RESEARCH ARTICLE Legume Research- An International Journal, Volume 47 Issue 1: 8-13 (January 2024)

An Efficient Hairy Root Transformation Method for Common Bean based on Petiole Explants

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10.18805/LRF-778

ABSTRACT

Background: Common bean (*Phaseolus vulgaris* L.) is a major food legume with high nutritional value and economic importance globally. A remarkable attribute of this plant is its propensity to generate adventitious roots from petioles of detached leaves when moisture is appropriate. This distinctive feature presents promising prospects for harnessing petioles in the facilitation of transgenic hairy root production.

Methods: We achieved the successful induction of transgenic hairy roots from common bean petioles through the utilization of *Agrobacterium rhizogenes* strain K599. Our experimentation encompassed a diverse array of common bean varieties and leaves of varying ages. Subsequently, we refined and optimized the procedure for hairy root induction.

Result: Our investigations have revealed that the most conducive conditions for hairy root transformation with K599 are attained using 5-day-old leaves from the cultivar 'Honghuaqingjia', exhibiting a remarkable induction efficiency of 59%. The usefulness of this system was demonstrated through a subcellular localization analysis of the transcription factor PvTCP2 protein in combination with GFP (Green Fluorescent Protein).

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

INTRODUCTION

Hairy root culture, also referred to as transformed root culture, represents a well-established technique in plant tissue culture widely utilized in plant research. This method harnesses the natural capabilities of *Agrobacterium rhizogenes*, a soil-dwelling bacterium carrying root-inducing plasmids (Ri plasmids), to infect and trigger abnormal root growth in plants. These atypical roots, brought about by *A. rhizogenes* infection, exhibit exceptional growth rates and maintain genetic and biochemical stability, making them a valuable asset in plant research. Transgenic hairy roots are extensively employed for exploring the biosynthesis and regulation of plant secondary metabolites (Farag and Kayser, 2015). They are also a valuable tool for gene function analysis, encompassing tasks such as protein expression, subcellular localization, protein-protein interactions and the assessment of gene editing systems (Aarrouf *et al*., 2012; Cheng *et al*., 2021; Du *et al*., 2018; Plasencia *et al*., 2016; Ron *et al*., 2014; Yan *et al*., 2023). Owing to their swift induction properties, transgenic hairy roots find use in the verification of off-target effects in Cas9 gene editing (Cheng *et al*., 2021). Recently, plant hairy roots have been instrumental in high-throughput screening for antimicrobial agents (Irigoyen *et al*., 2020).

A variety of commercial *A. rhizogenes* strains are commonly employed for hairy root induction, with K599 being a widely utilized strain recognized for its versatility. The genome of *A. rhizogenes* strain K599, also known as strain NCPPB2659, comprises 5,277,347 base pairs. The wild-type strain induces hairy root disease in dicotyledonous plants and effectively promotes hairy root formation in a broad spectrum of plant species. It has also been instrumental in generating transgenic hairy root cultures and composite plants, where non-transgenic shoots are paired with transgenic roots. Variants of this strain have been employed to produce stable transgenic plants, both monocotyledonous and dicotyledonous, such as *Arabidopsis thaliana*, maize, tomato and soybean (Mankin *et al*., 2007; Valdes Franco *et al*., 2016).

The common bean, scientifically known as *Phaseolus vulgaris*, holds a prominent place in the realm of staple foods (Pineda *et al*., 2023). Renowned for its high protein and micronutrient content, common beans do not necessitate extensive industrial processing and can be readily cooked and consumed, contributing significantly to human nutrition (Castro-Guerrero *et al*., 2016). They find a wide array of applications in the realms of food, medicine and agriculture and are a vital functional crop (Alfaro-Diaz *et al*., 2023;

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How to cite this article: Liu, Y.H., Cai, X.Q., Ning, K. and Xu, P. (2024). An Efficient Hairy Root Transformation Method for Common Bean based on Petiole Explants. Legume Research. 47(1): 8-13. doi: 10.18805/LRF-778.

