Chapter 3
Hairy Root Culture: An Efficient System for Secondary Metabolite Production

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3.1 Introduction

Plants are a potential source for many important drugs because they are able to produce various chemical entities and bioactive molecules through the process known as metabolism. Plant cell carries out both primary and secondary metabolism. Primary metabolism involves the synthesis of polysaccharides, proteins, lipids, RNA and DNA through utilization of sugars, amino acids, common fatty acids and nucleotides whereas secondary metabolism is activated during particular stages of growth and development or during periods of stress limitation of nutrients or attack by microorganisms (Yazaki et al. 2008). Secondary metabolites generally derived from primary metabolites through modifications, such as methylation, hydroxylation and glycosidation. Therefore, secondary metabolites are naturally more complex than primary metabolites and are classified on the basis of chemical structure (e.g., aromatic rings, sugars), composition (containing nitrogen or not), their solubility in various solvents or the pathway by which they are synthesized. They have been categorized into Terpenes (composed entirely of carbon and hydrogen), phenolics (composed of simple sugars, benzene ring, hydrogen and oxygen) and nitrogen and/or sulphur containing compounds (Chinou 2008) (Table 3.1). It has been observed that each plant family, genus and species produces a characteristic mixture of these metabolites.

These compounds usually have very complicated structures and/or exhibit chirality. Consequently, in many cases organic synthesis is not cost effective. Many of these natural products can be obtained by direct extraction from plants. However, this method is known to cause serious ecological problems. The large-scale production of valuable materials, by virtue of field grown plants and original habitats has been

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Table 3.1 Classification of secondary metabolites

Terpenes (composed of C and H)	Phenols (composed of sugars, benzene ring, H and O)	Nitrogen and/or sulphur containing compound
Monoterpenes: Limonene	Phenolic acids: Caffeic	Alkaloids: nicotine
Sesquoiterpenes: Farnesol	Coumarins: umbellifereone	Glucosinolates: Sinigrin
Diterpenes: Taxol	Lignas: podophyllin	
Triterpenes, cardiac glycosides: Digitogenin	Flavonoids: anthocyanin	
Tetraterpenoids: Carotene	Tannins: gallotannin	
Sterols: Spinasterol	Lignin	

limited, primarily by a variety of environmental factors, including low growth rates, restricted cultivation areas, climate dependency, pests, plant diseases, intense labor requirement and the overall time-consuming nature of the tasks inherent to the pursuit. Furthermore, the volume and range of phytochemicals used by modern society are continuously expanding due to explosive population rise. These challenges demand to develop new ways for the production of plant derived metabolites at commercial level.

Plant cell suspension culture has been considered an alternative source to agricultural process for producing valuable secondary metabolites, totally independent of geographical and climatic conditions. Although in vitro culture of plant cell is now a mature technology with successful applications in agricultural crop improvement, germplasm storage, and micropropagation, but the application for commercial production is limited. Notable exceptions include the commercial production of shikonin, berberine and ginseng cells in Japan and pilot-scale trials for the production of sanguinarine, rosmaric acid, digoxin, geraniol and immunologically active polysaccharides, which are currently underway in the USA, Canada, and Germany (Mavituna 1992; Giri and Narasu 2000; Lee et al. 2004). The biggest challenge for producing secondary metabolites from plant cell suspension culture is that secondary metabolites are usually produced by specialized cells and/or at distinct developmental stages (Balandrin et al. 1985). Some compounds are not synthesized if the cells remain undifferentiated (Berlin et al. 1985). Therefore, undifferentiated plant cell cultures often lose, partially to accumulate secondary products (Rokem and Goldberg 1985; Charlwood and Charlwood 1991). Thus, in vitro culture of differentiated and organized tissues (particularly the roots) was focused as their behavior have been claimed to be much more predictable when compared with that of cell suspension cultures (Parr 1989). The plant roots seem to be the most suitable for large scale cultivation since the roots are the site of synthesis and/or storage of certain chemicals of pharmaceutical importance. However, there are some reports of co-cultured differentiated tissues (e.g. shoots + roots) being used to produce secondary metabolites (Subruto et al. 1996; Mahagamasekera and Doran 1998). Slow growth rate due to highly organized nature of normal roots posed another serious limitation in commercialization of technology using root biomass as a source for secondary metabolite production. Recently root culture has been re-developed as an experimental tool making use of

natural ability of a soil bacterium *Agrobacterium rhizogenes* to transfer genes into the host plant genome. *A. rhizogenes*, a gram negative bacterium infects a wide range of plant species and causes the neoplastic plant disease syndrome known as 'hairy root disease'. Attentions are now being focused on genetic transformation of plants using this natural vector as an important alternative to intact plants as well as cell suspension cultures for the production of secondary metabolites.

3.2 Hairy Root Culture

3.2.1 Mechanism of Hairy Root Induction

The term 'hairy root' was first mentioned in the literature by Stewart et al. (1900) (see Srivastava and Srivastava 2007). The identity of the hairy root-causing organism remains uncertain for a long time. Riker et al. (1930) described and named the hairy root-causing organism as *Phytomonas rhizogenes* which was later renamed as *Agrobacterium rhizogenes* by the same group. A large number of small roots protrude as fine hairs directly from the infection site in response to *A. rhizogenes* attack, a phenomenon that gave rise to term 'hairy root'. The first directed transformation of higher plants using *A. rhizogenes* was made by Ackermann (1977).

The interaction between *A. rhizogenes* and plants involves a complex series of events, the temporal sequence of which is defined by cellular activities of the interacting partners like that of processes involved with related species *A. tumefaciens*.

Agrobacterium recognizes some signal molecules in the form of various phenolic compounds released by wounded plant cells such as acetosyringone and α-hydroxy acetosyringone and become attached to them (chemotactic response). After bacterial colonization and attachment to plant cells at or near wound site, the infection leads to insertion of T-DNA fragments of the T_i-plasmid (tumor inducing plasmid of *A. tumefaciens*) or R_i-plasmid (root inducing plasmid of *A. rhizogenes*) to the plant cells. Genes of T-DNA fragment mediate the formation of neoplastic crown gall tumor and hairy root tissues, followed by the synthesis of sugar and amino acid conjugates known as 'opines' which are used by the invading bacteria as a source of carbon and nitrogen (Binns and Thomashow 1988). Genes encoded in T-DNA have eukaryotic regulatory sequences, enabling their expression in infected plant cells. The transformation events are triggered by *vir* genes located in a 40-kbp region of Ri-plasmid called the virulence (vir) region. The *vir* genes are only expressed in the presence of acetosyringone. Various sugars also act synergistically with acetosyringone to induce high level of *vir* gene expression. Finally the expression of T-DNA genes coding for auxin synthesis and other rhizogenic functions results in root formation at the infection site of host plant.

