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·Review·

Hairy Root and Its Application in Plant Genetic Engineering

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Abstract

Agrobacterium rhizogenes **Conn. causes hairy root disease in plants. Hairy root-infected *A. rhizogenes* is characterized by a high growth rate and genetic stability. Hairy root cultures have been proven to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of differentiated plants. Furthermore, a transgenic root system offers tremendous potential for introducing additional genes along with the Ri plasmid, especially with modified genes, into medicinal plant cells with *A. rhizogenes* vector systems. The cultures have turned out to be a valuable tool with which to study the biochemical properties and the gene expression profile of metabolic pathways. Moreover, the cultures can be used to elucidate the intermediates and key enzymes involved in the biosynthesis of secondary metabolites. The present article discusses various applications of hairy root cultures in plant genetic engineering and potential problems associated with them.**

Key words: *Agrobacterium rhizogenes*; hairy root; plant genetic engineering; Ri plasmid; secondary metabolites.

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Hairy root is a plant disease caused by *Agrobacterium rhizogenes* Conn., a Gram-negative soil bacterium. When the bacterium infects the plant, the T-DNA between the TR and TL regions of the Ri-plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable by-product, hairy root, which will form at or near the site of infection. In addition, opines are produced and serve as specific food for the bacteria (Chilton et al. 1982). Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium. The transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites, whereas other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize very low levels of useful secondary metabolites (Rhodes et al. 1990; Merkli et al. 1997; Kittipongpatana et al. 1998). Most importantly, *A. rhizogenes* can transfer T-DNA from binary vectors and enable the production of transgenic plants containing foreign genes carried on a second plasmid. This property has been used to produce transgenic plants (Tepfer et al. 1984; Christey et al. 1997).

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Hairy Root Induction and Selection

Establishment of a hairy root culture system

To succeed in establishing a hairy root culture system for a certain plant species, several essential conditions should be taken into consideration. These conditions include the bacterial strain of *A. rhizogenes*, an appropriate explant, a proper antibiotic to eliminate redundant bacteria after cocultivation, and a suitable culture medium. Based on the types of opines produced, the strains of *A. rhizogenes* can be separated into five lines: octopine, agropine, nopaline, mannopine, and cucumopine (Zhou et al. 1998). Agropine strains are the most often used strains owing to their strongest induction ability. Most plant materials, such as hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, or tuber, can be used to induce hairy roots (Mugnier 1988; Han et al. 1993; Drewes et al. 1995; Giri et al. 2001; Krolicka et al. 2001; Azlan et al. 2002). However, for different species, the proper explant material may vary and the age of the material is most critical, with juvenile material being optimal. To induce hairy root, explants are separately wounded and cocultivated or inoculated with *A. rhizogenes*. Usually two or three days later, the explant

can be transferred into solid media with antibiotics, such as cefotaxime sodium, carbencilin disodium, vancomycin, ampicillin sodium, claforan, streptomycin sulphate, or tetracycline, ranging in concentration from 100 to 500 μg/mL, to kill or eliminate redundant bacteria (Spano et al. 1981; Drewes et al. 1995; Giri et al. 2001; Krolicka et al. 2001). The hairy roots will be induced within a short period of time, which varies from one week to over a month depending on different plant species. The decontaminated hairy roots can be subcultured on phytohormone-free medium.

Optimizing the composition of nutrients for hairy root cultures is critical to gain a high production of secondary metabolites. Factors such as the carbon source and its concentration, the ionic concentration of the medium, the pH of the medium, light, phytohormones, temperature, and inoculum are known to influence growth and secondary metabolism (Christen et al. 1992; Toivonen et al. 1992; Rhodes et al. 1994; Arroo et al. 1995; Bhadra et al. 1995; Vanhala et al. 1998; Morgan et al. 2000). Heavy metal ions and the concentrations of phosphate, nitrate, and ammonia have also been well studied (Payne et al. 1987; Toivonen et al. 1991; Christen et al. 1992; Sevon et al. 1992). The addition of auxin and elicitors often increases the levels of secondary metabolites (Dymov et al. 1997; Pittaalvarez et al. 1998; Rijhwani et al. 1998; Singh et al. 1998; Vanhala et al. 1998). Because of these factors and the fact that individual hairy roots may have different requirements for nutrient conditions, the culture conditions should be optimized separately for each species and for individual clones.