Submitted: 13-11-2023 **Accepted:** 16-12-2023 **Online:** 02-01-2024

Ayra *et al*., 2021; Calderón Guzmán *et al*., 2020; Carbas *et al*., 2020; Chillo *et al*., 2010; Chiozzotto *et al*., 2018; Thompson *et al*., 2017). For instance, common beans possess anti-obesity potential and under conditions of water scarcity, they can serve as a dietary intervention for obesity treatment (Salas-Lumbreras *et al*., 2023). Globally, common bean production is being constantly challenged by drought, salinity, insect pests and other stresses (Kouki *et al*., 2021; Kiymaz and Beyaz, 2019; Mukherjee *et al*., 2020). Given their rich nutritional profile and significance, research on common beans is imperative in addressing contemporary global food challenges, necessitating the establishment of a robust research framework for both fundamental functional studies and practical applications.

Previously, hairy root induction systems for common beans have been reliant on seedlings, involving strict requirements regarding seedling age, humidity levels and darkness (Estrada-Navarrete *et al*., 2007; Li *et al*., 2022; Nanjareddy *et al*., 2017; Wu *et al*., 2023). Cotyledons and hypocotyls have been the primary explants used for hairy root induction in these protocols.

Many legume plants have the propensity to generate adventitious roots from petioles of detached leaves when moisture is appropriate. This study introduces a swift and efficient hairy root induction system utilizing common bean petioles, which offers convenience and expediency. Given the numerous leaves produced by a single plant, our method presents an efficient means of generating a substantial quantity of hairy roots with minimal seed requirements.

MATERIALS AND METHODS

The experiment was conducted in Key Lab of Specialty Agri-Product Quality and Hazard Controlling Technology, School of Life Sciences, China Jiliang University, Hangzhou, Zhejiang Province, China, from April to November 2023.

Plant materials and preparation

Three common bean genotypes were employed: *Honghuabaijia* (trailing, sourced from Hualing Gaoke Seed Breeding Research Center in Mianyang, Sichuan Province, China), *Honghuaqingjia* (trailing, provided by Zhejiang Academy of Agricultural Sciences) and *Lvyoudiyoudou* (dwarf, obtained from Jiaxing Pioneer Seed Industry Co Ltd, Zhejiang Province, China). The full and healthy common bean seeds underwent a sterilization process involving a 30-second exposure to 75% ethanol. Subsequently, they were soaked in distilled water and placed in an oven at 30°C overnight. Following this, the seeds were arranged in square Petri dishes containing damp paper towels and left in darkness for 24 hours. The germinated seeds were then planted in a composite soil mixture of grass charcoal and vermiculite in a 3:1 ratio. These seeds were cultivated in an indoor environment with temperature set at 25°C (16 h light/8 h dark), humidity maintained between 50%-60% and light intensity of 100 µmol m^{-1} s^{-1}.

Construction of recombinant plasmids

The coding sequence (CDS) of *PvTCP2* (*Phvul.006G166600*), encoding a putative transcription factor, was obtained from Phytozome (https://phytozome-next.jgi.doe.gov/). Primer5 software was used to design primer and primers (Forward Primer: CAGGTCGACTCTAGAGGATCCATGGAAGAGGATGAGAT; Reverse Primer: GGGAAATTCGAGCTCGGTACCTTAGTTCTTTCCCTTGCC) were used to clone *PvTCP2.* The pMDC83 plasmid was digested with *KpnI* and *BamHI*. A positive single clone carrying the recombinant plasmid was selected for *Agrobacterium* transformation.

Agrobacterium transformation

Following established protocols for common bean (Estrada-Navarrete *et al*., 2007; Wu *et al*., 2023), *A. rhizogenes* strain K599 was used in this study. The recombinant pMDC83-PvTCP2-GFP plasmid was used for transformation, with GFP (Green Fluorescent Protein) in the plasmid serving as a marker for positive hairy root selection. *Agrobacterium* transformation was performed as per Li *et al*. (2022). In summary, 5 µl of plasmid was added to *A. rhizogenes* K599 and the mixture was subjected to an ice bath for 30 minutes, followed by a 5-minute treatment with liquid nitrogen and a 5-minute incubation in a 37°C water bath. The *A. rhizogenes* culture was then incubated at 28°C for 3 hours and subsequently grown on selected YEB (Yeast Extract Mannitol Broth) solid medium containing rifampicin and kanamycin. Positive single clones were confirmed through Polymerase Chain Reaction (PCR) analysis by using KOD One™ PCR Master Mix (TOYOBO, China). The thermocycling profile was as follow: 3 minutes at 98°C, then 10 seconds at 98°C, 10 seconds at 55°C, 30 seconds at 68°C with 30 cycles, finally 5 minutes at 68°C.

