REVIEW ARTICLE

Research advances on tobacco hairy roots

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As a bioreactor, the tobacco hairy root system has attracted considerable attention and is capable of producing a variety of valuable bio-active compounds. Hairy root cultures provide a useful platform for the production of plant secondary metabolites, particularly alkaloids and their derivatives, which are produced at high levels in hairy roots. For the purpose of verifying the function of related genes or producing secondary metabolic products, *Rhizobium rhizogenes* was used to induce hairy roots in tobacco. This technique has become very common in recent years. The analysis of tobacco hairy root induction mechanisms, induction factors, and related applications were investigated in this study. This review study focused on the research status of plant secondary metabolites produced by the tobacco hairy root system as well as significant conditions related to hairy root production and process problems that needed to be resolved in industrial production. In addition, some unsolved issues and key questions were also discussed.

Keywords: tobacco; hairy root; metabolic engineering; secondary metabolites.

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Background

The tobacco plant is one of the most important economic crops and model plants. A variety of important metabolites can be produced by the hairy root system of tobacco including nicotine, resveratrol, solanesol, geraniol, thaumatin, erythropoietin, flavonoids, bovine lactoferrin, cecropin, and antibacterial compounds such as peptides which may be used in medicine, cosmetics, perfumes, dyes, and fragrances. Secondary metabolites are less abundant during plant growth and are difficult to synthesize chemically, making their commercial production more costly. Furthermore, it is a waste of resources to extract these compounds from plants grown in the field. Induced hairy roots can be produced in large quantities without the addition of medium, eliminating the possibility of plant growth cycles. These genetically modified hairy roots can grow indefinitely and continuously produce the desired product. It is imperative that tobacco hairy roots are utilized for the production of these valuable compounds. Previous study had successfully constructed the C58C1-SPS strain [1], and its rooting rate and root length were greater than those of Agrobacterium A4 and ATCC 15834. To produce transgenic hairy roots, Anneli, *et al*. used the transgenic tobacco system [2]. The GC-MS analysis of hairy root extracts revealed 31.1 grams of geraniol per gram of dry weight (mean: 13.7 grams per gram). However, no geraniol was detected in the roots of the wild type. Hou, *et al*. used *Rhizobium rhizogenes* to induce tobacco hairy roots and treated tobacco hairy roots with 0.1% colchicine

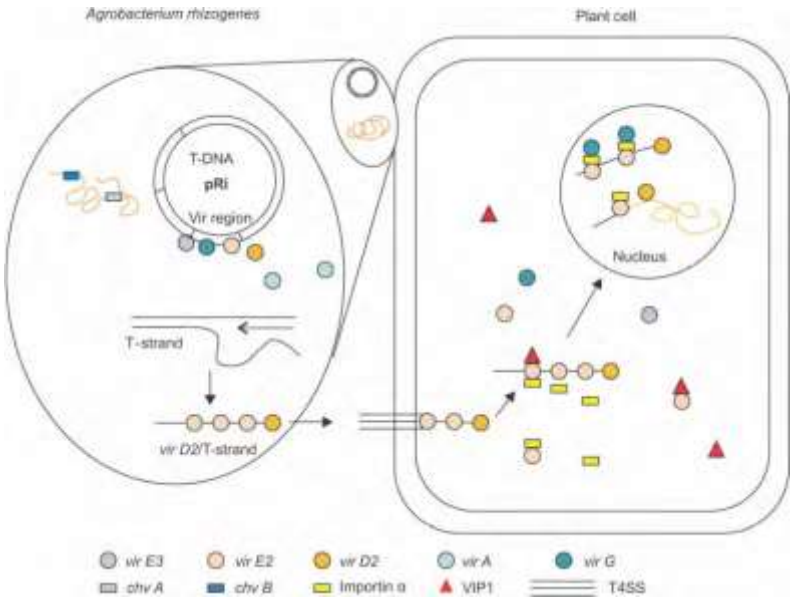

Figure 1. The infection of plant cells by *Rhizobium rhizogenes* [5]. The Vir region on the Ri plasmid contains proteins that reduce the sensitivity of the plant cell wall, which allows T-DNA to be inserted into the plant genome, resulting in the rapid growth of hairy roots.

solution [3]. The polyploid induction was successful in 64.71% of cases. There was a significant increase in polyploid tobacco content, which was 4.57 times greater than that of diploid plants that were regenerated. The results confirmed that tobacco hairy roots were of great practical and theoretical significance. With the efforts of scientists domestically and internationally, the induction system of tobacco hairy roots has become quite mature and perfect, making it possible to use tobacco as a bioreactor to produce important compounds.

