RESEARCH ARTICLE

Establishment of *Agrobacterium rhizogenes*-mediated tobacco genetic transformation system

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Tobacco (*Nicotiana tabacum L.*) is an important economic crop. *Agrobacterium rhizogenes* can induce the production of hairy roots in plants after infestation. However, no study has reported the induction of hairy roots and regeneration of plants from tobacco seedlings grown in soil. This study used *Agrobacterium rhizogenes* A4, 15834, K599, and R1601 to induce tobacco K326 soil seedlings to investigate the induction efficiency of *Agrobacterium rhizogenes* on cotyledons and hypocotyls. Regenerable roots were selected from the induced hairy roots for culture, and the regenerated plants were obtained after 60 days. Results showed that the induction rate of hairy roots was up to 81.33% after A4 infection of tobacco explant stems. While R1601 had a relatively low induction rate of 24.33%. Agrobacterium strain A4, suitable for inducing hairy roots in tobacco, was screened. The hairy roots of tobacco were white with abundant root hairs and could grow rapidly on solid medium without exogenous hormones. Moreover, regenerated plants with hairy roots of tobacco were obtained, and the regeneration rate was 80%. In addition, A4 could transfer T-DNA containing Ri plasmids into the explants. The results of PCR showed that *rolB* gene was stably expressed in tobacco hairy root genome with a 50% co-transformation frequency. The study implied that the hairy root regenerative plants could be induced by using tobacco soil culture seedlings.

Keywords: hairy root, *Agrobacterium rhizogenes*, regenerated plants, tobacco.

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Introduction

Secondary metabolism is a specific and complex type of metabolism existing in plants, animals, and microorganisms that is distinct from primary metabolism. Plant secondary metabolites not only provide abundant drugs, spices, and industrial raw materials for human beings but also play an important role in human life. Some secondary metabolites are associated with phenomena such as organismal resistance, signal transduction, adaptive regulation, growth and development, and plant color and fragrance [1]. The important secondary metabolites in tobacco include alkaloids and major aroma precursors such as terpenes, alcohols, acids, lipids, phenols, and other substances. Plant cell culture and hairy root culture are considered as important ways to obtain secondary metabolites. Compared with cell culture, hairy root culture has genetic and biochemical stability. However, during the root culture, the procedure to add 2,4-dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (6-BA), indole-3-acetic acid (IAA), and 1-naphthylacetic acid (NAA) into the tobacco rooting culture genetic transformation

system was cumbersome [2]. Hairy roots can grow rapidly on hormone-free media with more branches and are easier to scale-up than cell culture, and secondary metabolites with high economic value can be improved by changing culture conditions or using relevant biotechnology during hairy root culture. Therefore, hairy roots are an excellent raw material for obtaining plant secondary metabolites [3]. The tobacco hairy root system is used to produce various important secondary metabolites including nicotine, resveratrol, solanesol, geraniol, somatrone, erythropoietin, flavonoid, lactoferrin, cecropin, *etc*., and these compounds are used in medicine, cosmetics, perfume, dyes, and flavor industries. Therefore, overexpression of genes in hairy roots or regulating the biosynthesis of plant secondary metabolites through gene editing techniques has become a rapid and effective research method [4]. Tobacco hairy roots can therefore be used as a plant metabolic factory to specifically produce secondary metabolites.

IN previous study, an *Agrobacterium rhizogenes* C58C1-SPS strain was successfully constructed and was used to infect tobacco explants [1]. The results showed that the rooting rate and root length were higher than those of *Agrobacterium* A4 and ATCC15834, indicating that different strains of *Agrobacterium rhizogenes* had different transformation rates for tobacco. In general, alkaline *Agrobacterium rhizogenes* have a broader host range and higher induction rate, which include many common strains as R1601, A4, 15834, K599, *etc*. [5]. The suitability of the co-culture time of the explants with *Agrobacterium rhizogenes* directly affects the induction rate of hairy roots. Lee *et al*. established a system to induce hairy roots in tobacco with *Agrobacterium rhizogenes* [6]. It was found that the optimal time for both pre-culture and co-culture was 48 h, i.e., 2-3 days was the appropriate time for pre-culture and co-culture [7-9]. Wongsamuth *et al*. added 2,4-D auxin into tobacco hairy root culture, which contributed to the growth of tobacco hairy roots and the successful induction of hairy roots [10]. Gurusamy *et al*. added kinetin to promote the growth of hairy roots during the tobacco hairy root induction [11]. Acetosyringone is a type of phenolic substance widely used in improving the conversion rate of hairy roots. Moderate amounts of acetosyringone can improve the conversion rate [12]. The use of tobacco hairy roots in the succession process can directly differentiate tobacco regeneration plants by hairy roots to obtain regenerated tobacco plants, which is a better way to use hairy root technology for germplasm innovation. Hairy roots can directly form adventitious buds or produce further adventitious buds through callus stage to achieve plant regeneration. Hou et al. used induced tobacco hairy roots and artificial chromosome to finally induce tobacco with double chromosome and improved nicotine content [2]. Gurusamy *et al*. successfully transferred recombinant red blood cell gene into hairy roots using the genetic transformation system of *Agrobacterium rhizogenes*, and cultivated regenerated plants to obtain tobacco plants which could express human erythropoietin [11]. The hairy roots of many plants such as *Saussurea involucrata* and *Bupleurum chinense* can produce regenerated plants when grown in liquid medium without any added hormone [13]. There were very few studies on the induction of hairy roots by using tobacco soil grown seedlings, and the use of tobacco hairy roots to obtain regenerated plants has not been reported. Compared with ordinary plant roots, hairy roots have the advantages of easy observation and rapid growth and can be produced in large quantities without the addition of culture medium, overcoming the possibility of plant growth cycle. Genetically modified hairy roots can grow indefinitely and continuously produce target products. Therefore, using hairy roots for observation and research has become very important. This study investigated the tobacco hairy root induction system and established an Agrobacterium transformation system for tobacco soil culture, which provided technical support for tobacco germplasm innovation and improving secondary biomass content, and laid an experimental and technical foundation for future plant breeding using hairy roots.

