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ORIGINAL ARTICLE

# An attempt to establish an *Agrobacterium*-mediated transient expression system in medicinal plants

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## Abstract

Genetic transformation has always been an important method for studying medical plant secondary metabolic regulation, among which stable transformation has a good reproducibility. However, it was time-consuming to obtain a stable transformed hairy root or transgenic plants, which was difficult to satisfy the great demand of researches on medical plant secondary metabolism–related genes. Moreover, *Agrobacterium tumefaciens*–mediated transient transformation has been extensively applied in studies of functional genes because of its simpleness, low cost, and short period. However, presently, researches on medical plant functional genes commonly used stable genetic transformation and some high-cost and high-difficulty transient transformation methods, such as gene gun and protoplast transformation. Thus, in this study, we selected the seedlings of *Nicotiana benthamiana*, *Salvia miltiorrhiza*, and *Prunella vulgaris* as the experimental material, with the methods of *Agrobacterium tumefaciens* injection, fast *Agrobacterium*-mediated seedling transformation (FAST), and FAST and mechanical damage. The results demonstrated that the injection transient transformation system of pCAMBIA1301 vector mediated by *A. tumefaciens* and the transient transformation of seedling system were not established in *S. miltiorrhiza*. In addition, the instantaneous transformation system of *N. benthamiana* and *P. vulgaris* seedlings was basically set up by FAST method. Besides, using the method of FAST and mechanical damage, the transient genetic transformation system of *P. vulgaris* seedlings was established for the first time. *A. tumefaciens*–mediated transient transformation of seedlings with pEAQ vectors provided an effective way and reference for the further study of functional genes of the medicinal plants *N. benthamiana* and *P. vulgaris*.

**Keywords** Transient transformation · Medicinal plants · *Salvia miltiorrhiza* · *Prunella vulgaris*

### Abbreviations

FAST Fast *Agrobacterium*-mediated seedling transformation

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## Introduction

*Nicotiana benthamiana* L. is a herbaceous plant belonging to the genus *Nicotiana* in the Solanaceae family, which is not only an essential industrial crop worldwide but also a model plant for biological mechanism studies due to its advantages of short generation time, disease susceptibility, and ease of genetic transformation (Udawat et al. 2016). *Salvia miltiorrhiza* Bunge and *Prunella vulgaris* L. belong to the family of Labiatae, which has important economic and medicinal value in China. Modern pharmacological studies exhibit that these medicinal plants have the properties of being anti-tumor, anti-virus, and anti-inflammatory (Tian et al. 2015; Ma et al. 2019; Zhang et al. 2018).

Transient transformation system, a new transformation method, has been able to replace the stable transformation (McIntosh et al. 2004). At present, there were two main means of transient transformation. One was protoplast transformation by electric shock or PEG penetration (Sheen 2001). The other

was particle bombardment of plant epidermal cells or leaves (Sessa et al. 1998; Schweizer et al. 1999), also known as gene gun method. Due to the time-consuming and low success rate of protoplast culture and the need for special corollary equipment for gene gun, it was difficult for these two methods to be widely used. Compared with the two methods mentioned above, *Agrobacterium*-mediated transient transformation has the advantages of low cost, easy operation, and high success rate, which could be used in many research fields, including gene expression detection, gene silencing, subcellular localization, protein interaction analysis, and inhibitor function identification (Zhao et al. 2013). *Agrobacterium tumefaciens*, as a common tool for plant genetic transformation, mainly employed toothpick inoculation (Lu et al. 2003), injection (Fu et al. 2005), high pressure jet (Liu et al. 2002), and vacuum infiltration (Ekengren et al. 2003). These methods have been successfully used in many plant tissues, such as leaves of *Arabidopsis thaliana* (Clough and Bent 2010); petals of lilies and goldfish grass; leaves, petioles, and callus of *Pinellia ternata* (Jia et al. 2007); leaves and fruits of lettuce and tomato; leaves of grape (Yang et al. 2000); and fleshy fruits of fresh apples, pears, peaches, strawberries, and oranges (Zhao et al. 2013). Injection penetration transient transformation method was widely used to express exogenous genes in *N. benthamiana* leaves (Li and Zhang 2010). This was due to *N. benthamiana* has advantages like short growth cycle, weak disease resistance, or low rejection reaction that other medicinal plants do not have. However, in other medicinal plants, how to achieve effective instantaneous expression in a relatively short period of time has always been one of the bottlenecks of instantaneous means. Therefore, the selection of target plant materials (including growth status and tissue location) and *A. tumefaciens* strains was particularly important.