Reporter gene

The β-glucuronidase (*GUS*) gene is usually transferred into hairy roots as a reporter gene and it can be analyzed easily by histological assay (Jefferson et al. 1987; Hosoki et al. 1994). So far, the GUS reporter system is the most common means of monitoring plant systems. In other cases, neomycin phosphotransferase II (NPT-II) encoding the kanamycin-resistance enzyme has been used (Han et al. 1993; Qin et al. 1994). Sometimes, both GUS and NPT-II have been transferred into the hairy roots (Christey et al. 1992; Azlam et al. 2002). Recently, the gene for green fluorescent protein (GFP) was used successfully as a reporter gene in *Catharanthus roseus* L. hairy roots (Hughes et al. 2002).

Selection of hairy root line

Owing to the site uncertainty of T-DNA integration into the host plant genome, the hairy roots derived often show different accumulation patterns of secondary metabolites. Mano et al. (1989) analyzed 45 hairy root clones of *Duboisia leichhardtii* F. and found that there was considerable variation in growth rate, alkaloid content, and productivity among the clones. Generally, hairy roots are considered to be stable and easy to subculture. Nonetheless, hairy roots also possess a certain amount of heterogeneity, even if derived from a single root tip, and the repeated selection seems to be important to obtain high scopolamine-producing hairy root lines (Yukimune et al. 1994).

Application of Hairy Roots

Functional analysis of genes

Kumagai et al. (2003) studied transgenic lines of *Lotus japonicus* Regel. that express GUS by constitutive or nodule-specific promoters. *L. japonicus* were supertransformed by infection with *A. rhizogenes* containing gene constructs for the expression of hairpin RNAs (hpRNAs) with sequences complementary to the GUS coding region. The results indicated that the GUS activity in those lines decreased more than 60%. This suggests that transient RNA silencing by hairy root transformation provides a powerful tool for loss-of-function analyses of genes that are expressed in roots. In another case, a glucocorticoid-inducible promoter controlling the expression of GFP was transformed into hairy roots of *C. roseus* (Hughes et al. 2002). The inducible promoter showed a tightly controlled, reversible, and dose-dependent response to the glucocorticoid dexamethasone in the hairy roots. The *GUS* gene fused with the alcohol dehydrogenase (Adh) promoter was transformed into hairy roots of soybean. The function of the promoter was studied under different conditions, including cold temperature, wounding, anoxia, and abscisic acid treatment (Preiszner et al. 2001). A chimeric gene with a tobacco hydroxyproline-rich glycoprotein (*HRGPnt3*) gene promoter–*GUS* was expressed in the hairy roots of tobacco (*Nicotiana tabacum* L.; Vera et al. 1994). The results demonstrated the different expression pattern of the promoter. An antisense dihydroflavonol reductase (*DFR*) gene was introduced into the hairy roots of *Lotus corniculatus* L. and effectively downregulated tannin biosynthesis in two of the recipient genotypes (Carron et al. 1994).

Expressing foreign proteins

The production of industrial and therapeutic proteins by plants is an area of intense commercial interest. Three genes from *Ralstonia eutropha* Davis, a type of bacteria necessary for poly(3-hydroxybutyrate) (PHB) synthesis, were introduced into the hairy roots of sugar beet (Menzel et al. 2003). The 20 transgenic hairy root clones produced up to 55 mg high molecular PHB per gram dry weight. The pea *lectin* gene was introduced into white clover (*Trifolium repens* L.) hairy roots and correctly processed (Diaz et al. 1995a). Sharp and Doran (2001b) reported that murine IgG1 was produced in the hairy roots of tobacco and these authors improved the accumulation of the antibody by increasing the dissolved oxygen tension to 150% air saturation. Anti-viral traits are important economic factors in viticulture. Plants transformed with virus protein often show resistance to virus infection. Torregrosa et al. (1997) succeeded in obtaining grapevine hairy roots

transferred with the coat protein of grapevine chrome mosaic nepovirus. Unfortunately, the regeneration of plants from roots was not achieved. Nevertheless, grafted plants with transgenic roots could be established in the greenhouse.

Production of secondary metabolites

Normally, root cultures need an exogenous phytohormone supply and grow very slowly, resulting in the poor or negligible synthesis of secondary metabolites. The hairy root system is stable and highly productive under hormone-free culture conditions. The fast growth, low doubling time, ease of maintenance, and ability to synthesize a range of chemical compounds of hairy root cultures offer additional advantages as continuous sources for the production of valuable secondary metabolites. Hairy roots are also a valuable source of phytochemicals that are useful as pharmaceuticals, cosmetics, and food additives. These roots can also synthesize more than a single metabolite and, therefore, prove economical for commercial production purposes. Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, which are potentially important pharmaceutical products. Sevon (2002) has summarized the most important alkaloids produced by hairy roots, including *Atropa belladonna* L., *Catharanthus tricophyllus* L., and *Datura candida* L.