The principle of hairy root induction

As a result of the construction of tobacco hairy roots, secondary metabolites such as drugs, spices, and cosmetics can be produced, and the mechanism of induction of tobacco hairy roots has been clarified. First, an alkaline-type strain with strong induction ability, such as R1601, was chosen, which could be used as a strain to induce hairy roots in tobacco. It was demonstrated that the T-DNA of the Ri plasmid of Agrobacterium R1601 could be inserted into the genome of injured plants, causing hairy roots to develop. The T-DNA entered the induced plant cells with the assistance of the agrobacterium's virulence region (Vir) gene and was expressed, which contributed to the formation of hairy roots. Vir and T-DNA transfer region are essential for inducing hairy roots in plants. Liu, *et al*. illustrated the molecular mechanism through which agrobacterium induced plants [4]. The main functional regions of agrobacterium include the virulence and T-DNA transfer regions (Figure 1). The Vir region consists of multiple gene regions such as VirA, VirB, VirC, and VirD. Acetosyringone stimulates the Vir region in plant tissues and induces agrobacterium to approach the injured plant cells, during which time the cell walls of plant cells are affected. There are two distinct parts of the T-DNA, the TR-DNA and the TL-DNA. Ags and auxin synthesis-related genes are located in the TR-DNA region, whereas the rol gene group which can induce plants to form hairy roots is located in the TL-DNA region. The gene in the Vir region affects the pore size of the cell wall, allowing T-DNA to enter the cell smoothly and successfully integrating the gene region into the plant's genome, resulting in hairy roots. Taking into account the induction factors, the hairy root induction system can be optimized based on the

characteristics of the explants, the strain type, the exogenous hormones, and the conditions of the culture.

Induction factors for producing hairy roots

Morphology of explants

Selection of explants such as cotyledons and hypocotyls can generally induce hairy roots. Thus, the type of explants is an important factor in genetic transformation of tobacco. The type of explant determines whether the tobacco plant cells are still actively dividing. As of now, tobacco leaves are primarily responsible for inducing hairy roots. Only a few reports have been published regarding hypocotyl induction. Past study found that the induction rate of hairy roots in tobacco young leaves was higher than that in mature leaves, indicating that the rate of hairy root induction was closely related to the developmental stage and location of the explants [6]. According to most current reports, tobacco leaves were used as explants to induce hairy roots with the different sizes of 5 × 5 cm^2, 2 × 2 cm^2, and 1 × 1 cm^2, respectively [6-8]. Incisions of different leaf sizes have been found to induce adventitious roots. Thus, the size of the leaf did not determine rooting rate, and the incision of the leaf was primarily made to facilitate the infection with *Rhizobium rhizogenes*. Using the same strain, Song, *et al*. induced hairy roots in NC89, K326, and Shamson leaves and found that different varieties of plants had significantly different leaf induction rates [6]. Therefore, different tobacco varieties and different ages of explants will affect the induction rate of hairy roots.

Types of strains

Rhizobium rhizogenes strains convert tobacco at different rates. As a general rule, *Rhizobium rhizogenes* has a wider host range and a higher induction rate, and the more common ones include R1601, A4, 15834, K599, *etc*. [9, 10]. Huang, *et al*. studied the induction efficiency of *Rhizobium rhizogenes* on the hairy roots of tobacco [11]. Four types of agrobacteria (A4, 15834, R1601, LBA9402) were used to infect tobacco leaves. The induction efficiency and hairy root differentiation time of A4 were both delayed compared to the other three strains. However, LBA9402 exhibited the highest level of infection efficiency, and hairy roots were induced after one week, demonstrating that LBA9402 *Rhizobium rhizogenes* infected tobacco more efficiently to produce hairy roots.

Time required for pre- and co-cultivation

Co-cultivation time of explants and *Rhizobium rhizogenes* directly affect the induction rate of hairy roots. Lee, *et al*. established a tobacco hairy root system that was induced by *Rhizobium rhizogenes* and found the optimal pre- and co-cultivation periods were 48 hours, which was 2-3 days prior to cultivation and co-cultivation [12].

Exogenous hormones

The exogenous hormones include methyl jasmonate, 24D auxin, salicylic acid, kinetin, and heavy metals. Among them, methyl jasmonate can induce signal transduction in the process of secondary metabolite production by tobacco roots, thereby maximizing the level of stimulation. Wongsamuth, *et al*. added Auxin 24D to the tobacco hairy root culture in order to stimulate the growth of the tobacco hairy roots and to induce hairy roots successfully [13], while Gurusamy, *et al*. added kinetin during the induction process in order to promote the growth of tobacco hairy roots [14].

