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An *Agrobacterium rhizogenes* Strain R1000-mediated Efficient Hairy Root Transformation Protocol for Common Bean

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ABSTRACT

Background: Common bean (*Phaseolus vulgaris* L.) is a globally important grain and vegetable legume crop, providing a substantial portion of the diet protein and minerals for many people in the developing world. However, the genetic studies and improvement on this crop has long been impeded by its recalcitrance to Agrobacterium-mediated whole plant genetic transformation. Established *Agrobacterium rhizogenes*-based hairy root transformation in common bean heavily relies on the strain K599.

Methods: In order to develop an efficient alternative protocol for hair transformation in common bean, the efficiency of *Agrobacterium rhizogenes* strain R1000 in inducing hairy roots from 6-day-old seedlings with root below cotyledons excised by the soaking and smearing method were tested. The binary plasmid pBI121 with the reporter gene GUS (pBI121-GUS) or eGFP (pBI121-eGFP) driven by the constitutive promoter was used for transformation and rapid identification of the transgenic hairy roots.

Result: We established a strain R1000-based system for the induction of hairy roots in common bean. The plant receptor genotypes and infection methods were optimized, which led to a high transformation rate of hairy roots up to 60%. This method therefore provides a useful alternative means for functional genomic studies in common bean.

Key words: *Agrobacterium rhizogenes*, Common bean, Hairy root, Transformation.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important food and fresh vegetable legume in the world. Providing up to 70% of the total protein requirement, common bean is particularly important for people in Latin America and Africa where affordable animal proteins are scarce (Khandual, 2014). In addition to providing proteins, common bean also is an important source of iron, phosphorus, magnesium, manganese and to a lesser degree, zinc, copper and calcium (Stagnari *et al*., 2017). Thus, especially in developing countries, the excellent dietary microelement source can be complemented from common bean. Globally, the yield and quality of common bean are often constrained by diseases, pests and abiotic stresses such as drought, heat and high salinity (Partap and Godara, 2022; Priyanka *et al*., 2022; Xu *et al*., 2022). Conventional breeding is still the mainstream approach for genetic improvement of this crop, which, however, has many disadvantages such as long breeding cycle, labor intensity, low recombination rate of multiple genes and incompatibility of distant crosses (Asfaw and Blair, 2014). With the development of biotechnology, advances in breeding, functional research and targeted genetic modifications have become essential for studying common bean. Thus, efficient transformation systems are required to advance common bean research.

Genetic engineering is a powerful approach to accelerate crop improvement and overcomes the major limitations of traditional breeding (Mukeshimana *et al*., 2013; Varshney *et al*., 2012). Plant transgenic technology, as a means of genetic engineering, has shown its usefulness in breaking the linkage drag or species barrier in many crop taxa and become a common approach in crop breeding programs (Datta, 2013). Unfortunately, the genus *phaseolus vulgaris* that includes common bean has known to be recalcitrant to regenerate *in vitro,* imposing a bottleneck for efficient plant transformation (Malik and Saxena, 1991; Singh and Tiwari, 2012). To date, limited cases of success have been reported in common bean transformation efforts. Some authors reported direct gene transfer based on particle bombardment or electroporation has been documented, but these methods generally yield low transformation frequencies and high rates of chimeras and false positives (Bonfim *et al*., 2007; Vianna *et al*., 2004).

At present, Agrobacterium-mediated transformation is the method most frequently used for common bean transformation. Compared with *Agrobacterium tumefaciens*-mediated transformation of common bean, the *Agrobacterium rhizogenes*-mediated hairy root transformation system has a high transformation efficiency

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and short transformation period. The process can be completed within one month. Hairy root transformation technique is a commonly used alternative method for the generation of transgenic plants of legumes. Instead of using *A.tumefaciens*, hairy root transformation uses *A.rhizogenes*, a gram-negative soil bacterium belonging to the genus *Rhizobia* that infects most dicotyledonous plants, some monocotyledonous plants and a few gymnosperms (Veena and Taylor, 2017). *A. rhizosphere* has a root-inciting (Ri) plasmid, which can induce hairy roots formation at the injured site of the infected plant. During this process, the T-DNA in the Ri plasmid can be randomly transferred, integrated and inserted into the genome of the plant cells, thereby generating composite transgenic plants (Chilton *et al*., 1982). Usually, hairy roots are non-chimeric, because they are derived from single cells and each hairy root consists of uniformly transformed cells. Agrobacterium-mediated hairy root technique has many advantages such as low cost, high reproducibility, high transformation rate and wide range of receptor plants.

