Hairy Root Cultures as an Alternative Source for the Production of High-Value Secondary Metabolites

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M. S. Akhtar, M. K. Swamy (eds.), *Natural Bio-active Compounds*,
https://doi.org/10.1007/978-981-13-7438-8_10

Abstract

Hairy roots are rapidly growing, highly differentiated transformed root cultures induced by *Agrobacterium rhizogenes* infection usually at the infected site of the representative medicinal plant. Hairy roots have the ability to rapidly multiply in the culture medium devoid of any hormones. Unlike other plant cell cultures, hairy root cultures are genetically and biochemically stable and produce a variety of secondary metabolites. In the past three decades, researchers across the world have successfully initiated and cultured hairy roots in vitro for a large number of medicinal plants. Hairy root technology is becoming a promising source for the production of pharmaceutically and industrially important secondary metabolites. This is due to the characteristics of hairy roots, such as rapid growth, the lack of geotropism, extensive lateral branching, and, more importantly, genetic stability. This chapter explores the applications of secondary metabolites in drug formulation, cosmetic preparation, food processing, and the study of plant metabolic pathways. It also briefs about the recent advancements in the area of hairy root culture involving other biotechnological approaches like metabolic engineering or genetic engineering, elicitation, metabolic trapping, and phytoremediation. This chapter certainly benefits the researchers to further explore on the applications of hairy root culturing technology to produce desired plant secondary metabolites on a large scale.

Keywords

Agrobacterium rhizogenes · Genetic engineering · Hairy roots · Medicinal plants · Secondary metabolites

10.1 Introduction

Medicinal plants produce a variety of biologically active compounds, i.e., secondary metabolites which play a vital role in plant self-defense mechanisms. Especially, roots play major roles in plants, including anchoring plants to the soil, uptake of minerals and water from the soil, storage of nutrients in perennial plants, and defending themselves from other plants or microbes present in the soil by producing a wide variety of chemical compounds, popularly known as secondary metabolites. These secreted metabolites not only provide protection to plants from biotic and abiotic stresses like pathogens, insects, and other environmental stresses but also useful in improving human's and other animal's health (Tian 2015). These compounds are produced in trace amounts during the secondary metabolism, but not essentially necessary for plant growth and development. Plant-based compounds, including alkaloids, flavonoids, saponins, terpenes, anthraquinones, and anthocyanins, are the essential source for the preparation of drugs, food additives, dyes, oils, resins, and agricultural chemicals (Kim et al. 2002; Zhou et al. 2011; Bharati and Bansal 2014). Obtaining the chemical compounds directly from the wild- or field-grown plants is not promising as the yield obtainable is being very low and has limited availability in their habitat. Moreover, it may lead to the

destruction of the natural habitat due to over exploitation of these plants. The artificial synthesis of chemical compounds also has several disadvantages including high cost of production, the difficulties in the synthesis, unavailability of the optimized methods for the compound synthesis, and characterization. These problems can be overcome by the using the biotechnological approaches such as plant tissue culture, transgenic medicinal plants, etc. to enhance the synthesis of valuable phytochemicals from medicinal plants (Zhou et al. 2011). In this regard, the hairy root technology is widely preferred by biotechnologists for the large-scale production of diverse secondary metabolites from various medicinal plant resources (Veena and Taylor 2007).

Hairy roots are the by-products from the *Agrobacterium rhizogenes* (gram negative, soil bacterium)-infected sites, commonly known as hairy root disease or syndrome. This soil bacterium transfers its T-DNA segment from Ri (root-inducing) plasmid into the host plant genome. The T-DNA region contains a set of genes encoding for the specific enzymes, which control the biosynthesis of natural auxins and cytokinins. The new changes, i.e., insertion of new genes, cause hormonal imbalance in the host plant and induce the formation of proliferating roots (hairy roots) from the wounded sites infected with *A. rhizogenes* (Guillon 2006). Hairy roots are characterized by the abnormal multiplication on the phytohormone free medium by retaining genetic stability. Hairy roots have several unique properties including fast growth rate, able to accumulate vast variety of chemical compounds, no requirement of exogenous hormone in the medium, and genetic and biochemical stability (Giri and Narasu 2000). The schematic representation of hairy root induction and its application is shown in Fig. 10.1. Nowadays, many research groups are paying attention toward in vitro culturing of hairy roots for producing wide varieties of root-oriented plant secondary metabolites. Recent advancements have provided a better understanding about the molecular mechanisms involved in the T-DNA transfer and their integration into the host plant genome. This has paved a new way for producing plant secondary metabolites through employing metabolic engineering strategies. Also, hairy roots have shown the capability of absorbing some of the threatening recalcitrant pollutants and thus can be used to clean the environment (phytoremediation). In this chapter, detailed information about hairy roots and their applications in the production of valuable plant secondary metabolites are discussed. Further, more recent advances in the field of hairy root culture technology are highlighted.