FAST method is an *A. tumefaciens*–mediated transient transformation system based on plant seedlings (Li et al. 2009). Compared with *A. tumefaciens* injection method, FAST method has the advantages of simple operation, no special equipment, and could complete transformation and analysis in a very short experimental cycle (about a week). The acquisition of plant seedlings was usually fast, and the self-defense system of seedlings might not be fully mature, which provided favorable conditions for the invasion of *A. tumefaciens* and the expression of exogenous genes. Therefore, this technique was quickly applied by many researchers in different plant materials such as *N. benthamiana* (Ratcliff et al. 2001), tomato, rice (Li et al. 2009), orange, banana, lentil, sugarcane (Subramanyam et al. 2013), and a variety of woody plants (Zheng et al. 2012). In addition, using plants as expression systems to produce recombinant proteins has obvious advantages over traditional bacterial and yeast expression systems. Recombinant proteins produced in plants undergo post-translational modification by higher-level proteins, which have better safety and fidelity when used as drugs or in research (Kusnadi and Nikolov 1997; Lico et al. 2008). Moreover, some medicinal plants such as *N. benthamiana* could grow at high density in a short time and produce high biomass in a few weeks.

The pEAQ vector transient transformation system was based on cowpea mosaic virus (CPMV) RNA-2 (CPMV-*HT*) expression system, which added 5′-untranslated region (UTR) and 3′-UTR of CPMV virus to both sides of the target gene and co-expressed with virus silencing inhibitor P19 (Supplementary fig. 1), thus achieving the effect of large amount of expression of the target protein (Sainsbury et al. 2009). At present, pEAQ vectors have been successfully used to express various recombinant proteins, including human gastric lipase (hGL) (Vardakou et al. 2012) and recombinant human serum albumin (HSA) (Sun et al. 2011). Importantly, these exogenous proteins were expressed in *N. benthamiana*. Hence, we can speculate that the system can express one or more enzymatic genes related to secondary metabolite synthesis in *N. benthamiana*, *S. miltiorrhiza*, *P. vulgaris*, and other medicinal plants, so as to realize heterologous construction of secondary metabolite synthesis pathway.

## Materials and methods

### Plant materials and sample preparation

*N. benthamiana*, *S. miltiorrhiza*, and *P. vulgaris* seeds were collected from the Tianshili Plant Pharmaceutical (Shaanxi Province, China) Co., Ltd. When *S. miltiorrhiza* seeds germinated, they should be evenly sprayed in a culture dish covered with three layers of wet filter papers and placed in a light incubator. The incubation conditions in the light incubator were as follows: the temperature was at 25 ± 2 °C, the illumination time was about 12–14 h, and the light intensity was at 2000–3000 lx. The germination method of *N. benthamiana* and *P. vulgaris* seeds was the same as that of *S. miltiorrhiza*. After a week of germination, these seedlings were selected for *Agrobacterium*-mediated transient transformation of seedlings.

The cotyledons, stems, and roots of *S. miltiorrhiza* seedlings for total component analysis were prepared according to the following steps: (1) After the seeds of *S. miltiorrhiza* germinated on the gauze for 7 days, the cotyledons of the seedlings were carefully picked up with ophthalmic surgical scissors. The collected cotyledons were washed with distilled water and dried on filter paper then packed them into kraft envelope and dried in an oven at 40 °C until constant weight. (2) Used ophthalmic surgical scissors to carefully cut bare seedling stems along the gauze surface. And then dried and preserved them according to the treatment method of cotyledons. (3) The roots of the seedlings remaining on the back of the gauze were cut with ophthalmic surgical scissors. The

treatment method was same with cotyledons, too. (4) The cotyledons, stems, and roots of *S. miltiorrhiza* seedlings dried to constant weight were ground into powder in a mortar for high-performance liquid chromatography (HPLC) analysis.

## Chemicals and reagents

4-methyl umbrella ketone (4-MU), 4-methyl umbrella ketone-D-glucuronide (MUG), X-Glucucuc, Silwet L-77, TaKaRa protein quantitative kit, 2 × buffer (0.1 M sodium citrate, 0.2 N HCl, pH 7.0), β-glucuronidase (GUS) tissue-staining reagent, GUS enzyme extract, GUS enzyme termination solution (0.2 M $Na_2CO_3$), surface disinfectant (0.05% sodium hypochlorite), cleaning buffer (10 mM $MgCl_2$, 100 uM acetyleugenone), and co-culture buffer were obtained.

## Strains and vectors

*E. coli* DH5α competent cells were purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd. *A. tumefaciens* EHA105 was preserved in our laboratory. The pEAQ vectors include plant expression vectors pCAMBIA1301 and pCAMBIA1304, which contain reporter genes GUS and green fluorescent protein (GFP) respectively.