Successful legumes studies have been performed using *Agrobacterium rhizogenes*. Aggarwal *et al*. (2018) demonstrated an efficient, high-throughput and genotype-independent method of root transformation in chickpea using *A.rhizogenes* K599. The efficiency of transformation was higher (73.33%) using immersion method than the cotyledonary injection method (38.66%). Estrada-Navarrete *et al.* (2007) described a fast, reproducible and efficient common bean root transformation protocol with the *A. rhizogenes* strain K599. This method has worked successfully in generating high efficiency (70-90%) of hairy roots in multiple genotypes and landraces of common bean and other *phaseolus vulgaris* subspecies. To date, the *A.rhizogenes*-mediated hairy root transformation system has been applied in gene functional analysis, promoter analysis and plant stress response studies in common bean (Carrasco-Castilla *et al*., 2018; Estrada-Navarrete *et al*., 2007; Nanjareddy *et al*., 2017; Yao *et al*., 2014). However, in most reported cases the *A. rhizogenes* strain used was K599, which was assumed to be critical by some researchers (Estrada-Navarrete *et al*., 2007).

In this study, to overcome the limitation of *A.rhizogenes* strain selectivity in current common bean hairy root transformations, we established an alternative, *A. rhizogenes* strain R1000-mediated hairy root transformation protocol for common bean, which showed up to 60% of transgenic rate in a wide range of receptor genotypes.

MATERIALS AND METHODS

The experiment was conducted from October 2020 to March 2021 in the Lab of Plant Quality and Safety Biology, College of Life Sciences, China Jiliang University, Hangzhou, China.

Plant materials

Twenty common bean genotypes provided by Zhejiang Academy of Agricultural Sciences were used for *A.rhizogenes* strain R1000-mediated transformation. These genotypes include *LP089, P331024017, P331024004, LP093, LP074, LP047, LP096, LP020, LP011, LP053, 289332211, 2018334327, Hongjinyuanjia, Honghuaqinjia, Cuiyu, Yudouwang, Yinmanjia, Chunqiujiadouwang, Huazihonghuaqingjia* and *Pan'an common bean.*

Seeds disinfection and germination

The seeds were rinsed twice with sterile water before being surface-sterilized with 70% ethanol for 30 s. The seeds were thoroughly washed with sterile water. Disinfected seeds were placed on plates containing sterile wet filter paper for germination. The plates were kept in 23 to 26°C temperatures in the dark. After germination, the germinated seedlings were sown into sterilized growing substrate (a mixture of 3:1 peat and vermiculite) and were cultured in a plant growth room of 16 h light/8 h dark cycle at 25±2°C under a 60% relative humidity condition with an intensity 600 µmol $m^{-1}s^{-1}$. Three days after sowing, seedlings with green cotyledons unfolded were selected for hairy roots induction.

Plasmid

The binary plasmid pBI121 with the reporter gene GUS (pBI121-GUS) or eGFP (pBI121-eGFP) driven by the constitutive promoter cauliflower mosaic virus (CaMV) 35S were used for transformation and rapid identification of the positive hairy roots.

Agrobacterium strains and inoculum preparation

The plasmid vector was transferred into *A.rhizogenes* strain K599 and R1000 *via* electroporation. The strains were grown at 28°C in solidified Luria Bertani (LB) medium with appropriate antibiotics (kanamycin for R1000, kanamycin and rifampin for K599) to produce a lawn in the plate. The plates were cultured for 2 days at 28°C, then the strains were re-streaked onto fresh plates with appropriate antibiotic selection and incubated for 3 days at 28°C before inoculation.

Induction of hairy roots

Healthy and uniform seedlings with unfolded green cotyledons were selected for agro-infiltration by the soaking and smearing methods as described below:

Soaking method

The cultures of strains were suspended in MS buffer (10 mM MES-KOH, pH 5.8 and 100 µM acetosyringone) until the OD_{600} value reached 1.2 and then 15 ml of the bacterial suspension was pipetted onto a beaker with a volume of 25 ml. Healthy 7-day-old plants were taken out of chamber and the roots were excised approximately 3 cm below the cotyledons using a sterile razor blade. The plants were soaked into the bacterial suspension in the darkness at 22°C for 1 hour, then were transplanted into seed germinating bag containing 5% 1/2 MS culture liquid. The humidity level was maintained at 95%. Three days later, fluorescent light at 600 µmol $m^{-1}s^{-1}$ of photosynthetic photon flux density (PPFD) in a 16 h photoperiod was resumed. After 5-7 days,

calluses production was noticed. After 14 to 15 days after excision, the transformed roots started emerging from the excision site.