Materials and methods

Agrobacterium rhizogenes strain activation

Agrobacterium rhizogenes strains A4, 15834, K599, and R1601 were obtained from Zhengzhou University of Light Industry (Zhengzhou, Henan, China). All strains were Rif-resistant and maintained at -80°C on the YEP medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) plate containing rifampicin. The monoclonal clones were obtained by incubation at 28°C for 6-8 h before inoculated into 50 mL of YEB (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) liquid medium supplemented with rifampicin at 28°C, 200 rpm, for 8-12 h. After centrifugation at 5,000 rpm for 15 mins, the bacteria were resuspended in 50 mL of MS medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to an OD_{600} value of 0.8, which equaled to 10^9 colony forming unite (CFU)/mL.

Induction of hairy roots and regenerated tobacco plants

Tobacco strain k326 provided by Zhengzhou University of Light Industry (Zhengzhou, Henan, China) was employed as the plant material for this study. Tobacco seeds were collected and vernalized at 4°C. One week later, the seeds were removed and put into the nutrient soil for cultivation. After four weeks, tobacco seedlings were obtained and cut into 1 × 1 cm^2 squares and co-cultured with Agrobacterium rhizogenes after disinfected with 70% alcohol for 60 seconds in a super clean workbench and soaking in 5% hypochlorite solution for 14 minutes. The disinfected plant was then rinsed with sterile water three times to remove any residual hypochlorite solution on the surface. Cotyledons and hypocotyls of tobacco seedlings cultured for 28 days were used as explants to co-culture with *Agrobacterium rhizogenes* in GXZ-160B intelligent light incubator (Ningbo Jiangnan Instrument Factory, Ningbo, Zhejiang, China). After 5 mins incubation with *Agrobacterium rhizogenes* infection solution, the plant was dried on sterile filter paper and cultured on MS solid medium at 25°C in the dark for two days. The explants were then rinsed three times with MS liquid medium containing 50 mg/L cefotaxime and dried on filter paper before cultured on MS agar medium containing 50 mg/L cefotaxime at 25°C under the light. After 2-3 weeks of incubation, single growing roots were excised and inoculated on MS solid medium containing 50 mg/L cefotaxime. Hairy roots were cultured at 25°C under the light and subcultured every five weeks. About 100 mg roots (3 cm in length) were inoculated into a 150 mL conical flask containing 40 mL liquid MS medium and cultured in the dark at 25°C, 100 rpm. Hairy roots with regenerated plants were selected and transferred to hormone-free MS medium for succession. Single plant with height of 0.5 cm was selected for succession. The regenerated plants with a height of 1.0 cm were transplanted and cultured in nutrient soil to obtain regenerated plants, and then cultured in a light incubator at a constant temperature of 25°C for 12 hours light-dark cycle.

Optimization of conditions for induction and regeneration of plantlets of tobacco hairy roots

To find the best strain of *Agrobacterium rhizogenes* to promote tobacco hairy roots, A4, R1601, 15834, and K599 strains were used to infect tobacco explants with the bacterial concentration of 10^9 CFU/mL, respectively. The effects on hairy root rate and regenerated plants were evaluated by the rooting rate of explants, which were calculated as follows:

$$\text{Hairy root induction rate} = \frac{\text{number of rooting explants}}{\text{number of infected explants}} \times 100\%$$

$$\text{Transformation rate of regenerated plants} = \frac{\text{number of regenerated plants}}{\text{number of hairy roots}} \times 100\%$$

Identification of hairy roots and regenerated plants

The total DNA of induced hairy roots and regenerated plants were extracted by using TransZol Up Plus DNA Kit (Beijing TransGen Biotech Co., Ltd., Beijing, China). The primers of *rolA* and *VirD* genes were synthesized by Zhengzhou Qingke Biotechnology Co., Ltd.