## Main instruments

Vacuum freeze dryer LL3000 (Somerfly Shier Company, USA), constant temperature shaker TSQ-280 (Shanghai Jinghong Experimental Equipment Co., Ltd., China), constant temperature incubator DNP-9082 (Shanghai Jinghong Experimental Equipment Co., Ltd., China), constant temperature incubator ZPW-350 (Heilongjiang dongtuo Instrument Manufacturing Co., Ltd., China), pH meter (Shanghai Instrument and Electrical Science Instrument Co., Ltd., China), electric heating constant temperature blast drying Box 101-2AB (Tianjin Tester Instruments Co., Ltd., China), electronic balance AUW120 (Shimadzu Corporation, Japan), electric converter (Eppendorf Company, Germany), high-performance liquid chromatography (Waters Company, USA), and high-intensity ultraviolet flaw detection lamp FC-100/FA (SPECTROLINE Company, USA) were required.

## Analysis of active ingredients in S. miltiorrhiza leaves by HPLC

There were two main components of *S. miltiorrhiza*: rosmarinic acid and salvianolic acid B. The two active ingredients in the cotyledons, stems, and roots of *S. miltiorrhiza* were determined by HPLC. The method of extraction and detection has been slightly changed (Liang et al. 2012). When extracting the active ingredients, the powder of the above-mentioned dried sample should be weighed at 0.05 g, added 10 mL 70% methanol-water solution, ultrasounded for 60 min, centrifugated for 10 min at 12,000 rpm, and then the supernatant should be filtered through a 0.45-μm membrane for reserve. Waters 1525 binary chromatographic pump, waters 2996 PAD detector, and waters sun fire C18 (250 mm × 4.6 mm, 5 μm) were used in this experiment. The chromatography was performed under the following conditions: the flow rate was kept at 1 mL/min, the column temperature was maintained at 30 °C, and the injection volume was 20 μL. The detection wavelength was 288 nm (water-soluble component) and 270 nm (fat-soluble component). The mobile phase consisted of solvent A (0.02% phosphoric acid water, v/v) and solvent B (acetonitrile) was set in a gradient elution program: 0–10 min (5–20% B), 10–15 min (20–25% B), 15–20 min (25% B), 20–25 min (25–20% B), 25–28 min (20–25% B), 28–37 min (25–30% B), 37–45 min (30–45% B), 40–50 min (45–50% B), 50–60 min (50% B), 60–65 min (50–60% B), 65–70 min (60% B), 70–80 min (60–70% B), and 80–85 min (100% B).

## Electrical transformation of *A. tumefaciens* EHA105

The vectors pCAMBIA1301 and pCAMBIA1304 were transformed into *A. tumefaciens* EHA105 competent cells by electric shock. Firstly, 1-μL recombinant plasmid was added into the 100-μL EHA105 competent cells, gently mixed and ice-bathed for 30 min. The voltage of the electrometer was adjusted to 2.5 KV and electric shock to 5 ms. Secondly, the mixture of plasmid and competent cells was transferred to the bottom of the shock cup and quickly put into the electrometer. The electric pulse was started according to the set parameters. Then samples were taken out as soon as possible after electric shock, supplementing 1-mL fresh LB liquid medium. The treated solution was slightly shaken at 28 °C for 4–6 h. Subsequently, the bacterial solution was coated on solid medium containing rifampicin (50 mg/L) and kanamycin (50 mg/L) for 2–3 days in the darkness at 28 °C. Finally, the single colony was selected, the plasmid after shaking the bacteria was extracted, and the vector was verified by sequencing.

## The *A. tumefaciens* injection and FAST method

The main method of *A. tumefaciens* injection was leaf injection. The above suspension of *Agrobacterium* with a sterile injection syringe of 1 mL was taken and then injected into the leaves.

The FAST method was performed by the following steps (Li et al. 2009): (1) The monoclonal of *A. tumefaciens* was selected and cultured in 2-mL LB liquid medium, which contains 50 mg/mL Rif and 50 mg/mL Kan. The culture conditions were at 28 °C for 18–24 h on a shaker at 220 rpm; (2) the 2-mL bacterial solution was transferred into 100-mL LB liquid medium with the same antibiotics and grown at 28 °C on a