Most *Agrobacterium* strains contain only one type T-DNA, but some (like those carrying agropine type R_i-plasmids) transfer two independent T-DNA denoted as T_L-DNA (left handed T-DNA) and T_R (right handed T-DNA). T_R-DNA has high homology to the T-DNA of the T_i-plasmid of *A. tumefaciens* while, T_L-DNA is

strikingly different and has homology to the T-DNA carried by the R_i-plasmid of mannopine *A. rhizogenes* strains (Nilsson and Olsson 1997). Both T_L-DNA and T_R-DNA are transferred and integrated independently into the host plant genome. Previously, it was assumed that the synthesis of auxin can be ascribed to the T_R-DNA, but the genes of T_L-DNA direct the synthesis of a substance that induces the cells to differentiate into roots under the influence of endogenous auxin synthesis (Ooms et al. 1986; Shen et al. 1988). But now it is clear that, the transfer of T_L-DNA is essential for induction of hairy root syndrome, and transfer of T_R-DNA does not provoke formation of roots from transformed cultures (Nilsson and Olsson 1997; Sevon and Oksman-Caldentey 2002). T_R-DNA contains two genes (*iaa*M and *iaa*H) responsible for the biosynthesis of auxin and genes are responsible for the synthesis of theopines, mannopine (*mas1′* and *mas2′*) and agropine (*ags*). T_L-DNA carries 18 open reading frames (ORF), four of which are essential for hairy root formation; ORF10, ORF11, ORF12 and ORF15 are corresponding to gene *rol*A, *rol*B, *rol*C and *rol*D respectively. The *rol*B gene is absolutely essential for hairy root induction. Even when expressed alone, the *rol*B gene can induce significant hairy root production (Nilsson and Olsson 1997). Conformation that a plant cell is transformed can be obtained by transformed root morphology exhibited by hairy root cultures and their transformed regenerants. The hairy roots have altered phenotype and these roots show high degree of lateral branching, profusion of root hairs and lack of geotropism.

Different strains of *A. rhizogenes* vary in their transforming ability (Kumar et al. 1991; Giri et al. 1997). Hairy roots obtained by various infections with different bacterial strains exhibit different morphologies. The differences in virulence and morphology can be explained by the different plasmid harbored by the strain (Nguyen et al. 1992). [(*Detailed information regarding the mechanism involved in gene transfer can be found in the report of* Gelvin (2000))].

According to opine synthesized by hairy roots and utilized by bacterium, *A. rhizogenes* strains were grouped into two main classes (Petit et al. 1983):

- **Agropine-type strains** (**e.g.**, **A4**, **15834**, **HR1**, **LBA 9402**)-which induce roots to produce agropine, mannopine and corresponding acids.
- **Mannopine-type strains** (**e.g.**, **8196**, **TR7**, **TR101**)-which elicit roots containing only mannopine, mannopinic acid and agropinic acid.

While, Zhou et al. (1997) classified the strains of *A. rhizogenes* into five classes: octopine, agropine, nopaline, mannopine, cucumopine.

3.2.2 Establishment of Hairy Root Culture

For successful establishment of hairy root culture system for a certain plant species, several essential conditions should be taken into consideration. These conditions include the selection of best bacterial strain of *A. rhizogenes*, an appropriate explants, a proper antibiotic to eliminate redundant bacteria after co-cultivation and a suitable culture medium. Amongst, Agropine are most often used strains owing to their

strongest virulence. 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 (Giri et al. 2001; Krolicka et al. 2001; Azlan et al. 2002; Sevon and Oksman-Caldentey 2002). However, for different species, the proper explants material may vary and the age of the explants is most critical, generally juvenile material being optimal.

To induce hairy roots, explants are separately wounded and infected with *A. rhizogenes* strain either by direct inoculation with a thick, viable bacterial suspensions and incubation on a solid medium or by co-cultivation in liquid medium. Two or three days later, the infected explants are subsequently transferred to a solid medium with antibiotics, such as cefotaxime sodium, carbencilin disodium, vanocomycin, ampicilicin sodium, claforan, streptomycin sulphate or tetracycline, ranging in concentration from 100 to 500 µg/mL, generally for 3 days to kill or eliminate redundant bacteria (Giri et al. 2001; Krolicka et al. 2001; Pavlov et al. 2002a, b; Rahman et al. 2004). The neoplastic hairy roots will be emerged at the site of infection within a short period of time, which varies from 1 week to over a month depending on different plant species. Thereafter, roots are individually cut off and subculture to a hormone-free nutrient medium e.g., MS (Murashige and Skoog 1962) or B_5 (Gamborg et al. 1968) where they grow in a profusely branch manner with abundant lateral branches. The whole process of hairy root induction can be explained by Fig. 3.1. Successful genetic transformation can be demonstrated in either of two ways, directly or indirectly detecting T-DNA or opine respectively. The direct way is preferred, as in some cases opine production is not stable and may even cease (Sevon and Oksman-Caldentey 2002). To detect T-DNA, either polymerase chain reactions (PCR) (Le Flem-Bonhomme et al. 2004; Palazon et al. 2003) or Southern blot hybridization (Nin et al. 1997; Xie et al. 2001) can be used.

3.2.3 SAAT: A New Approach of Transformation

Recently, a new technique named sonication-assisted *Agrobacterium*-mediated transformation (SAAT) has also been developed to induce hairy roots in those plant species which are difficult to transform. Trick and Finer (1997) observed that SAAT treatment produces small, uniform fissures and channels in tissues of various plants which facilitate the access of *A. rhizogenes* to the internal plant tissues. Recently, this technique was successfully used by Le Flem-Bonhomme et al. (2004) for transforming the hypocotyls of *Papaver*.

3.2.4 Characteristics of Hairy Root Culture

Hairy roots have various attractive properties for secondary metabolite production like:

- Roots are plagiotropic (grow away from the vertical) and neoplastic (cancerous) in nature, therefore do not require external supply of growth hormones. The plagiotropic characteristic is advantageous; as it increases the aeration in liquid medium and thereby leading an elevated accumulation of biomass.