Phenolic substances

Currently, acetosyringone (AS) is an important phenolic substance for improving the conversion rate of hairy roots. An appropriate amount of acetosyringone can result in increased conversion rates [15]. Researchers added 200 μmol/ml AS to the media in order to determine the rooting rates of K326, NC89, and Shamson tobacco. In contrast to the other two varieties, the rooting rate of K326 was found to increase, but not those of the other two varieties. The results confirmed that acetosyringone had little effect on the tobacco rooting rate [16].

Culture conditions

Many literatures reported that MS, 1/2MS, and B5 media could be used to cultivate tobacco hairy roots. MS (or B5 medium) has been found to be an ideal medium type for the growth of hairy roots [17, 18]. Hashemi, *et al*. cultured hairy roots at 24°C after successful transformation [19]. Lee, *et al*. generally maintained co-cultures at a temperature of 22℃ to 26℃ [12]. Hairy root cultures were incubated at 25°C in the dark following successful transformation. Cephalosporins were added at the beginning of the culture to inhibit the growth of agrobacterium. Three subcultures were used to screen out the rapid and stable production of hairy root systems. The last step was to culture the specimen in a culture medium. Xiang, *et al*. examined the effect of sucrose concentration on the hairy root rate induced by *Rhizobium rhizogenes* C58C1 on tobacco hairy roots [7]. To induce tobacco roots, different concentrations of sucrose were used in the experiments. As a result of this study, a medium sucrose concentration of 25 g/L was optimal for C58C1-induced NC82 hairy roots as well as 15 g/L for Va116-induced hairy roots. The results showed that different strains and varieties of tobacco required different levels of production to induce tobacco hairy roots.

Microorganisms

The advantages of hairy roots are their rapid growth and ease of observation. Consequently, tobacco hairy roots can be used to investigate the relationship between root microorganisms and roots. A fungus called *Arbuscular Mycorrhizal* fungus (AMF) is capable of forming mutualistic symbioses with plant roots, thereby promoting plant growth and development as well as improving crop yield and resistance. The AMF cannot, however, be cultivated solely *in vitro*. Taking advantages of rapid growth of hairy roots, *in vitro* culture, and simplicity, Lu, *et al*. observed the presence of arbuscules and vesicles on tobacco hairy roots through the exploration of symbiotic culture [20]. The results demonstrated that the dual culture system of the bacterium and tobacco hairy roots had been successfully established *in vitro*. Additionally, this enabled the production of *Claroideoglomus etunicatum* on a large scale. A dual culture system of tobacco hairy roots and AMF of the *Genus Megaspora* was developed by Yang [8], which enabled the scale-up of AMF agents of the G*enus Megaspora*.

Induction of hairy roots with a target gene

In order to transform an agrobacterium containing the target gene, it must be transformed into the corresponding carrier by either heat shock or electric shock, and then positively screened to obtain a bacterial solution containing the target gene. In accordance with the transformed genes, *Rhizobium rhizogenes* containing different target genes were obtained. It was used to test tobacco infection and resistance, and finally, the hairy root of the plant containing the gene of interest was obtained [14]. In a recent study, Diego, *et al*. constructed an overexpression vector containing the AtMB12 gene, which was successfully transformed into tobacco hairy roots and was capable of being stably expressed and inherited [21].

The applications of hairy roots

The method of obtaining regenerated plants

A hairy root system is used to regenerate tobacco plants, which is a more effective method for germplasm innovation using hairy root technology. It is possible to regenerate adventitious buds directly from hairy roots or to regenerate adventitious buds through the callus stage in order to achieve plant regeneration. A study conducted by Hou, *et al*. used induced hairy roots of tobacco and doubled the artificial chromosomes to increase the nicotine content of the tobacco [3]. Using *Rhizobium rhizogenes*' genetic transformation system, Gurusamy, *et al*. successfully transferred the recombinant erythrocyte gene into hairy roots [14]. The regenerated plants were then bred to express human erythropoietin, thus achieving tobacco plants capable of expressing human erythropoietin.

The production of metabolites

Table 1. The secondary metabolites produced by tobacco hairy roots.