Hairy root induction and transgenic verification

Positive single clones of K599 were cultivated on YEB solid medium containing rifampicin and kanamycin. The bacterial solution was shaken overnight and coated in solid medium for two days and validated by PCR by using 2xTaq Master Mix (CWBIO, China), the thermocycling profile was as follow: 10 minutes at 94°C, then 30 seconds at 94°C, 45 seconds at 55°C, 30 seconds at 72°C with 30 cycles, then 2 minutes at 72°C. Then the *A. rhizogenes* culture was then applied to the petioles of detached leaves at different stages of development. The treated leaves were kept in darkness for two days and then transferred to Petri dishes with dampened paper, under normal growth conditions (25°C, 16 hours of light and 8 hours of darkness, with humidity maintained between 50% and 60%). Petioles treated with K599 carrying no pMDC83 vector were used as controls. Hairy roots were induced 12 days after treatment and a portable fluorescent lamp (LUYOR-c3415RG) was employed to identify positive hairy roots based on GFP fluorescence. Roots displaying GFP fluorescence were considered transgenic hairy roots.

Subcellular localization analysis

After 12-15 days of hairy roots induction, the transgenic hairy roots showing the GFP fluorescent were excised from the petioles and were mounted on a slide and examined for the subcellular localization of the target proteins by using an ECLIPSE Ti2 inverted confocal microscope (Nikon, Japan). The excitation wavelength used was 488 nm.

RESULTS AND DISCUSSION

A. rhizogenes K599 can induce transgenic hairy root formation from petioles

Based on our previous study, we noted that leaves of common bean, when treated with water, had the ability to induce root formation at the base of the petiole. Building upon this knowledge, we endeavored to induce hairy roots using the petioles of common bean, following a previously established hairy root induction system. For this study, we utilized '*Honghuaqingjia*,' a commonly employed variety within our laboratory. Specifically, we treated 5-day-old first true leaves with water, K599 without a vector and K599 carrying the pMDC83 vector. Within 5-7 days of infestation, we observed the formation of calluses at the petiole across all treatments. Subsequently, at 12-14 days after infestation, the development of roots became apparent. As depicted in Fig 1, the treatment involving water induced a significant number of roots without exhibiting GFP fluorescence. In contrast, the roots induced by K599 carrying the pMDC83 vector displayed distinct green fluorescence.

The influence of varieties and leaf age on hairy root formation efficiency

The K599 strain of *A. rhizogenes* demonstrates remarkable efficacy in inducing hairy root formation. To explore this further, we examined the impact on different varieties and the age of leaves on this process. We selected three varieties ('*Honghuaqingjia*', '*Lvyoudiyoudou*' and '*Honghuabaijia*') and three different leaf ages (5-day-old, 10-day-old and 15-day-old) for in this study. Notably, '*Honghuaqingjia*' at 5 days, '*Lvyoudiyoudou*' at 5 days and '*Honghuaqingjia*' at 10 days exhibited a high induction efficiency exceeding 50%. These findings highlight '*Honghuaqingjia*' as a particularly suitable variety for inducing hairy roots with the K599 strain. Additionally, younger leaves consistently displayed a higher induction efficiency (Fig 2 and Table 1).