shaker at 220 rpm until the concentration of bacterial solution was about $OD_{600} = 0.5$; (3) the bacterial solution was diluted with fresh antibiotic-free LB liquid medium to $OD_{600} = 0.3$ and cultured at the same conditions with step (2) until $OD_{600} > 1.0$ (about 10–12 h); (4) the bacterial liquid was transferred into 50-mL centrifugal tube with 5000 rpm centrifugation for 5 min, and LB liquid medium was discarded; (5) the suspension was washed with 10-mL cleaning buffer, centrifuged with 5000 rpm for 5 min, and discarded the supernatant; (6) the suspension was resuspended with 1-mL cleaning buffer; (7) 100-μL concentrated bacterial liquid which was obtained in step (6) was taken, diluted 10 times with cleaning buffer, and calculated the volume of concentrated bacterial liquid; (8) some 7-day plant seedlings (*N. benthamiana*, *S. miltiorrhiza*, *P. vulgaris*) were placed in sterile dishes and 20-mL co-culture buffer solution was added; (9) the calculated volume of concentrated bacterial liquid and adjusted $OD_{600} = 0.5$ (the calculation method is as follows: adding bacterial liquid volume = total volume of co-culture × $0.5/OD_{600}$ value of concentrated bacterial liquid) was added; (10) the cultured seedlings were slowly shaken at room temperature for 24–72 h (*N. benthamiana* was cultured for 24 h, *P. vulgaris* and *S. miltiorrhiza* were cultured for 40–72 h); (11) The co-cultured seedlings were soaked in surface disinfectant for 5 min, then washed repeatedly with sterile water for more than 5 times; (12) The seedlings were transferred to a culture dish containing 0.5 × MS liquid medium and cultured for 3 to 5 days for observation. The procedures of the negative control groups were the same as the above, but there was no concentrated bacterial solution added to the co-culture medium in step (9).

### Mechanical damage treatment

Mechanical damage included cotyledon puncture and vacuum infiltration. The former was to place the seedlings in a culture dish and punctured cotyledon surface lightly with syringe needle (to avoid lateral force and scratch), each cotyledon has about five micro holes. The latter was to put the culture dish containing calculated volume of concentrated bacterial liquid in step (9) and seedlings into a vacuum dryer, vacuumed for 10 min with a vacuum pump, and then began the following infection steps.

### GUS staining and quantitative detection

GUS staining and quantitative detection were according to the previous method (Jefferson 1987).

### Data analysis

All experimental data were calculated by Microsoft Excel 2010.

## Results and discussion

### Analysis of effective components in cotyledons, stems, and roots of S. miltiorrhiza seedlings

In order to comprehensively analyze the effects of *A. tumefaciens*–mediated transient infection on the secondary metabolites of *S. miltiorrhiza* seedlings, the active components of the cotyledons, stems, and roots of *S. miltiorrhiza* seedlings were detected by HPLC. At the same time, the same HPLC condition was used to measure the secondary metabolites in the seedlings of *N. benthamiana* and *P. vulgaris*. The results are shown in Fig. 1. In our study, it was found that there were significant differences in secondary metabolic species and contents among roots, stems, and cotyledons of *S. miltiorrhiza* seedlings. Rosmarinic acid was the main secondary metabolites of the three water-soluble components, but the content of rosmarinic acid was quite different, showing root > stem > cotyledon. Water-soluble salvianolic acid B was mainly found in the roots of *S. miltiorrhiza* seedlings, and a small amount of salvianolic acid B was detected in stems, but not in cotyledons, indicating that cotyledons were not the main accumulation sites of secondary metabolites in *S. miltiorrhiza* seedlings. The fat-soluble components were not accumulated in the roots, stems, and cotyledons of *S. miltiorrhiza* seedlings, which was consistent with previous experimenters (Li 2008). Therefore, it was suggested that water-soluble components should be the main research area if secondary metabolites of *S. miltiorrhiza* are considered, and the focus should be on the root. These two secondary metabolites were not detected in the seedlings of *N. benthamiana* and *P. vulgaris*.

### Transient transformation with pCAMBIA1301 vector

To ensure the effective infection of *A. tumefaciens* EHA105 carrying binary vector pCAMBIA1301, which was containing GUS reporter gene, we first transformed *N. benthamiana* and *S. miltiorrhiza* leaves by *A. tumefaciens* injection. After GUS staining, the results are shown in Fig. 2. Compared with the control group, *N. benthamiana* leaves in the transformation experiment group reflected obvious blue color. It indicated that pCAMBIA1301 vector could successfully express GUS gene in *N. benthamiana* leaves. The staining results of *S. miltiorrhiza* leaves displayed that neither negative control nor infected experimental group has showed a blue staining. The research validated that pCAMBIA1301 vector could not be successfully transformed into *S. miltiorrhiza* leaves by *Agrobacterium* injection.