However, sometimes the efficiency of secondary metabolic production is not so desirable. Metabolic engineering offers new perspectives for improving the production of secondary metabolites by the overexpression of single genes. This approach may lead to an increase of some enzymes involved in metabolism and consequently results in the accumulation of the target products.

Normally, there are two different categories of transformation methods based on the type of genes to transfer. One method utilizes the foreign genes that encode enzyme activities not normally present in a plant. This may cause the modification or diversion of plant metabolic pathways. Two direct repeats of a bacterial lysine decarboxylase gene expressed in the hairy roots of *N. tabacum* markedly increased cadaverine and anabasine (Fecker et al. 1993). The production of anthraquinone and alizarin in hairy roots of *Rubia peregrina* L. was enhanced by the introduction of isochorismate synthase (Lodhi et al. 1996). The hairy roots of *A. belladonna* transformed with the rabbit *P450 2E1* gene displayed increased levels of the metabolites (Banerjee et al. 2002). *Catharanthus roseus* hairy roots harboring hamster 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase (HMGR) cDNA without the membrane-binding domain were found to produce more ajmalicine and cantharanthine or serpentine and campesterol than the control (Ayora-Talavera et al. 2002).

The other transformation method enhances the overexpression of enzymes that are already located in a plant. The tobacco putrescine *N*-methyltransfersase (PMT) was transformed into *Datura metel* L. and *Hyoscyamus muticus* L. (Moyano et al. 2003). The enzyme catalysed the first committed step in the tropane alkaloid pathway and stimulated the growth of transgenic roots and the accumulation of tropane alkaloid.

Oxygen deficiency is a usual problem in hairy root culture caused by poor mixing and mass transfer conditions. To improve the low oxygen conditions that affect growth during fermentation, two enzymes, namely Adh and pyruvate decarboxylase, were transferred into the hairy roots of *Arabidopsis thaliana* L. The transformant root lines maintained a similar growth rate under conditions of low oxygen to the rate achieved with full aeration (Shiao et al. 2002).

Production compounds not found in untransformed roots

Transformation may affect the metabolic pathway and produce new compounds that cannot be produced normally in untransformed roots. For example, the transformed hairy roots of *Scutellaria baicalensis* Georgi. accumulated glucoside conjugates of flavonoids instead of the glucose conjugates accumulated in untransformed roots (Nishikawa et al. 1997).

Changing composition of metabolites

Bavage et al. (1997) reported the expression of an *Antirrhinum* L. dihydroflavonol reductase gene resulted in changes in condensed tannin structure and accumulation in root cultures of *L. conrniculatus*. The analysis of selected root culture lines indicated the alteration of monomerlevels during growth and development without changes in composition.

Regeneration of whole plants

Regeneration of whole plants from hairy roots has been reported in several plant species. The successful regeneration of transgenic plants depends mostly on the *in vitro* culture conditions for each particular species. However, the genotype and juvenility of proper explants are also very important. Transformed roots can regenerate somatic embryos following the addition of the appropriate phytohormone. Cho and Wildholm (2002) reported that when cultured in medium with 7.5–10.0 mg 2,4-dichlorophenoxyacetic acid, the hairy root of *Astragalus sinicus* L. developed somatic embryos. Shoots were regenerated from hairy roots of *Robinia pseudoacacia* L. following the addition of 10 μmol/L α-naphthaleneacetic acid and 5 μmol/L 6-benzyl-aminopurine (Han et al. 1993).

Potential Problems

As a new method in the study of plant genetic engineering, hairy root culture shows many useful functions. However, at the same time, it also has some potential problems that remain to be solved.

Different regulation of secondary metabolism in related species

Although the secondary metabolism in related species may share the same pathway, their regulation differs according to different patterns. For example, the tropane alkaloid was increased in the hairy roots of both *D. metel* and *H. muticus* (Moyano et al. 2003). However, different accumulation patterns were found in the two plants. Both hyoscyamine and scopolamine were accumulated in the hairy roots of *D. metel*, whereas only hyoscyamine levels increased in *H. muticus*. This result indicates that the same pathway in two related species is regulated differently. 4-Hydroxycinnamoyl-CoA hydratase (HCHL) was expressed in hairy root cultures of *Datura strmonium* L. (Mitra et al. 2002). However, no 4-hydorxybenzaldehydes were found. In contrast, there was some decrease in the availability of feruloyl-CoA for the production of feruloyl putrescine and coniferyl alcohol.