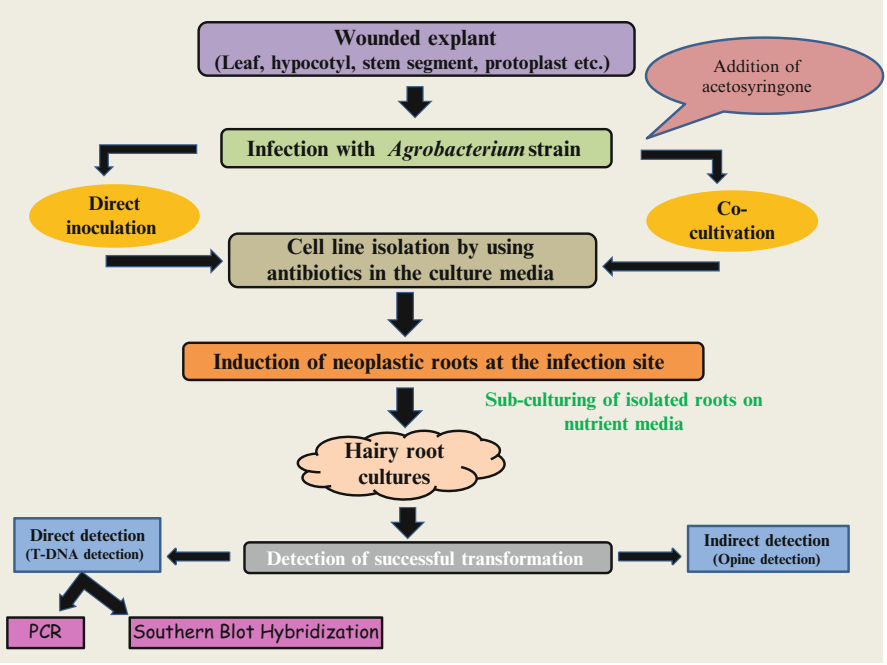

Fig. 3.1 Establishment of hairy root culture

- They often exhibit about the same or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Banerjee et al. 1998; Kittipongpatana et al. 1998).
- Genetic stability is another important characteristic of hairy roots. For example, hairy root culture of *Hyoscyamus muticus* showed equal or higher levels of hyoscyamine synthesis compared to the roots of a whole plant (Flores and Filner 1985) and maintained the same biosynthetic capacity for more than 15 years (Flores et al. 1999).
- Various useful products are only synthesized and accumulated in organized in vitro tissue (root), but not formed in suspension or callus culture of shoots and leaves; in such a critical situation hairy root culture is the only approach to obtain these useful chemicals at commercial level.
- In nature certain metabolites are only produced in aerial parts not in roots. Hairy root culture is found to be fruitful for extraction of such chemicals. For example, accumulation of lawsone, a napthoquinone derivative, is restricted to the aerial parts of wild-type henna (*Lawsonia inermis*); however, in hairy roots cultures, lawsone has been found in significant quantities in (Bakkali et al. 1997). Similarly, Artemisinin was successfully produced in hairy root culture (Weathers et al. 1997; Jaziri et al. 1995; Liu et al. 1999) which was previously thought to accumulate only in the aerial parts of *Artemisia annua* plant (Wallaart et al. 1999).

3.3 Hairy Root Culture: 'Productive Vehicle' for Secondary Metabolites

The high biosynthetic potential of hairy root culture was largely neglected for years and the investigations that were performed mainly focused on the mechanism of hairy root syndrome. However, the investigations of mid-1980s and early-1990s on the production of biologically active substances, especially alkaloids revealed the potential of transformed root systems. Now, hairy root culture technique is being interestingly adapted as a new research line for the production of bioactive compound. With several attractive features, the production of more than a specific compound synthesis, it acquires a great commercial importance (Fig. 3.2). Many medicinal plants have been transformed successfully by *A. rhizogenes* for enhanced production of secondary metabolites (Table 3.2). Following approaches are being adapted for enhanced secondary metabolite production through hairy root culture:

3.3.1 Increased Biomass Yield

Some secondary metabolites are growth-associated (Bhadra and Shanks 1997; Bhadra et al. 1998). Thus, the manipulation of extracellular environment to improve the growth has been used as a strategy to increase the productivity of valuable metabolites. The culture medium, particularly its nutrient content has a major impact on hairy root growth; it has become a target for maximizing phytochemical accumulation in hairy root cultures (Condori et al. 2010; Shinde et al. 2010). In a study on *Plumbago zeylanica*, MS was found to be best for hairy root growth in dark condition as compared to other nutrient media (B5 and SH) tested (Sivanesan and Jeong 2009). However, the medium composition being modified often with respect to its concentration of carbon, nitrogen and phosphorous sources (Wilhelmson et al. 2006) and other macronutrients (Sivakumar et al. 2005). A general approach for media optimization is to use statistical analysis (Sung and Huang 2000; Wilhelmson et al. 2006). In the first stage of this approach, the components of the nutrient media (independent variables) are varied, and the resulting culture growth and biosynthesis parameters of the desired metabolites are determined. Multivariate analysis is then applied to explore the interactive 'nutrient media-biomass-product' relationships between compounds in the biological systems. In parallel to experimental testing, interdisciplinary computational approaches were also adopted to predict the optimal growth conditions for high biomass and phytochemical production. Two artificial neural network models (regression and back propagation) were applied to estimate biomass yield in a licorice (*G. glabra*) hairy root cultures derived from *A. rhizogenes* transformed leaf explants (Prakash et al. 2010). Variables including volume, pH, sugar content of the culture medium, and the density of the inoculums were tested by both models for fresh biomass production. The robustness of the models was verified by experimentally obtained data. More accurate results were found using the regression neural network as compared to the back propagation

