of Novel Binary Expression Vectors for Plant Gene Function Analysis and Genetic Transformation. *Front. Plant Sci.* **2023**, *13*, 1104905. [[CrossRef](#)] [[PubMed](#)]
18. Zhang, S.; Kondorosi, É.; Kereszt, A. An Anthocyanin Marker for Direct Visualization of Plant Transformation and Its Use to Study Nitrogen-Fixing Nodule Development. *J. Plant Res.* **2019**, *132*, 695–703. [[CrossRef](#)] [[PubMed](#)]
19. Wang, D.; Zhong, Y.; Feng, B.; Qi, X.; Yan, T.; Liu, J.; Guo, S.; Wang, Y.; Liu, Z.; Cheng, D.; et al. The RUBY Reporter Enables Efficient Haploid Identification in Maize and Tomato. *Plant Biotechnol. J.* **2023**, *21*, 1707–1715. [[CrossRef](#)]
20. Sadowska-Bartos, I.; Bartosz, G. Biological Properties and Applications of Betalains. *Molecules* **2021**, *26*, 2520. [[CrossRef](#)]
21. Vogt, T.; Jones, P. Glycosyltransferases in Plant Natural Product Synthesis: Characterization of a Supergene Family. *Trends Plant Sci.* **2000**, *5*, 380–386. [[CrossRef](#)] [[PubMed](#)]
22. Esatbeyoglu, T.; Wagner, A.E.; Schini-Kerth, V.B.; Rimbach, G. Betanin—A Food Colorant with Biological Activity. *Mol. Nutr. Food Res.* **2015**, *59*, 36–47. [[CrossRef](#)] [[PubMed](#)]
23. Gandía-Herrero, F.; Escibano, J.; García-Carmona, F. Biological Activities of Plant Pigments Betalains. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 937–945. [[CrossRef](#)] [[PubMed](#)]
24. Saito, S.; Nishihara, M.; Kohakura, M.; Kimura, K.; Yashiro, T.; Takasawa, S.; Arimura, G. Metabolic Engineering of Betacyanin in Vegetables for Anti-inflammatory Therapy. *Biotech. Bioeng.* **2023**, *120*, 1357–1365. [[CrossRef](#)] [[PubMed](#)]
25. Grützner, R.; Schubert, R.; Horn, C.; Yang, C.; Vogt, T.; Marillonnet, S. Engineering Betalain Biosynthesis in Tomato for High Level Betanin Production in Fruits. *Front. Plant Sci.* **2021**, *12*, 682443. [[CrossRef](#)]
26. Lu, J.; Li, S.; Deng, S.; Wang, M.; Wu, Y.; Li, M.; Dong, J.; Lu, S.; Su, C.; Li, G.; et al. A Method of Genetic Transformation and Gene Editing of Succulents without Tissue Culture. *Plant Biotechnol. J.* **2024**, *22*, 1981–1988. [[CrossRef](#)]

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