Smearing method

Each plant was gently pricked with a needle tip for six or eight times around the cotyledon node. The cultures of strains that were suspended in the above-mentioned MS buffer were smeared around the wounded sites of the plants. The culture condition of infected plants was the same as that used in the soaking method. Typically, calluses were emerged from the wounding sites 5-7 days after infection, while hairy roots generated about 2 weeks.

Detection of transgenic hairy roots

According to reporter gene in the designed vector, reporter gene can be used to detect transgenic hairy roots. GFP can be assessed by fluorescence, whereas GUS can be assessed by GUS staining.

Visual detection of GFP

GFP fluorescence in the transgenic hairy roots were observed by using a portable fluorescence lamp (LUYOR-c3415RG) at the excitation wavelength of 488 nm and an LP 505-nm emission filter. Transgenic hairy roots showed GFP fluorescence labeling.

Histochemical GUS assay

The histochemical assay for the reporter gene GUS activity was performed following the established method (Jefferson, 2007). Briefly, the hairy roots were cut off and placed in a centrifuge tube containing GUS staining buffer [50 mmol L^{-1} sodium phosphate, 0.5 mmol L^{-1} potassium ferrocyanide, 0.5 mmol L^{-1} potassium ferricyanide, 0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 0.1% Triton X-100 and 20% methanol, pH = 7.0] for 12 h. The reaction mixture was incubated at 37°C overnight. Expression of the *GUS* gene in hairy roots was visually assayed and the percentage of transformed roots was calculated.

RESULTS AND DISCUSSION

Development and efficiency of the soaking method using R1000

We first tested the efficiency of R1000 in inducting hairy roots from 6-day-old seedlings with root below cotyledons excised by the soaking method according to Ali *et al.* (2012) with little modifications. A total of 20 genotypes were screened in this test. In general, after about 5-7 days of growth following inoculation, callus generated around the excision points. After 14 days of growth, small hairy roots emerged from the callus (Fig 1). From the 20 genotypes, transgenic hairy roots with green fluorescence of eGFP were observed from five genotypes (*LP089, P331024017, LP074, Cuiyu* and *Honghuaqinjia*) under the irradiation of a fluorescence detector. The genotype *'Cuiyu'* had the highest transformation rate, up to 33%, followed by *'LP089'*. Our attempt to improve the transformation efficiency in five randomly selected genotypes namely *LP089, Pan'an common bean, Hongjinyuanjia, honghuaqinjia* and *LP074* by adjusting the infection concentration of *A. rhizogenes* from OD_{600} 0.8 to 1.2 failed to raise the positive transformation rate.

Development and efficiency of the smearing method using R1000

To investigate if the infection method had a major effect on transformation rate, we then established a smearing-based transformation protocol using R1000 according to a published method with the K599 strain (Estrada-Navarrete *et al*., 2007). Four genotypes, namely *LP089, P331024004, LP074* and *Honghuaqinjia,* were used in this analysis. After 5-7 days, callus generated around the smearing point and after 14 days of growth, hairy roots were developed from the callus. Through GUS staining, transgenic hairy roots that expressed β-glucuronidase were identified. As follow, the transformation efficiency of genotype *'Honghuaqinjia'* increased drastically from 4% by soaking infection to 59% by smearing infection and that of the genotype *'LP089'* increased from 25% to 47%, respectively. Under the soaking condition, the transformation efficiency of *'P331024004'* was