The genotypes of recipients may affect the expression of transferred genes. The antisense DFR downregulated tannin biosynthesis in two genotypes (S33 and S50) of the hairy roots derived from *L. corniculatus* (Carron et al. 1994). However, in the third genotype (S41), the transgenic hairy roots accumulated high levels of condensed tannins.

Overexpression of key enzymes does not always improve secondary metabolism

Two key enzymes encoding chorismate pyruvate-lyase and HMGR, which were assumed to involve in the biosynthesis of shikonin, were transformed into the hairy roots of *Lithospermum erythrorhizon* Sieb. (Koehle et al. 2002). However, shikonin accumulation remained unchanged, even with the high expression of the two enzymes.

Possible reduction of chromosome numbers during subculture

The primary hairy roots of *Onobrychis viciaefolia* Scop. had normal chromosomes (2n=28; Xu et al. 1996). However, after four months of subculture, the percentage of hairy root cells with a normal chromosome number was reduced from 85.0% to 23.5%. Eight months later, only 4.1% of cells had a normal chromosome.

Cosuppression of endogenous and foreign genes

More copies of the transformant genes do not result in a greater expression of the task enzymes and a corresponding increase in the products. *Catharanthus roseus* hairy roots harboring hamster HMGR cDNA expressed a different alkaloid production pattern (Ayora-Talavera et al. 2002). Clone 236, with more HMGR copies, had the lowest HMGR activity but increased levels of ajmalicine and catharanthine. Clone 19, with low HMGR copies, expressed more HMGR and produced more campesterol and serpentine, but had a low level of ajmalicine and showed no accumulation of catharanthine. Transgenic silencing is evident in some species. Hairy roots of *Cinchona officinalis* L. transformed with tryptophan decarboxylase (TDC) and strictosidine synthase (STR) produced high amounts of tryptamine and strictosidine at the beginning (Geerlings et al. 1999). However, one year later, they had completely lost their capacity to accumulate alkaloids without changes in growth and morphology.

Morphological alterations of regenerated plants

Typically, the plants regenerated from hairy roots often have wrinkled leaves, an extremely abundant and plagiotropic root system, reduced apical dominance, reduced internode length and leaf size, and an increased ability of leaf explants to differentiate roots in phytohormone-free medium (Tepfer 1984; Tayler et al. 1985; Cardarelli et al. 1987; Spano et al. 1988; Hamamoto et al. 1990). In addition to these phenotypes, asymmetrical leaflets, variegated leaves, and reduced spine length have also been observed (Han et al. 1993). These abnormal phenotypes possibly originate from genomic disturbances due to either the insertion of foreign DNA or somaclonal variation, rather than from the expression of T-DNA genes in the transformants (Han et al. 1993). Transgenic plants often show higher mortality than normal, non-transformed plants.

Conclusions

The hairy root culture system is a potential approach for the production of secondary metabolites, especially pharmaceuticals, because it has many good traits, such as rapid growth rate, easy culture and genetic manipulation, and, most importantly, an increased ability to synthesize useful metabolites that cannot be produced by unorganized cells even higher than plant roots. Potential problems with the application of the hairy root culture system, including a variation in the synthesis abilities among different clones, a possible loss of chromosomes, and cosuppression between endogenous and exogenous genes, should also be taken into consideration when the system is used.

Several decades have passed since the hairy root culture system was first established. However, the system has not been utilized globally in practical industry production. The present established culture systems are based on flasks or small-scale bioreactors. So far, no successful example has been found to be applicable for scaled-up commercial production. Many attempts have been made to develop economical bioreactors containing airlifting, bubble column, mist, dual, and wave reactors (Du et al. 2003; Lin et al. 2003; Palazon et al. 2003; Souret et al. 2003; Kintzios et al. 2004; Suresh et al. 2004). The existing culture systems met with the same problem (i.e. they could not resolve the

contradiction between investment and consumption, which made them impractical for commercial use). To resolve the bottleneck of the application of hairy root culture systems, future research should focus on the establishment of effective and economical scaled-up culture systems that can reduce the consumption but obtain the biggest benefits. If such a breakthrough is achieved, the application of hairy root culture systems will be more likely.

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