Therefore, we adopted FAST method to further investigate the reasons why the pCAMBIA1301 vector could not transform *S. miltiorrhiza* seedlings successfully. The result was demonstrated in Fig. 3. *S. miltiorrhiza* seedlings in the negative control group and pCAMBIA1301 transformation

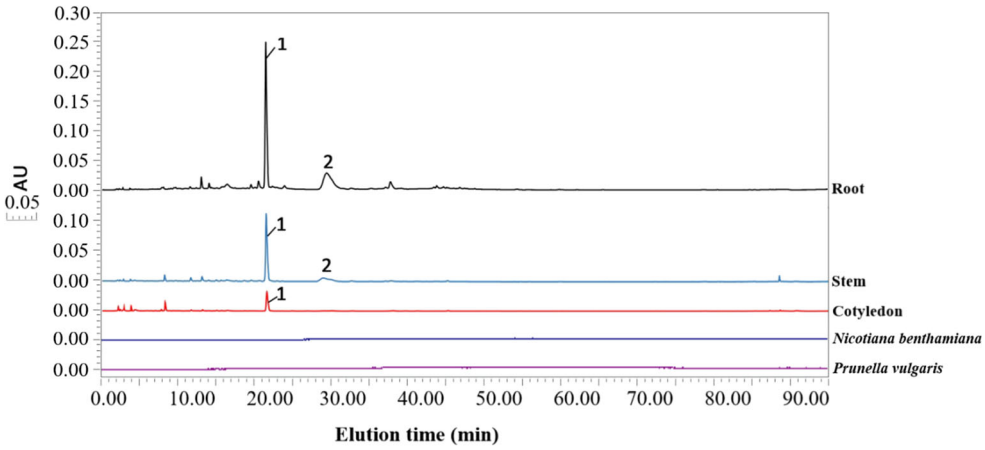


**Fig. 1** The HPLC map of active ingredients in *S. miltiorrhiza* root, stem, cotyledon, *N. benthamiana*, and *P. vulgaris*. Component 1: Rosmarinic acid, 2: Salvianolic acid B

experiment group showed a blue color reaction on the stem, which might be due to the GUS background staining of *S. miltiorrhiza* seedlings. This characteristic of *S. miltiorrhiza* species was semblable with other plants (Richard 1987). Therefore, a specific GUS activity related to the transformation was not detected in the infection experimental group. The results were still the same after multiple repetitions, so it was judged that there were other unknown factors influencing the transient transformation of *S. miltiorrhiza* seedlings by plant expression vector pCAMBIA1301, which led to the fail transformation of *S. miltiorrhiza* seedlings. Consequently, the pCAMBIA1301 vector carried by *A. tumefaciens* EHA105 could not achieve effective transient transformation in *S. miltiorrhiza* leaves and seedlings by FAST method.

## Transient transformation with pCAMBIA1304 vector

PCAMBIA1304 containing GFP reporter gene was used as transformation vector, and the seedlings of *N. benthamiana* and *P. vulgaris* were transformed by FAST method. *N. benthamiana* seedlings after infection were cultured in 0.5 × MS liquid medium for 3 days and observed under a fluorescence microscope with the excitation wavelength of 488 nm. GFP expression was observed in roots, stems, and cotyledons of *N. benthamiana* seedlings (Fig. 4). The green fluorescence was detected in all the seedlings of the infected group, and the infection efficiency was perfect. But there was no green fluorescence in the negative groups. These results indicated that pCAMBIA1304 vector could express exogenous genes in *N. benthamiana* by FAST method. Under the same infection conditions, most of the infected *P. vulgaris* seedlings did not show green fluorescence. Fluorescence signals were acquired only in the damaged parts of seedlings, such as root injured (Fig. 4c) and root tip injured (Fig. 4d). Therefore, we regarded that mechanical damage can improve transformation efficiency in the transient transformation of *P. vulgaris* seedlings.

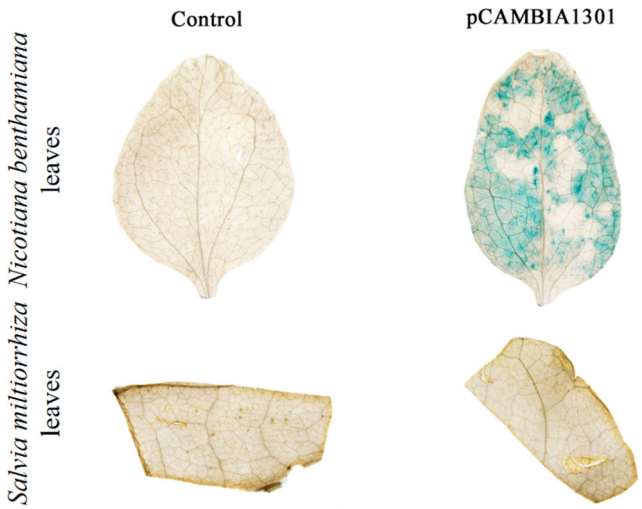


**Fig. 2** GUS histochemical analysis of transient expression in *N. benthamiana* and *S. miltiorrhiza* leaves (5 days after inoculation)