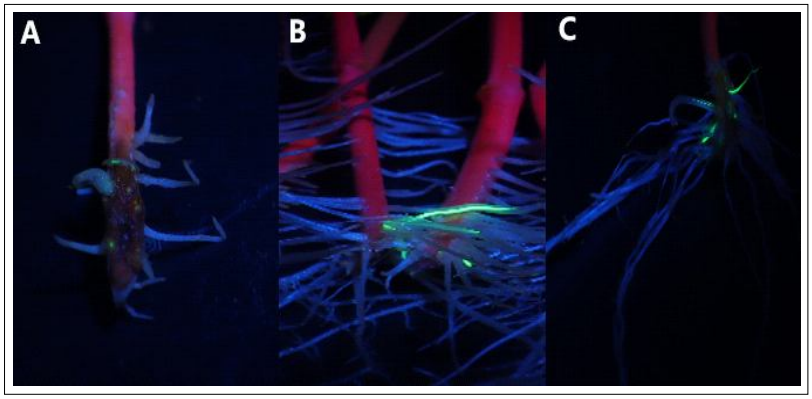

Fig 1: R1000-mediated transformation into common bean (*Phaseolus vulgaris* L. cv *'LP089'*) hairy roots by the soaking method. (A) Induction of callus after 14 days of growth. (B) Induction of hairy roots after 25 days of growth. (C) Transgenic hairy roots expressing the CaMV 35S-driven *eGFP* gene.

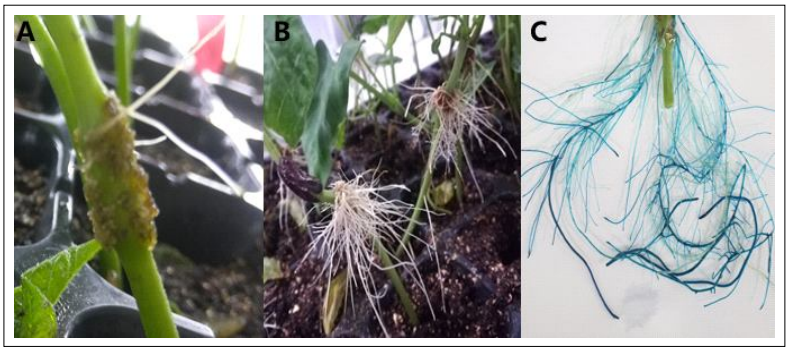

Fig 2: R1000-mediated transformation into common bean (*Phaseolus vulgaris* L. cv *'Cuiyu'*) hairy roots by smearing. (A) Induction of callus after 14 days of growth. (B) Induction of hairy roots after 25 days of growth. (C) Transgenic hairy roots expressing the CaMV 35S-driven *GUS* gene.

Table 1: Hairy root induction efficiency of genotype *'LP089'* by different infection ways.

Strain	Infection way	Hairy roots quantity	Transgenic roots quantity	Transformation efficiency (%)
R1000	Smear	32	17	53
R1000	Soak	13	3	23
K599	Smear	6	1	17
K599	Soak	12	2	17

0%, but under the smearing condition, the efficiency was 43%. However, the genotype *'LP074'* had a transformation rate of 0% by smearing, whereas showed a rate of 17% by soaking. Overall, the transformation efficiency by using the smearing infection method was higher than that using the soaking infection. These results taken together suggest that the efficiency of *A. rhizogenes* strain R1000-mediated hairy root transformation is generally satisfying with the smearing method but is dependent on the genotype of the common bean plant.

Comparison of using the strains R1000 and K599

We next compared the transformation efficiency in the common bean genotype *'LP089'* of using the two *A. rhizogenes* strains (R1000 and K599) containing the empty-plasmid pBI121-GUS. In our experiment, K599 appeared to be no better than R1000 to induce positive transgenic hairy roots. As show in Table 1, there was no obvious difference in the transformation rate between the two strains when the soaking infection method was used. Under the smearing infection method, the strain R1000 even exhibited a higher transformation efficiency of 53% as compared to that with the strain K599, which was only 17%. Therefore, for this specific common bean genotype, R1000 represents a good choice of *A. rhizogenes* strain.

To further test the superiority of the combination of strain R1000 and the smearing method, this hairy root induction protocol was then used in the genotype *'Cuiyu'* as well (Fig 2). The transformation efficiency was found to be similar (~50%) to that in *'LP089'*. Therefore, the combination of the strain R1000 and smearing infection method could be considered as a quite versatile, useful system for hairy root transformation in common bean.

CONCLUSION

In the present study, the *Agrobacterium rhizogenes* strain R1000 harboring pBI121 was proved to be able to effectively induce hairy roots in common bean. Our data demonstrate that using an optimized method and appropriate common bean genotype such as *'LP089'* and *'Cuiyu'*, a high efficiency of transgenic hairy root formation (~60%) can be achieved, which offers a new choice of common bean genetic transformation protocol. Further efforts to improve this protocol could be made by screening more genotypes of common bean for higher sensitivity to R1000 infection.

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Conflict of interest: None.

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