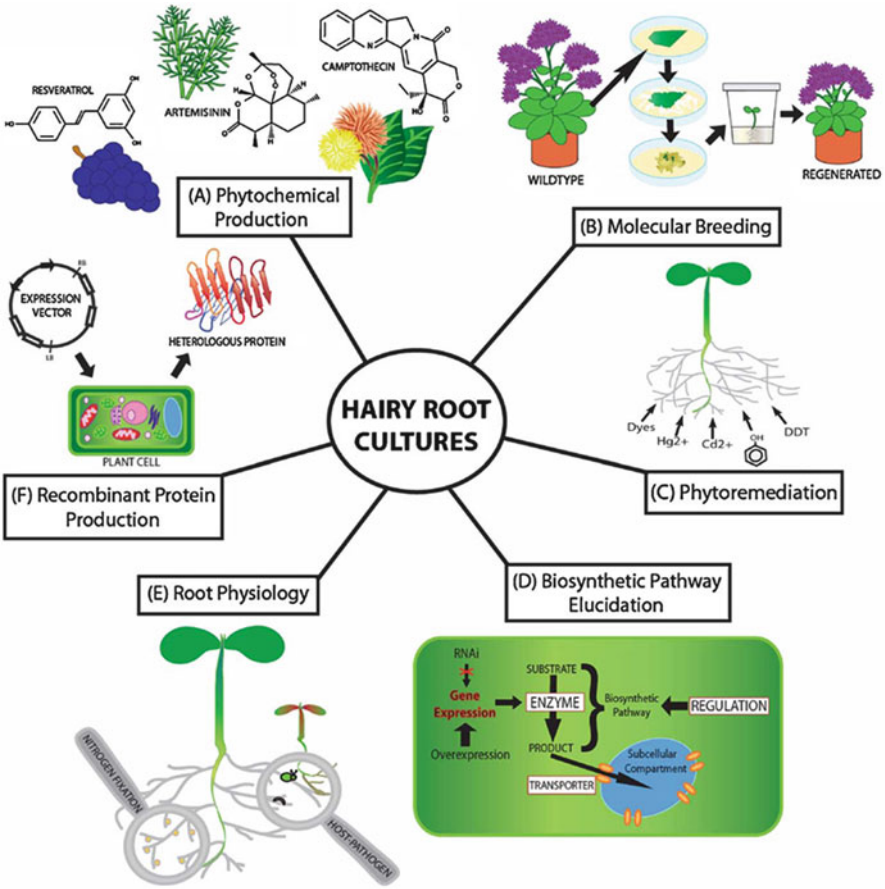

Fig. 3.2 The diverse and abundant uses of hairy root cultures. (**a**) Phytochemical production in hairy roots is a major topic of study that spans several classes of phytochemicals, including alkaloids, terpenoids, and phenolics. (**b**) Molecular breeding by infection of ornamental plants with *Agrobacterium rhizogenes* and regeneration of whole plants from hairy roots yields plants with desirable phenotypes, such as compact size, for horticultural purposes. (**c**) Hairy root culture has been used as a model system for studying Phytoremediation of toxic substances and reactive dyes. (**d**) Molecular, biochemical and genetic studies in hairy roots have accelerated Biosynthetic pathway elucidation for phytochemicals, which, in turn, facilitates metabolic engineering in hairy root cultures. (**e**) Root physiology studies ranging from nitrogen fixation, iron-deficiency, aluminum toxicity, to host–pathogen interactions have been conducted in hairy root cultures. (**f**) Recombinant protein production in this system has been explored as a rapid, contained, low-cost, genetically stable means of producing human antibodies, cytokines, and other protein therapeutics (Taken from the report of Ono and Tian (2011). With permission)

neural network, presumably due to the better learning potential of the regression neural network (Prakash et al. 2010). In another modeling study, mathematical equations were proposed to predict the growth of hairy roots in relation to nutrient distribution in the medium and within dense hairy root networks (Bastian et al. 2008).

Table 3.2 List of secondary metabolites production through hairy root culture

Plant	Family	Metabolite	Medicinal importance/effective against	References
Ammi majus	Apiaceae	Xanthotoxin (furococumarin)	Leucoderma	Krolicka et al. (2001)
Ophiorrhiza pumila	Rubiaceae	Camptothecin	Antitumor, AIDS, falciparum malaria	Sato et al. (2001)
Solanum aviculare	Solanaceae	Solasodine	Used as a base material for the production of steroid contraceptives	Koehle et al. (2002)
Glycyrrhiza pallidiflora	Fabaceae	Flavonoids	Meant for the treatment of gastric ulcers, anti-inflammatory and anti-tussive	Li et al. (2002)
Beta vulgaris	Chenopidiaceae	Betalains	Strong aphrodisiac, laxative	Pavlov et al. (2002a, b)
Catharanthus roseus	Apocynaceae	Indole alkaloids (vinblastine, vincristine (in aerial part)) Ajmalicine, serpentine and reserpine (roots)	Anti-cancerous	Ayora-Talavera et al. (2002)
Physalis minima	Solanaceae	Physalins	Diuretic, febrifuge, vermifuge	Azlan et al. (2002)
Datura metel; *Hyoscyamus muticus*	Solanaceae	Tropane alkaloids	Nacrotic, anti-cholinergic and anti-spasmodic activity	Moyano et al. (2003)
Papaver somniferum	Papaveraceae	Benzylisoquinoline alkaloids (Morphinan, codeine and sanguinarine)	Analgesic; antibiotic	Park and Facchini (2000), Le Bonhomme et al. (2004)
Rauvolfia micrantha	Apocyanaceae	Ajmalicine, ajmaline	Antihypertensive	Sudha et al. (2003)
Hyoscyamus niger	Solanaceae	Tropane alkaloids	Nacrotic, anti-cholinergic and anti-spasmodic activity	Zhang et al. (2004)

(continued)

Table 3.2 (continued)

Plant	Family	Metabolite	Medicinal importance/effective against	References
Gmelina arborea	Lamiaceae	Verbascoside	Claimed to be stomachic, galactagogue laxative and anthelmintic; improve appetite, useful in hallucination, piles, abdominal pains, burning sensations	Dhakulkar et al. (2005)
Datura innoxia	Solanaceae	Tropane alkaloids (Scopolamine and hyoscyamine)	Nacrotic, anti-cholinergic and anti-spasmodic activity	Dechaux and Boitel-Conti (2005)
Atropa belladonna	Solanaceae	Tropane alkaloid (hyoscyamine, atropine and hyoscine)	Used against Parkinson's disease	Richter et al. (2005)
Gmelina arborea	Verbenaceae	Verbascoside	Anti-inflammatory, wound healing, inhibit platelet aggregation	Dhakulkar et al. (2005)
Linum album; *Linum persicum*	Linaceae	6-methoxy-podophyllotoxin	Anticancer	Wink et al. (2005)
Artemisia annua	Asteraceae	Artemisinin	Antimalarial	Weathers et al. (2005)
Saussurea involucrata	Asteraceae	Rutin, hispidulin and syringin	Anti-inflammatory; antifungal	Fu et al. (2005)
Stizolobium hassjoo	Leguminoceae	3,4-Dihydroxyl-L-phenylalanine	Therapeutic agent against Parkinson's disease	Sung and Huang (2006)
Harpagophytum procumbens	Pedaliceae	Iridoid glycosides	Anti-inflammatory; analgesic; antidiabetic	Georgiev et al. (2006)
Arachis hypogaea	Papilionaceae	Resveratrol	Anti-inflammatory, antioxidant, anti-infective, anti-cancerous	Kim et al. (2008)
Datura stramonium	Solanaceae	Hyoscyamine	Nacrotic and anti-spasmodic activity , Used against Parkinson's disease	Pavlov et al. (2009)
Glycyrrhiza uralensis	Fabaceae	Flavonoids	Anti-mutagenic, anti-ulcer, anti-tumor, anti-microbial	Zhang et al. (2009)