Our research elucidated that the transformation of *N. benthamiana* seedlings and some damaged *P. vulgaris* seedlings could be successfully achieved by using *Agrobacterium tumefaciens* EHA105 and pCAMBIA1304 combined with FAST infection. The infection efficiency of *N. benthamiana* seedlings was higher than that of *P. vulgaris* seedlings. As shown in Fig. 4e, the individual comparison between week-old *P. vulgaris* seedlings and *N. benthamiana* seedlings showed that the discrepancy of infection efficiency might be closely related to the size of the two individuals. It was apparently reflected that there were significant divergences in the instantaneous infection efficiency of diversity plant materials (including different plant types and physiological states) when using the same strains and various carriers in the *Agrobacterium*-mediated transient transformation system. Furthermore, mechanical damage can improve the efficiency of infection, and similar verdict has been drawn by McIntosh's experiment (McIntosh et al. 2004).

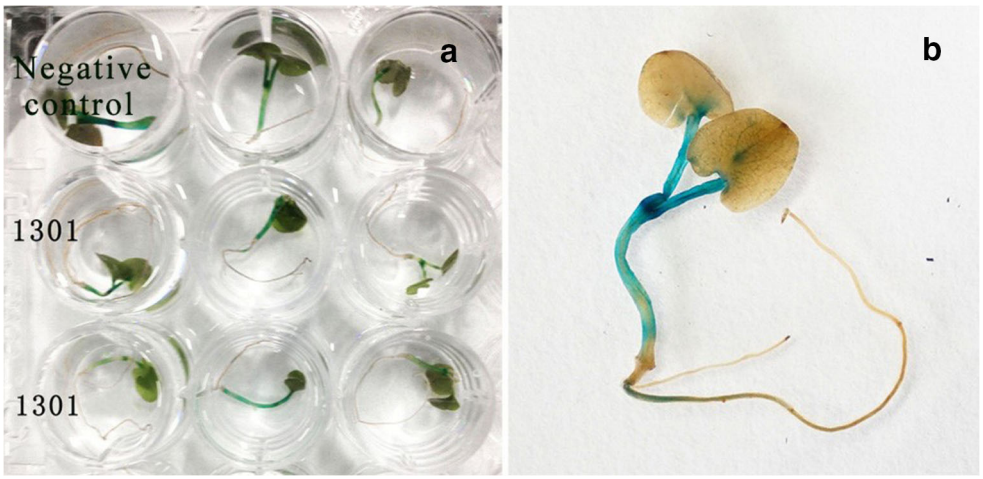


**Fig. 3** GUS expression of 1-week old *S. miltiorrhiza* seedlings infected with EHA105. **a** Negative control and pCAMBIA1301 transformed *S. miltiorrhiza* seedlings with GUS activity; **b** Negative control *S. miltiorrhiza* seedlings of A