Metabolites of secondary origin	Involvement of biologically active substances	References
solanesol	Ensure the health of liver cells, support liver metabolism, and facilitate liver detoxification.	[23]
geraniol	Flavors of the day and food flavors	[24]
human erythropoietin	Tonify the kidneys and strengthen the yang, tonify the spleens and strengthen the stomachs.	[14]
Reservatrol	Anti-cancer and antioxidant properties	[21]
bovine lactoferrin	Antibacterial and antioxidant properties	[25]
neonicotinoids	Inhibit the growth of fungi.	[26]
M12 antibody	Enhance blood circulation, improve blood flow, and treat cardiovascular disease.	[27]
human erythropoietin	Increase the production of red blood cells and relieve anemia.	[14]
thaumatin	Non-caloric sweetener	[28]
interleukin 12 (IL 12)	Anti-infection and anti-tumor properties	[29]
cecropin	Inhibit bacterial growth	[30]
flavonoid	The antioxidant and anti-aging properties	[31]
ricin	Anti-tumor property	[32]

There are currently a number of problems associated with medicinal plants such as deteriorating growth conditions, overexploitation, and low yields. It is possible to avoid the constraints imposed by the environment and other factors on medicinal plants through the use of genetic engineering technology. For instance, by using tobacco hairy root technology to induce medicinal plants to produce secondary metabolites and then improving the system for separating and purifying drugs, the secondary metabolites of medicinal plants can play a more prominent role in drug development. In order to produce recombinant proteins from tobacco hairy roots, Cao developed a system for the induction and cultivation of tobacco hairy roots [22]. Tobacco's rapidly growing hairy root was used as a reactor in this method. *Rhizobium rhizogenes* with the target gene was used to infect tobacco to produce foreign proteins that were expressed, and then secreted into the medium, thereby establishing a fast expression system for recombinant proteins. Table 1 demonstrated the studies on the secondary metabolites produced by tobacco hairy roots.

Validation of the function of tobacco genes

Hairy roots have several advantages including rapid growth, ease of induction, convenience of material extraction, and ease of production of secondary metabolites. Additionally, hairy roots can be used to investigate plant regulatory mechanisms and secondary metabolic signals in plant metabolic pathways on a single molecular level. Wang, *et al*. demonstrated successful transformation in tobacco hairy roots [33]. However, tobacco hairy roots lacking rolB or rolC showed less branching and lost the capacity for long-term differentiation. Lee, *et al*. linked a heat shock protein promoter to tobacco hairy roots and found that GUS protein expression was highest at 42°C, thus providing an alternative method for studying heat shock physiology in plants [12]. To promote the extracellular secretion of the fusion protein, Zhang, *et al*. transformed the gene of novel hydroxyproline (Hyp)-O-glycosylated peptides (HypGPs) into

tobacco hairy roots [34]. The results showed that the expression of recombinant protein increased, proving that the gene was indeed capable of increasing protein expression. Alderete, *et al*. overexpressed the circadian clock gene (CCG) in hairy roots and found that NtLHY, NtTOC1, NtFKF1, NtGI, and NtPRR9 genes also changed, demonstrating that CCG was involved in the regulation of plant circadian rhythms [35].

An environment conducive to the super repair of plant roots

By using induced tobacco hairy roots, Talano, *et al*. selected hairy roots that grew rapidly and were easily accumulating 2,4-dichlorophenol (2,4-DCP) in water. The results demonstrated that hairy roots were capable of removing 2,4-DCP pollutants from water with high efficiency (98%, 88%, and 83%) within a short period of time [36]. Transgenic hairy roots expressing alkaline peroxidase were identified in tobacco hairy roots by Alderete, *et al*. [35]. Additionally, using the hairy roots produced under this system could effectively remove phenol from water.

Discussion and developmental concerns

This study further enhanced the tobacco hairy root resource bank, contributed to a deeper understanding and application of the tobacco hairy root system, and provided a reference for further development and utilization of this resource. The tobacco hairy root system has been favored by many researchers domestically and internationally, primarily because it can be utilized to produce valuable secondary metabolites and exogenous proteins. A major advantage of tobacco hairy root systems is their ability to express high-quality secondary metabolites. In addition, it is capable of growing rapidly without the use of plant hormones, as well as serving as a biochemical reactor for expanded cultivation. It is still possible to conduct research on tobacco hairy roots in a variety of directions. In the first place, low yields of secondary metabolites in some medicinal plants are largely due to their own characteristics. The mechanisms of secondary metabolite pathways in plants are still uncertain, and regulation studies at the DNA, RNA, and protein levels are also relatively limited. Secondly, in order to promote the production of the target product more effectively, multiple bio-synthetic genes or global transcription regulation genes must be expressed simultaneously to promote the expression of the target product. Changing only one gene is a good idea, but there are some limitations. It may therefore be necessary to optimize the expression of each enzyme in the pathway in order to maximize the yield of metabolites. Finally, the culture conditions for tobacco hairy roots need to be further optimized such as the type of medium, the type of agrobacterium, the stability of tobacco hairy roots, and the exploration of the best conditions.

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