Plumbago zeylanica	Plumbaginaceae	Plumbagin	Diuretic, antibacterial and used against leprosy	Sivanesan and Jeong (2009)
Fagopyrum esculentum	Polygonaceae	Rutin	Antioxidant, anti-carcinogenic, antithromobotic, cytoprotective, vasoprotective	Kim et al. (2010)
Abrus precatorious	Fabaceae	Glycyrrhizin	Diuretic, tonic, alexitric, anti-fertility	Dixit and Vaidya (2010)
Przewalskia tangutica	Solanaceae	Scopolamine and hyoscyamine (tropane alkaloids)	Parasympatholytic	Lan and Quan (2010)
Nepeta cataria	Labiatae	Rosmarinic acid	Astringent, antioxidant, anti-inflammatory, antimutagenic, antimicrobial, antiviral	Yang (2010)

Effect of Cytokinins and auxins on growth and morphogenesis of hairy roots indicates that auxins play an important role in hairy root growth. The sensitivity of hairy root tips to exogenous auxin was found to be 100–1,000 times higher than that of untransformed material (Ohkawa et al. 1989). To enhance growth and rosmarinic acid production in *Nepeta cataria*, hairy root cultures were grown for 15 days in media supplemented with various concentrations of auxins (IAA, IBA, and NAA). Cultures treated with IBA induced maximum biomass enhancement (13.5 g/L) and production of rosmarinic acid (19.2 mg/L) (Yang 2010).

While, recent studies have shown that inoculum size and age strongly influenced the growth of *Panax ginseng* hairy root cultures (Jeong et al. 2004) and betalains production form *Beta vulgaris* hairy root cultures (Pavlov et al. 2003). Jeong et al. (2004) found that growth rate of *P. ginseng* hairy roots was maximum when a 0.7 % (w/v) inoculum was used and significantly lowered with 0.4 % (w/v) inoculum. The optimal duration of subculture cycle was found to be 10 days for *P. ginseng* (Jeong et al. 2004) and 14 days for hairy roots of *B. vulgaris* (Pavlov et al. 2003).

In general, artificial polyploidy enhances the vigor of determinate plant parts and may be favorable where vegetative organs and biomass constitute the economic product. Recently, artificial polyploidy has been considered as a potential method for increased secondary metabolite production (Lavania 2005). When analyzing the alkaloid profiles, similar types of major metabolites were detected in hairy root cultures induced from diploid and tetraploid *Datura stramonium* plants (Pavlov et al. 2009). However, the concentration of compounds significantly enhanced in tetraploid-derived hairy root cultures as compared to diploid-derived hairy root cultures. In this study, the hairy root cells underwent endo-reduplication and a large fraction of the nuclei contained double the number of chromosomes of the parental cells (Pavlov et al. 2009). Similarly, colchicine induced stable tetraploid hairy root clones of *Artemisia annua* showed major differences in growth and development compared to diploid clones. Artimisinin yields of these tetraploid clones were 2–5 times higher than that of the diploids (De Jesus-Gonzalez and Weathers 2003).

There are so many reports showing increased metabolite production through biomass enhancement of hairy root cultures. In a preliminary study on hairy root induction in *Gmelina arborea*, about sevenfold biomass increment was achieved at the end of 4 weeks as compared to non-transformed seedling roots and suggesting the potential ability of hairy roots to synthesize verbascoside, a phenylpropanoid glycoside of medicinal value (Dhakulkar et al. 2005). In *Saussurea involucrata*, increased biomass yield of hairy roots (66.7 g/L fresh weight) and flavonoid (102.3 mg/g dry weight) were achieved after 20 days of incubation (Fu et al. 2005). Enhanced scopolamine (0.68 mg/g dry weight) and hyoscyamine (1.13 mg/g dry weight) production as compared to wild type roots was reported through hairy root culture of *Przewalskia tangutica*. It was the first time that hairy root cultures of *P. tangutica* were established to produce tropane alkaloids (Lan and Quan 2010). Another study on *A. rhizogenes*-mediated transformation of *Abrus precatorious* showed significant increment (5.25 times) in fresh weight of hairy roots from initial fresh weight. A maximum of 700 mg of glycyrrhizin was obtained from 20 g roots of field grown plant of *A. precatorious* giving 35 mg/g dry weight concentration

of glycyrrizin (Dixit and Vaidya 2010). Kim et al. (2010) propagated *Fagopyrum esculentum* hairy roots for enhanced production of rutin, an important flavonol glycoside. The biomass of hairy roots (12.6 g dry weight L^{-1}) was around 2.4 times more than that of wild-type roots (5.3 g dry weight L^{-1}). The content of rutin in hairy roots was found to 1.3 mg/g dry weight which was 2.6 times more than that of wild-type roots where the amount of rutin was 0.5 mg/g dry weight.

Le Flem-Bonhomme et al. (2004) have been established hairy root cultures of *Papaver somniferum*, a natural source of morphinan, codeine and sanguinarine alkaloids. The total alkaloid content was higher in transformed roots (0.46 % dry weight) than non-transformed roots (0.32 % dry weight). The transformed roots accumulated 3-times more codeine (0.18 % dry weight) than intact roots (0.05 % dry weight). Morphine (0.255 % dry weight) and sanguinarine (0.014 % dry weight) were found in the liquid culture medium. While, Kim et al. (2008) studied five different strains differing in their ability to induce peanut (*Arachis hypogaea*) hairy roots and also showed varying effects on the growth and resveratrol production in hairy root cultures. *A. rhizogenes* R1601 is the most effective strain for the induction (75.8 %), growth (7.6 g/L) and resveratrol production (1.5 mg/g) in hairy root of peanut.