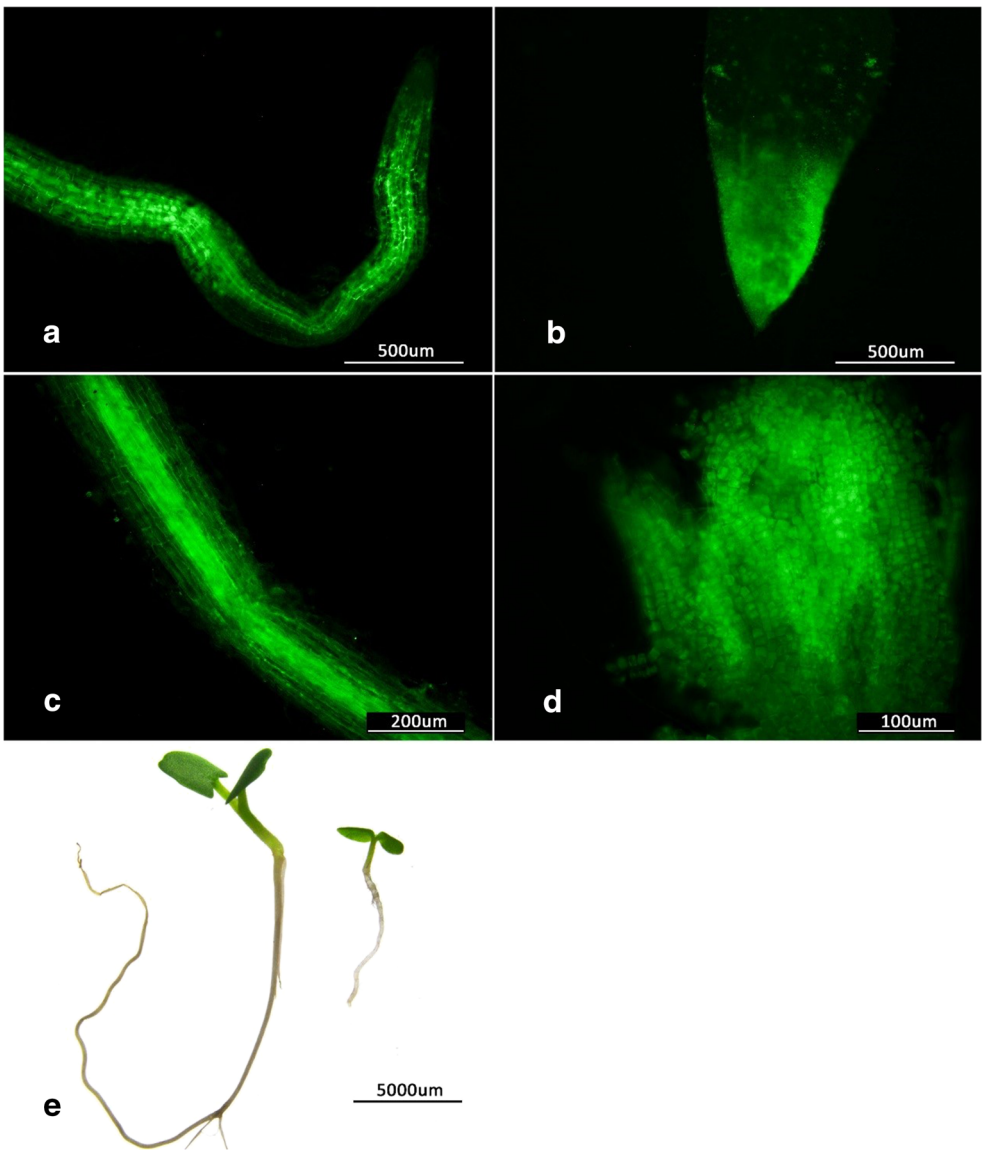


**Fig. 4** FAST method in *N. benthamiana* and *P. vulgaris* seedlings with pCAMBIA1304. GFP was observed under a fluorescent microscope. **a** Root and stem of *N. benthamiana* seedlings. **b** Cotyledon of *N. benthamiana* seedlings. **c** The middle of *P. vulgaris* seedlings root. **d** The top of *P. vulgaris* seedlings root. **e** Overall comparison of *N. benthamiana* and *P. vulgaris* seedlings

### Transient transformation of *P. vulgaris* seedlings by FAST and mechanical damage

As described in the results from the "Transient transformation with pCAMBIA1304 vector" section, green fluorescent protein was expressed in damaged *P. vulgaris* seedlings. Therefore, we carried out the infection experiment of *P. vulgaris* seedlings by FAST and mechanical damage method. After co-cultured with *A. tumefaciens* for 72 h and 0.5 × MS liquid medium for 3 days, fluorescence microscopy was used to watch the reaction results. Clear and well reproducible green fluorescent protein expression was examined in cotyledons of *P. vulgaris* seedlings (Fig. 5a–c). It was displayed that FAST and mechanical damage method could successfully express exogenous genes in *P. vulgaris* seedlings. As reflected in Fig. 5e, f, a highly efficient green fluorescent protein expression was observed after injection of *A. tumefaciens* EHA105 carrying pCAMBIA1304 vector into the leaves of *N. benthamiana* as a positive control, which verified the effectiveness of pCAMBIA1304 vector. This exhibited that we have successfully established the transient transformation system of *P. vulgaris* seedlings, which provided an effective means for the study of functional genes in *P. vulgaris,* whereas the co-culture of bacterial solution and the addition of mechanical damage in the system were both a kind of injury stress for plant seedlings. It was implied and insinuated that the system was not suitable for some studies of stress-related functional genes (Jessica et al. 2014).