Table 1. Effects of different explants on hairy root induction rates of tobacco.

Explants	Number of infected explants	Number of explants rooted	Induction rate (%)
Hypocotyl	30	26.2±0.3[a]	87.3±0.01[a]
Cotyledon	30	12±0.5[b]	40±0.02[b]

Note: the different lowercase letters indicated significant differences ($P < 0.05$).

(Zhengzhou, Henan, China) with the primer sequences of rolA-F: 5'-AAG TGC TGA AGG AAC AAT C-3', rolB-R: 5'-CAA GTG AAT GAA CAA GGA AC-3', VirD-F: 5'-ATG TCG CAA GGA CGT AAG CCC A-3', VirD-R: 5'-GGA GTC TTT CAG CAT GGA GCA A-3'. The polymerase chain reaction (PCR) was performed by using PCR reaction kit (Beijing TransGen Biotech Co., Ltd., Beijing, China) with the total reaction volume of 20 µL including 10 µL of reaction mixture, 1 µL of each primer, 1 µL of DNA template, 7 µL of ddH_2O, and the reaction program of 94°C for 5 mins followed by 31 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 10 mins. The PCR products were subjected to 1% agarose gel electrophoresis.

Statistical analysis

Microsoft Excel (Microsoft, Redmond, WA, USA) was employed for the data analysis in this study. The data were expressed as the mean ± standard deviation (SD). The difference between the sample groups was expressed by *P* value with *P* less than 0.05 as the significant difference.

Results and discussion

Induction process of hairy root system

After co-culture of tobacco explants with *Agrobacterium rhizogenes* for two days, hairy roots grew after 14 days incubation on MS agar medium containing 50 mg/L cefotaxime, and vigorous hairy roots were obtained after 21 days incubation. The effects of different explants on the induction rate of tobacco hairy roots were shown in Table 1. The results showed that the induction rate of tobacco hairy roots by the hypocotyl was higher than that of cotyledon, reaching 87.3%.

***Agrobacterium rhizogenes* strain, infection mode, and dark treatment**

To obtain hairy roots of tobacco, four strains of *Agrobacterium rhizogenes* (A4, K599, 15834, R1601) were selected to infect tobacco explants. The induction rate of A4 was significantly higher than the other three strains with the highest induction rate of 81.33% in leaves (Figure 1). Among the four strains of *Agrobacterium rhizogenes*, the root rates were 25%, 53.67%, 81.33%, and 24.33% for 15834, K599, A4, and R1601, respectively. It had been determined that A4 had a higher induction rate of hairy roots.

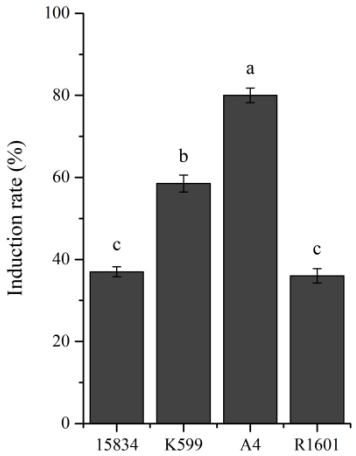

Figure 1. Comparison of hairy root induction efficiency of different *Agrobacterium rhizogenes* strains. (the different letters indicated significant differences ($P < 0.05$) between different strains.

Comparing the effects of different dark treatment times of 12 h, 24 h, 48 h, and 72 h on the induction rate of hairy roots, the results showed that the lowest positive root rate was 7.5% after 12 h co-culture, while the highest one was 55.00% after 48 h co-culture (Table 2). There was no significant difference between 24 h and 72 h treatments in positive root rate. However, the long dark treatment time might increase the

Table 2. Effects of hairy roots induction rate under different dark treatment time.

Dark treatment time	Total number of induced blades	Total number of hair root leaves	Average induction rate
12 h	120	9	7.50±1.44[c]
24 h	120	24	20.00±1.44[b]
48 h	120	66	55.00±3.82[a]
72 h	120	29	24.17±3.00[b]

Note: the different lowercase letters indicated significant differences ($P < 0.05$).

Table 3. Effects of hairy roots induction rate under different infection time.