3.3.2 Elicitation and Precursor Feeding

Stimulation of biosynthetic activity using elicitation and precursor feeding is the most studied approach to optimize product accumulation in plant cell cultures. Elicitation strategies are compounds or treatments that induce plants to synthesize phytoalexins at elevated levels. Since little is known of the biosynthetic pathways of most secondary metabolites in plants, the effect of elicitation on a plant cell/tissue culture cannot be easily predicted. Therefore, elicitation approaches are performed by trial and error. The effect of elicitors depends on many factors, such as, the concentration of elicitor, the growth stage of culture at the time of elicitation and contact time of elicitation. Both biotic and abiotic elicitors can be used to stimulate secondary metabolite biosynthesis in plant cell/tissue culture, thereby reducing the processing time necessary for high product yields. Elicitors of non-biological origin (abiotic elicitors), such as heavy metals and ultraviolet light, which induce phytoalexin synthesis, are actually designated as abiotic stresses (Lu et al. 2001; Ramachandra and Ravishankar 2002). Secondary metabolite production through elicitation will be discussed in detail in following chapter of '**Elicitation**'.

3.3.3 Over-Expression of Foreign Gene

Over-expression of multiple biosynthetic genes or transcription factors that control the expression of enzymes in pathways targeted by bioengineering is a promising

strategy to improve accumulation of certain secondary metabolites by enhancing rate-limiting steps or by blocking competitive pathways. In this regard, *Agrobacterium*-mediated transformation provides a rapid and simple means. Several studies reveal the key role of *pmt* (putrescine *N*-methyl transferase) gene in tropane alkaloid biosynthesis and there have many attempts to increase the scopolamine production by over-expressing *pmt* gene. In most cases, the plant material has been transformed with this heterlogous gene (*pmt*) from tobacco, under the control of CaMV 35-S promoter, with the advantages of no feedback inhibition by downstream products and a high affinity for the substrate (Zhang et al. 2005). The *pmt* over-expressing plants of *Atropa belladonna* and *Nicotiana sylvestris* have already been produced by Sato et al. (2001). No changes were observed in *Atropa* alkaloid content, while the nicotine content in *N. sylvestris* leaves increased significantly. Similar behavior has been expressed by engineered roots of *Hyoscyamus muticus* and *Datura metel* (Moyano et al. 2003). However, in both species the over-expression of *pmt* gene from tobacco increased the hyoscyamine content, but the production of scopolamine improved significantly only in *D. metel*, in *Hyoscyamus* tropane alkaloid level remained similar to that of wild type hairy roots. As already mentioned, in *Atropa belladonna* over-expression of *pmt* gene only increased the accumulation of the direct metabolite *N*-methyl putrescine (Rothe et al. 2001) while the effect on total alkaloid level was marginal. Similar to *pmt* gene, engineered *A. belladonna* root lines with strong over-expression of *tr* gene (tropane reductase) from *D. stramonium* showed more enzyme activity of the respective reductase and a high level of the enzyme products, tropane and pseudotropine (Richter et al. 2005).

Researchers have worked on genetic engineering of pharmaceutically important tropane alkaloids, in which the conversion of hyoscyamine to much more valuable alkaloid, scopolamine is the major goal. A rough correlation has been found between H6H (hyoscyamine 6-β-hydroxylase) activity and the ratio of scopolamine to hyoscyamine in scopolamine producing cultured roots (Oksman-Caldentey and Strauss 1986). H6H, therefore, is a promising target enzyme that, if over expressed in hyoscyamine accumulating tissues, would result in increased scopolamine levels in transgenic plants or roots. In this way, several unattractive hyoscyamine-rich, but scopolamine-poor plants may now become promising candidates for large scale scopolamine production by means of cultured roots.

The first successfully altered plant by metabolic engineering was *Atropa belladonna* (Yun et al. 1992). The *h6h* gene from *H. niger* was over-expressed in the target plant and the progenies of single primary transformed plant showed elevated scopolamine contents, resulting in near to complete conversion of hyoscyamine to scopolamine in the mature plants. Similar results were achieved with *H. muticus* hairy roots over-expressing the same gene, in which the best transgenic clone had a 100-fold enhancement of scopolamine, while hyoscyamine content remained same (Jouhikainen et al. 1999). Zhang et al. (2004) reported the simultaneous introduction and over-expression of genes encoding the rate-limiting upstream enzyme PMT and H6H of scoplolamine biosynthesis. The best line produced 411 mg/L scopolamine which was over nine times more than that in the wild-type (43 mg/L) and more than twice the amount in the highest scopolamine producing *h6h* single-gene transgene line (184 mg/L).

They concluded that the pulling force of the downstream enzyme, H6H plays a more important role in stimulating scopolamine accumulation in *H. niger*, whereas the functioning of the upstream enzyme PMT increased proportionally.

Similar to tropane alkaloids, the regulation of flavonoid biosynthesis together with indole alkaloids is quite well understood in comparison with other secondary metabolites and a lot of transgenic materials over-expressing *chi* (chalcone isomerase) and *chs* (chalcone synthase) were constructed (Cain et al. 1997; Shul'ts et al. 2000; Li et al. 2002; Ralston et al. 2005). Zhang et al. (2009) observed enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* by combining the over-expression of *chi* gene with the elicitation treatment and reported a maximum of 2.838/100 g dry weight of total flavonoids than 0.842/100 g dry weight in wild-type hairy roots.

3.4 Other Applications

3.4.1 Hairy Root Culture for Molecular Farming: Expression of Foreign Proteins

However, plants are the potential source of industrial and therapeutic proteins, but the extraction and purification of complex proteins from plant tissues require a laborious and costly method. Thus, the possibility to express functional animal proteins in hairy roots makes this plant material attractive for molecular farming, with several advantages over field-cultured plants. These advantages are mainly based on the fact that hairy roots are cultured in confined recipient, avoiding transgene or pharmacologically active protein dissemination in the environment, and in controlled conditions of growth, avoiding pollution of the produced proteins. Moreover, animal proteins expressed in hairy roots are often secreted in the culture medium which provides an easy way of their extraction than intact plant cells. The proteins, those can be produced by hairy roots include medicinally important enzymes such as superoxide dismutase, peroxidase, phytase (Kim and Yoo 1996; Hyon and Yoo 2002; Jin et al. 2005), foreign proteins such as monoclonal antibodies (Sharp and Doarn 2001), the human secreted alkaline phosphatase (Gaume et al. 2003) and ribosome-inactivating proteins (Thorup et al. 1994). Three genes from *Ralstonia eutropha*, a type of bacteria necessary for polyhydroxybutyrate (PHB) synthesis, were introduced into the hairy roots of sugar beet (Menzel et al. 2003). It was observed that the 20 transgenic hairy root clones produced up to 55 mg high molecular PHB per gram dry weight. Wongsamuth and Doran (1997) reported the production of monoclonal antibodies by hairy roots. They initiated hairy roots from transgenic tobacco plants expressing a full-length I_g G monoclonal antibody and tested the long-term stability of antibody expression in hairy roots, variation between clones, the time course of antibody accumulation in batch culture and the effect of different factors on antibody accumulation and secretion. Antibody degradation in the medium was