## Conclusions

Genetic transformation has always been an important means to study the regulation of secondary metabolites in medicinal plants. Moreover, stable transformation has the advantage of good reproducibility. Nonetheless, obtaining stable hairy root system or transgenic plants was often time-consuming and laborious, which could not meet the needs of a large number of studies on secondary metabolic regulation–related functional genes of medicinal plants. Therefore, recently, *Agrobacterium*-mediated transient transformation system has been widely used in the study of plant functional genes owing to its easy operation, low cost, and short cycles.

The research suggested that the *Agrobacterium*-mediated instantaneous transformation system had distinct infection efficiency in different plant seedling materials. In this experiment, *S. miltiorrhiza* as a material was not successful in the transient transformation of pEAQ vector mediated by *A. tumefaciens* and the transient transformation of seedlings. This might be associated to the defense mechanism of *S. miltiorrhiza* against the transient expression of exogenous genes. The data of instantaneous transformation between *N. benthamiana* and *P. vulgaris* seedlings also implied that the discrepancy of seedlings' instantaneous infection efficiency might be linked to the diversity of individual size and tissue density of seedlings. GFP and GUS exogenous genes were validly expressed in *N. benthamiana* leaves by pEAQ vector. This provided a powerful approach for rapid and efficient expression of

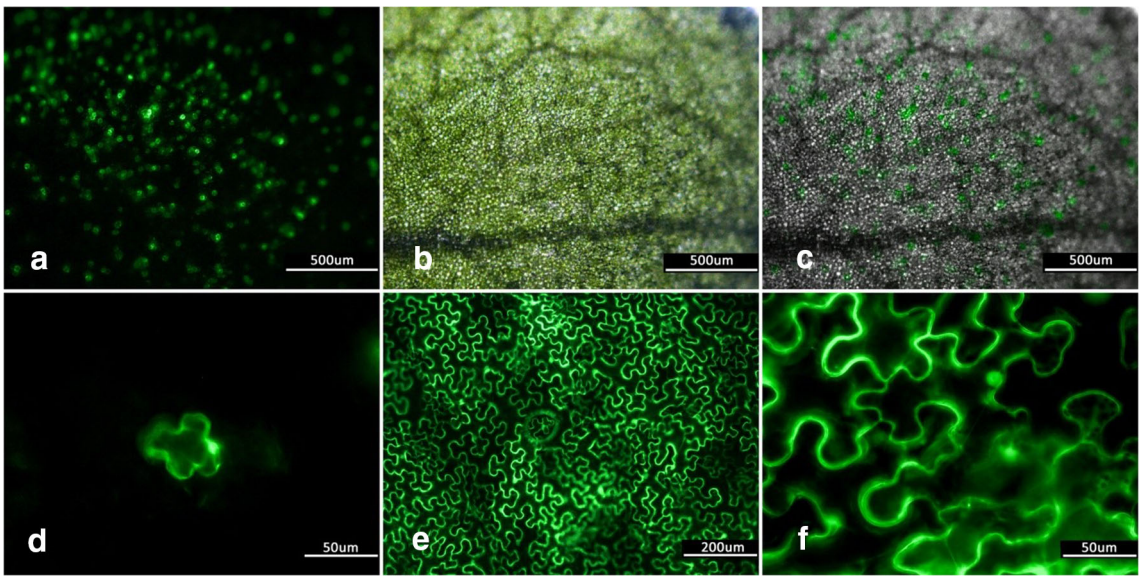


**Fig. 5** FAST and mechanical damage method in *P. vulgaris* seedlings with pCAMBIA1304. GFP was observed under a fluorescent microscope. **a** Cotyledon of *P. vulgaris* at 488-nm excitation light (× 50 magnification). **b** The same position with a in bright field. **c** Merge of a and b. **d** The cotyledon cells in a (expanded up to 8 times). **e** *N. benthamiana* leaf at 488-nm excitation light (× 50 magnification). **f** *N. benthamiana* leaf epidermal cells in e (expanded up to 8 times)

exogenous genes in *N. benthamiana* and further probe of their functions. The highly efficient co-expression of multiple exogenous genes also offered the probability of heterologous construction of secondary metabolic pathways in *N. benthamiana*. At the same time, the instantaneous transformation system of *P. vulgaris* seedlings was preliminarily established by FAST and mechanical damage method, which exhibited a significant means for the study of functional genes in *P. vulgaris*. However, the co-culture of material and bacterial solution and the addition of mechanical damage in the system were all stress treatments for plant seedlings. These factors restricted the utilization of this system in the study of stress-related functional genes.

**Author contributions** P.X. and Z.L. conceived and designed the research. W.H., T.L., and P.X. analyzed the results. D.Y. collected the plant samples. T.L. and P.X. preformed the experiments. W.H. and P.X. wrote the manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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