It's well-established that the choice of plant genotype and *Agrobacterium* strain can significantly influence the transformation efficiency, as has been reported in various studies (Bakhsh, 2020; Kavitah *et al.*, 2010; Liu *et al.*, 2023; Sahoo and Tuteja, 2012). This emphasizes the importance of selecting the right combination to optimize transformation outcomes. For instance, the transformation efficiency of 'LP089,' a common bean genotype, was 53% with strain R1000 and 17% with K599 (Li *et al.*, 2022). In the case of *Arabidopsis*, the Wassilewskija ecotype combined with the EHA101 strain yielded the highest transformation and bud regeneration efficiency among different ecotypes and *Agrobacterium* strains (Akama *et al.*, 1992). Similarly, the choice of *Agrobacterium* species influenced the transformation rate in tomato (*Solanum lycopersicum* L.) var. Micro-Tom, with GV3101 achieving the highest transformation rate at 65% (Chetty *et al.*, 2013). The selection of explants also plays a pivotal role in determining transformation efficiency. Young and tender explants are typically favored for infestation, as reported in several studies (Hadfi and Batschauer, 1994; Liu *et al.*, 2013; Sriskandarajah *et al.*, 2004). In potato, leaf and internode explants from various cultivars were infected with *Agrobacterium tumefaciens* strain LBA4404, yielding a transformation efficiency of 22% for internode explants and 15% for leaf discs in the Lady Olympia cultivar (Bakhsh, 2020). Hence, based on the results of this study, the combination of '*Honghuaqingjia*' and K599 is strongly recommended for use in future studies of common bean.

Application of the hairy root induction system for subcellular localization

With the establishment of our highly efficient hairy root induction system, we embarked on exploring its utility in the



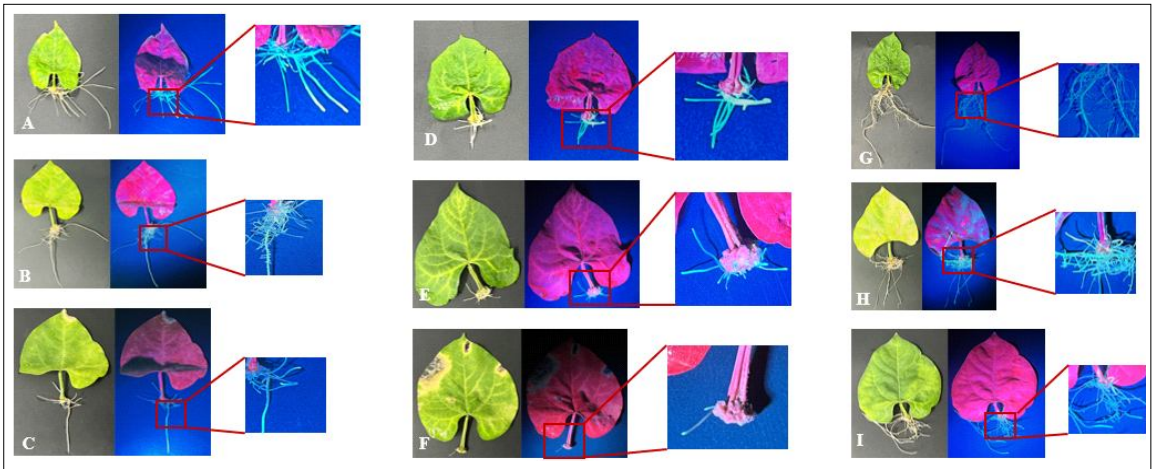
(**A**) No transgenic root was induced after 15 days of water treatment. (**B**) No transgenic root was induced after 15 days of infection by K599 carrying no vector. (**C**) Transgenic Root induction after 15 days of infection by K599 carrying the pMDC83 vector. In each panel, the left image is the plant under white light irradiation, while the right image is the plant under UV-light irradiation. Roots exhibiting green fluorescence are transgenic hairy roots.

Fig 1: Transgenic hairy roots induction.

Table 1: Statistical data related to different varieties and various leaf ages after a 15-day induction.

Variety - leaf age (days)	Average number of roots	Average transgenic root rate (fluorescence rate)
Honghuaqingjia-5	7.33	59%
Honghuaqingjia-10	15.33	52%
Honghuaqingjia-15	22.67	42%
Lvyoudiyoudou-5	3.00	56%
Lvyoudiyoudou-10	10.33	35%
Lvyoudiyoudou-15	2.67	38%
Honghuabaijia-5	2.67	0%
Honghuabaijia-10	13.33	33%
Honghuabaijia-15	22.00	38%