Infection time	Total number of induced blades	Total number of hair root leaves	Average induction rate
10 min	120	23	19.17±2.20[c]
20 min	120	95	79.17±3.00[a]
30 min	120	37	30.83±0.83[b]

Note: the different lowercase letters indicated significant differences ($P < 0.05$).

risk of *agrobacterium* contamination, which was not conducive to the rapid induction of hairy roots. Therefore, 48 h was the best dark treatment time. In the induction of hairy roots, different *Agrobacterium rhizogenes* infection times had a great effect on the hair root rate. The effects of different infection times of 10, 20, and 30 min on hairy roots induction were compared, and the results showed that 10 mins and 30 mins infections had a low effect on hair root rate, while the induction rate of 20 min infection reached 79% (Table 3).

Induction of regeneration system

Hairy roots were grown in MS medium. Some of them were induced to regenerate plants and produce roots after 20 days of culture. Regenerative buds were obtained from 3 of the 13 tobacco hairy root lines induced by A4. The vigorous hairy root plantlets were obtained by subculture after 21 days. The regenerated buds were inoculated into the rooting medium for rooting culture and rooted after 10–20 days. However, compared with the wild-type seed germination plants, tobacco hairy root regeneration plants had more clumps of buds and an extremely well-developed root system with a 90% rooting rate. Moreover, 54% of hairy roots regenerated plants could flower in tissue culture bottles. The obtained seeds were removed, and three plants were randomly selected from the regenerated plants with six roots.

PCR detection of *VirD* and *rolA* genes in regenerated plants

Both *VirD* and *rolA* genes promote the production of hairy roots and are the indicators for the successful transformation of hairy roots. Three transgenic tobacco regeneration lines were selected and tested by using PCR technology. The results showed that all 3 tobacco regenerated plants demonstrated both *VirD* and *rolA* genes (Figure 2), which indicated that both genes had been successfully integrated into regenerated plants.

Optimization of hairy root induction conditions

The establishment of a fast and stable tobacco hairy root system and gene transformation system facilitates the transfer of exogenous genes and is important for the functional validation of transgenes and the research of secondary metabolite synthesis. The results of this study found that the induction rate of hairy roots differed greatly between different *Agrobacterium rhizogenes* infesting tobacco leaves with A4 significantly higher than 15834, K599, and R1601. In addition, A4 could induce hairy root system better than other strains,

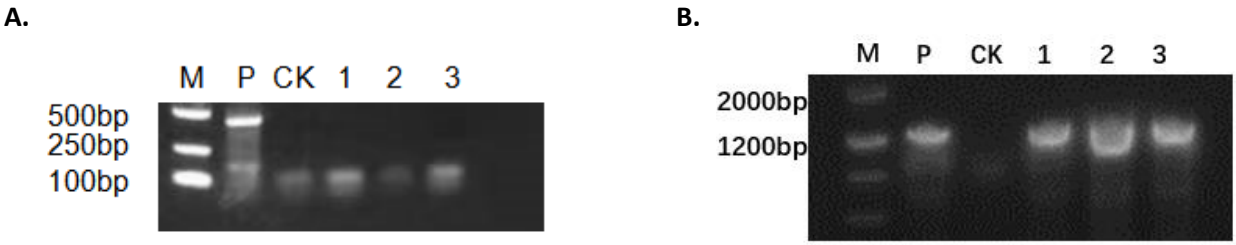

Figure 2. Identification of *VirD* gene (A) and *rolA* gene in regenerated plants**.** M: marker. P: plasmid (positive control). CK: negative control. Lanes 1-3: tobacco regenerated plants.

which indicated that explants of tobacco soil-grown seedlings could be transformed into hairy roots. The traditional tobacco genetic transformation system is a method of inducing callus to transform regenerated plants, which has a long cycle time and low transformation efficiency taking more than six months to obtain T1 generation transgenic seedlings. In this study, a stable root system could be induced in 40 days, and the hairy root system was simple to operate without the addition of plant growth regulators, providing a reference for the establishment of a rapid transgenic system for tobacco. The tobacco hairy root system established in this study could also be used to rapidly analyze the expression of genes involved in the secondary metabolic pathway of tobacco growth.

Induction and optimization of regeneration system

During the transformation of tobacco leaves, *Agrobacterium* A4 transformed more efficiently than that of R1601, 15834, and K599 with an induction rate of 80.33%. It has not been previously reported that the simultaneous induction of regenerated plants during the induction of tobacco hairy root. The use of hairy roots to directly induce tobacco regenerated plants can greatly reduce the time of the tobacco regeneration system, which can be used to functionally verify the relevant genes and can provide a relevant basis for tobacco breeding. Under the tobacco regeneration system, it can grow and develop into transplantable seedlings with root culturing on MS medium without the addition of any hormones, which greatly decreases the cultivation time of tobacco tissue culture seedlings, and improves the efficiency of rapid propagation, providing a new way for the establishment of tobacco rapid propagation system. In this study, a regeneration system was established by using *Agrobacterium rhizogenes* to induce tobacco soil-grown seedlings and was confirmed by using PCR. A rapidly and efficient regenerated plant system was established.

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