a significant problem however, affecting the final titers and this remains to be resolved. Later on, Sharp and Doran (2001) reported that murine I_g G1 production in hairy roots of tobacco and improved the accumulation of the antibody by increasing the dissolved oxygen tension to 150 % air saturation. While, the non-toxic lectin subunit ricin B, fused to GFP, was expressed in tobacco hairy roots and secreted in the culture medium (Medina-Bolivar et al. 2003). This fusion protein was tested in mouse as an antigen, showing that protein fused to ricin B (a mucosal adjuvant in mammalian immune responses) can be efficiently produced by hairy roots. Recently, Doran (2006) reviewed the current status and problems associated with the production of foreign protein by hairy root cultures (such as low accumulation levels, instability in the culture medium etc.) and outlined strategies to minimize their degradation and losses. Although some of these problems have not yet been fully resolved, there is a little doubt that these application will continue to expand in the future.

3.4.2 Functional Analysis of Genes

Several recent reports highlight the important contribution of hairy root cultures to identification of biosynthesis and regulatory genes as well as transporter. For example, the stress hormone methyl jasmonate (MeJa) which often as a role as secondary messenger in elicitor transduction pathways, is also efficient in inducing or increasing the production of valuable secondary metabolites in hairy root cultures (Palazon et al. 2003; Yaoya et al. 2004; Komaraiah et al. 2003; Nakanishi et al. 2005). This inducible system was also used to discover unsown genes involved in the metabolite pathways. Such a strategy was also recently applied to *Catharnathus roseus* cell cultures and made possible the definition of a gene-to-metabolite network (Rischer et al. 2006). Similarly, the treatment of ginseng hairy roots with MeJa enabled the identification and the study of 3134 expressed sequence tags (ESTs) (Choi et al. 2005). By this means, it was possible to characterize several genes encoding enzymes such as squalene synthatse, squalene epoxydase, oxidosqualene cyclase, cytochrome P450 and glycosyltransferase, all of which are involved in the biosynthesis of the triterpene glycoside ginsenosides (Choi et al. 2005). Such studies are needed to gain information and new tools to design metabolite engineering strategies. Owing to their capacity for fast growth in vitro and the ease of being elicited, the hairy root cultures will be increasingly used for such studies in non-model plants, including medicinal plants.

Another powerful technique for identifying new gene functions is the T-DNA activation tagging. It consists of random integration of a T-DNA carrying a constitutive enhancer promoter element (often the cauliflower mosaic virus 35S enhancer element) into the plant genome. When this enhancer element integrates near to a gene, it will increase the expression of this gene and give rise to a gain of functional mutant. This strategy has been successfully applied to a number of plant species such as, *Arabidopsis thalina*, *Solanum tuberosum* and *Nicotiana tobaccum* (Seki et al. 2005). To facilitate the application of a forward genetcics

approach for gene discovery in hairy root cultures, binary vectors were designed and constructed specifically for activation tagging in hairy roots. T-DNA activation-tag technology can be applied to plant recalcitrant for regeneration (e.g., tree species), to characterize new genes important for the root biology, including those involved in biotic and abiotic stress resistance, developed or in the regulation of biosynthesis pathways. AtTT2 is a MYB transcription factor that controls expression of several proanthocyanidins and flavonoid biosynthesis genes (Nesi et al. 2001). Microarray analysis of genes induced in *Medicago truncatula*. Hairy root cultures over-expressing AtTT2 led to cloning of UGT72L1, a glycosyltransferase with specificity for (−)-epicatechin (a proanthocyanidin biosynthesis precursor) (Pang et al. 2008). The expression of UGT72L1 was associated with the accumulation of proanthocyanidins and epicatechin glycosides in developing *M. truncatula* seeds (Pang et al. 2008).

Over-expression studies in hairy root cultures can be complimented by employing RNA interference (RNAi) to knockdown gens of interest. Two back-to-back publications described the use of an RNAi approach in tobacco hairy root cultures (*Nicotiana tobaccum* and *N. glauca* respectively) to elucidate the role of NgA662, a NADPH-dependent reductase, in pyridine alkaloid biosynthesis in tobacco (Deboer et al. 2009; Kajikawa et al. 2009).

While, Kumagi and Kouchi (2003) studied transgenic lines of *Lotus japonicus* that express GUS by constitutive or nodule-specific promoters. *L. japonicus* were super transformed by infection with *A. rhizogenes* containing gene constructs for the expression of hairpin RNAs (hp RNAs) 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 roots transformation provides a powerful tool for loss-of-function analysis of genes that are expressed in roots (Kumagi and Kouchi 2003).

3.4.3 Regeneration of Whole Plants

Hairy roots are able to whole plant regeneration in several plant species. Generally, these transgenic plants are genetically stable. However, in some cases, transgenic plants have shown an altered phenotype compared to controls. These plants display 'hairy root syndrome' due to combined expression of the *rol*A, B and C loci of the R_i plasmid. Each locus is responsible for a typical phenotypic alternation, i.e., *rol*A is associated with internodes shortening and leaf wrinkling, *rol*B is responsible for protruding stigmas and reduced length of stamens, *rol*C responsible for internode shortening and reduced apical dominance. Some of the altered phenotypes have proven to be useful in plant breeding programs. Dwarfing, altered flowering, wrinkled leaves, increased branching due to reduced apical dominance may also be useful for ornamentals. Dwarf phenotype is an important characteristic for flower crops such as *Eustoma grandiflorum* and *Dianthus* (Giovanni et al. 1997). Transformed roots can also 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-dichlorophenoxy acetic acid (2, 4-D), the hairy roots of *Astragalus siniensis* developed somatic embryos.