investigation of gene function. In this context, we cloned *PvTCP2* into the pMDC83 vector to express the fusion protein PvTCP2-GFP. To observe subcellular localization, we employed '*Honghuaqingjia*' 5-day-old leaves for obtaining transgenic hairy roots, utilizing empty pMDC83 as a control. As depicted in Fig 3, no fluorescence was detected in the wild-type (WT) hairy roots, while the fusion protein PvTCP2-GFP was distinctly localized within the nucleus. This outcome suggests the potential of our hairy root induction system for subcellular localization studies. Moreover, our induction system holds promise for investigating gene functions related to various aspects of common bean biology, such as metabolism, plant-pathogen interactions, symbiotic nitrogen fixation and nodulation, biotic and abiotic stress tolerance, mycorrhizal interactions, phytoremediation, root-



(A) images of 'Honghuaqingjia' at 5 days, **(B)** images of 'Honghuaqingjia' at 10 days, **(C)** images of 'Honghuaqingjia' at 15 days, **(D)** images of 'Lvyoudiyoudou' at 5 days, **(E)** images of 'Lvyoudiyoudou' at 10 days, **(F)** images of 'Lvyoudiyoudou' at 15 days, **(G)** images of 'Honghuabaijia' at 5 days, **(H)** images of 'Honghuabaijia' at 10 days and **(I)** images of 'Honghuabaijia' at 15 days. In each panel, left image: plant under white light; middle image: plant under UV light; right image: regional enlargement.

Fig 2: Images of hairy root formation with various varieties and leaf ages after 15 days of treatment.

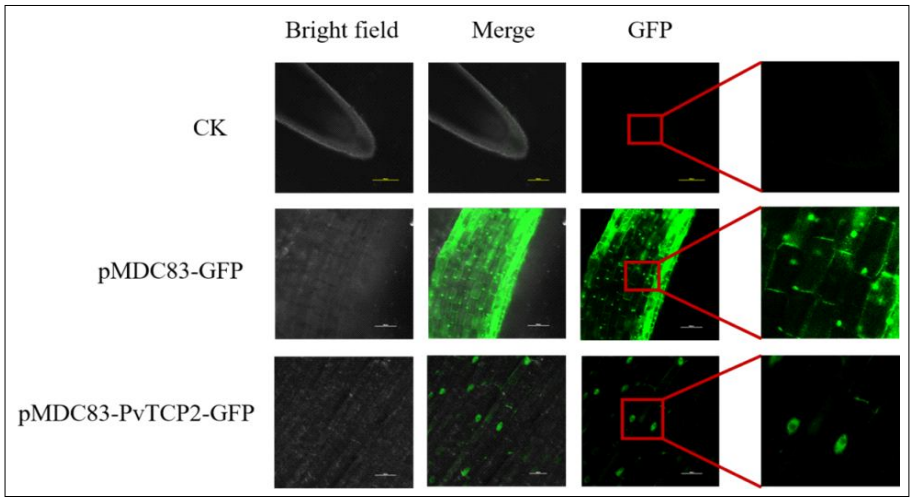


Fig 3: PvTCP2 subcellular localization analysis by using the established hairy root induction system. CK is the hairy root of 'honghuaqingjia' grown from 5-day-old leaves treated with water.

shoot interactions, nutrient uptake and hormone transport (Alagarsamy *et al.*, 2018; Aragão *et al.*, 2023; Kim *et al.*, 2012; Niazian *et al.*, 2022; Wang *et al.*, 2023).

CONCLUSION

This study represents the successful establishment of a novel hairy root induction system. We initiated our investigation by identifying the efficacy of the *Agrobacterium* strain K599 in inducing hairy roots in common bean leaves. Subsequently, we meticulously optimized the system through the utilization of three different bean varieties and leaves of varying ages. Our findings underscore that 5-day-old leaves of the *'Honghuaqingjia'* variety are particularly well-suited for hairy root induction with K599. Finally, our ability to observe subcellular localization of PvTCP2 serves as compelling evidence that our system holds great utility for molecular biology studies.

ACKNOWLEDGEMENT

This work was supported by National Natural Science Foundation of China (32372718) and State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products (2021DG700024-KF202403).

Conflict of interest

The authors declare that there are no conflicts of interest.

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