3.4.4 Phytoremediation

Phytoremediation is an emerging technology that uses green plants to remove, accumulate or otherwise render metals or organic contaminants present in soils and ground-waters benign. In this technology, plant roots are central to the remediation action. Hairy root cultures have been a valuable model root system to elucidate the transformation processes and fate of the contaminants without interference from microbes. Hairy root cultures of plant hyper accumulators are being used to study heavy metal uptake. Removal of polychlorinated biphenyls from the culture medium has been monitored in *Solanum nigrum* hairy roots (Khas et al. 1997). Significant progress on the transformation processes and fate of the nitro aromatic explosive 2, 4, 6-trinitrotoluene (TNT) in plants has been made in mass balance and fate experiments using *Catharanthus roseus* hairy roots as a model system. TNT was shown to be reduced to mono aminio dinitrotoluenes, and then conjugated at the amine groups with at least a six-carbon unit, and finally incorporated into un-extractable 'bound' residues in the cell wall material (Bhadra et al. 1999). The occurrence of the same conjugate in a diversity of plant species (aquatic versus terrestrial) reiterated the importance of conjugation as a fate process in TNT metabolism.

3.4.5 Production of Novel Compounds

The use of hairy root cultures in biotransformation processes for the production of novel compounds has also been reported (Wilson et al. 1987; Ushiyana and Furuya 1989; Kawaguchi et al. 1990; Asada et al. 1993; Flores et al. 1994). For example, transformed hairy roots of *Scutellaria baicalensis* accumulated glucoside conjugates of flavonoids instead of the glucose conjugates accumulated in untransformed roots (Nishikawa and Ishimaru 1997). Li et al. (1998) isolated a new compound named licoagrodione from *Glycyrrhiza glabra*, which was shown to possess strong antimicrobial activities. However, other two novel isoprenylated flavonoid compounds with anti-microbial and anti-oxidant activities were detected by Asada et al. (1998) in hairy root cultures of *G. galbra*. In another interesting study, a novel benzoquinone, hydorxyechinofuran B, was found to be secreted from *Lithospermum erythrorhizon* hairy root cultures when media conditions were altered (Fukui et al. 1998). This species produces the well known red dye and antibacterial compound, shikonin, when cultivated in ammonium-ion-free liquid medium. In this study, the addition of low levels of ammonium was found to induce the synthesis of the brown compound benzoquinone. Berkov et al. (2003) reported the biosynthesis of a new tropane alkaloid ester in tetraploid hairy roots of *Datura stramonium*.

3.4.6 Germplasm Conservation

Hairy root culture can also be used for the production of artificial seeds thus, providing an effective tool for ex vitro germplasm conservation. Hairy roots in the form of artificial seeds are a reliable delivery system for clonal propagation of elite plants with genetic uniformity, high yield and low production cost. Nakashimada et al. (1995) produced artificial seeds of horseradish. In *Ajuga reptans* GUS-transformed hairy roots been used for producing artificial seeds (Uozumi 2004). While, root tips of hairy roots of *Panax ginseng* (Yoshimatsu et al. 1996) and shoot tips of hairy roots regenerants have been cryopreserved in horseradish (Phunchindawan et al. 1997).

3.5 Problems of Hairy Root Culture

Although, hairy root culture is an emerging technique of genetic transformation with a wide range of applications, but it also has some major problems still remain to be solved such as;

- Different regulation of secondary metabolism in related species (Moyano et al. 2003).
- Over-expression of key enzymes does not always improve secondary metabolism (Koehle et al. 2002).
- Co-suppression of endogenous and foreign genes (Ayora-Talavera et al. 2002).
- Silencing of transgenes (Sivakumar 2006).
- Morphological alteration of regenerated plants (Han et al. 1993).
- Possible reduction of chromosome numbers during sub-culture (Xu and Jia 1996).
- Hairy roots usually produce opine-like substances which are lethal to mammalian cells (Yoshikawa and Furuya 1987) probably this is one of the reason that GMOs are not always accepted in several countries, specially in regards no medicines containing live genetically-modified organisms have been approved for use.

3.6 Conclusion and Future Prospects

Hairy root culture 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 efficient ability of enhanced production of secondary metabolites than unorganized cells and wild-type root. Hairy root cultures induced from rare medicinal plant species can be used for regenerating whole plants, making it an alternative and complementary ex situ biodiversity conservation method to seed banks. Long-term preservation of hairy root

cultures is critical for germplasm conservation and maintaining clonal lines for high-level phytochemical or recombinant protein production. In this regard, hairy root cultures have been stored at ambient, low and sub-zero temperatures with success. Stable alkaloid production was also observed in transgenic *Catharanthus roseus* hairy roots after 5-year maintenance in liquid cultures (Peebles et al. 2009). Still, there are many serious problems in metabolite production through hairy root culture which require further attempts for their resolution. One of the biggest challenges for commercial phytochemical and recombinant therapeutic protein production in hairy root culture is the production bottleneck. Further exploration into inexpensive novel elicitors and bioreactors will aid their industrial implementation by increasing yields and driving down production costs. Further, enhancement of knowledge regarding plant metabolic pathways and the mechanisms of their regulation in the near future should give us powerful tools for exploiting the biosynthetic potential of hairy roots.

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Glossary

Agrobacterium *Agrobacterium* is a genus of Gram-negative bacteria established by H. J. Conn that uses horizontal gene transfer to cause tumors in plants. *Agrobacterium* is well known for its ability to transfer DNA between itself and plants, and for this reason it has become an important tool for genetic engineering.

Plasmid An extra-chromosomal, autonomous circular DNA molecule found in certain bacteria, capable of autonomous replication. Plasmids can transfer genes between bacteria and are important tools of transformation.

T-DNA Transferred DNA of the tumor-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. It derives its name from the fact that the bacterium transfers this DNA fragment into the host plant's nuclear DNA genome.

Hairy root A phase of crown gall (especially in apples) during which there is abnormal development of fine fibrous roots.

Secondary metabolite Organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism. Secondary metabolites often play an important role in plant defense against herbivory and other interspecies defenses. Humans use secondary metabolites as medicines, flavorings, and recreational drugs.

Phytoremediation The treatment of environmental problems (bioremediation) through the use of plants that mitigate the environmental problem without the need to excavate the contaminant material and dispose of it elsewhere.

Opines Low molecular weight compounds found in plant crown gall tumors or hairy root tumors produced by parasitic bacteria of the genus *Agrobacterium*. Opine biosynthesis is catalyzed by specific enzymes encoded by genes contained in a small segment of DNA (known as the T-DNA, for 'transfer DNA'), which is part of the Ti plasmid, inserted by the bacterium into the plant genome. The opines are used by the bacterium as an important source of nitrogen and energy. Each strain of *Agrobacterium* induces and catabolizes a specific set of opines.

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