10.2 Production of Secondary Metabolites Through Hairy Root Cultures

From several decades to now, worldwide population is still depending on plants and plant-derived products for their daily needs. Even today, around 80% of the human population depends on plants as a traditional medicine to cure several diseases (Ekor 2014; Swamy et al. 2016). Terrestrial plants are the greatest source for several chemical compounds with wide-ranging pharmaceutical applications. As these compounds occur in trace amounts in plants, they generally do not meet the huge demand in the pharmaceutical industry. Hence, this has raised a curiosity among researchers to make use of biotechnological approaches to commercially produce

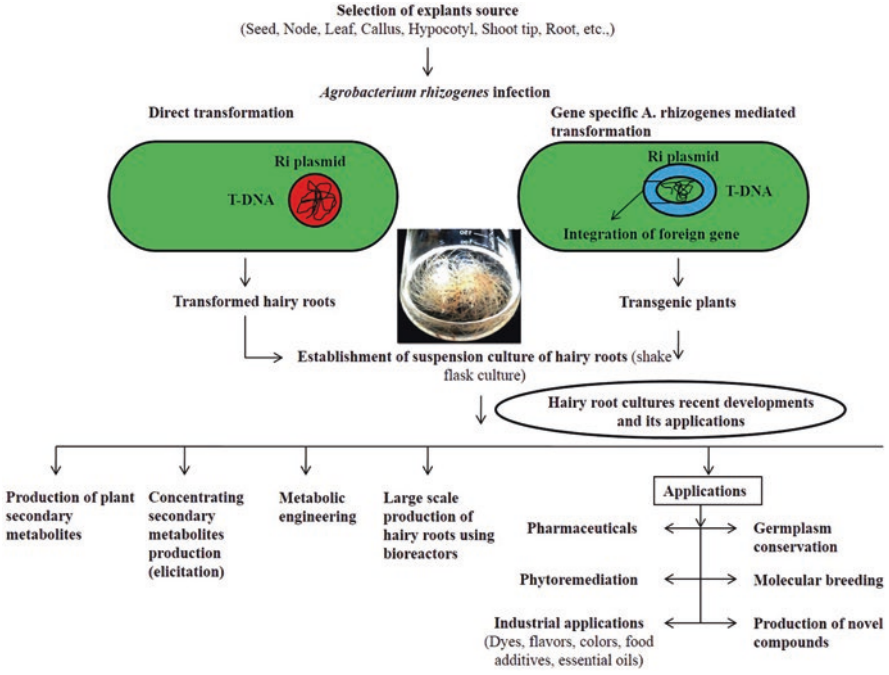

Fig. 10.1 The schematic representation of hairy root induction and its application

these valuable compounds using plant sources (Verpoorte et al. 1999). In search of this is the hairy root culture technology, an alternative approach which offers the production of secondary metabolites in a large scale. Moreover, hairy roots have the unique characteristics of fast growth, and also levels of secondary metabolites produced are equal to or superior than the parent plants (Roychowdhury et al. 2013). The genetic and biosynthetic stability of hairy roots is another advantage for the production of valuable secondary metabolites. In addition to that, transformed hairy roots can be proficient to regenerate into entire viable plants and also preserve their genetic stability throughout and further successive subculturing and plant regeneration (Giri and Narasu 2000). There are several important secondary metabolites produced through hairy root cultures in many medicinal plant species which are endangered and pharmaceutically important. The list of few important secondary metabolites produced through hairy root cultures from various medicinal plants has been described in Table 10.1. In the recent era, hairy root cultures are not only used for secondary metabolite production but also widely used as model systems for studying plant physiology and metabolism, regulation of metabolic pathways, and identification of key genes for production and regulation of particular metabolite (Shanks and Morgan 1999; Sharma et al. 2013; Tian 2015). For example, the roots of *Panax ginseng* plants were rich in ginsenosides, saponin which possesses immunomodulatory, adaptogenic, and antiaging properties. The hairy roots of *P. ginseng* produce twofold increased concentration of ginsenosides than the wild-type roots

Table 10.1 Establishment of hairy root cultures for plant secondary metabolite production

Plant species	Secondary metabolite	Biological properties	References
Artemisia annua	Artemisinin	Antimalarial	Weathers et al. (2005)
Beta vulgaris	Betalains	Antioxidant, colorant	Pavlov and Bley (2006)
Bixa orellana	Stigmasterol	Antimalarial	Zhai et al. (2014)
Chlorophytum borivilianum	Stigmasterol and hecogenin	Antioxidant	Bathoju et al. (2017)
Clitoria ternatea	Taraxerol	Anticancer	Swain et al. (2012)
Datura innoxia	Scopolamine and hyoscyamine	Anticholinergic	Dechaux and Boitel-Conti (2005)
Echinacea sps.	Alkamides	Anti-inflammatory, immune-stimulatory	Romero et al. (2009)
Eschscholzia californica	Benzylisoquinoline	Antimicrobial, anticancer	Vázquez-Flota et al. (2017)
Fragaria x ananassa cv. Reikou	Polyphenols (proanthocyanidins, flavonoids, hydrolyzable tannin)	Antioxidant, anticancer	Motomori et al. (1995)
Gingko biloba	Ginkgolide	Against cardiovascular and aging diseases	Ayadi and Tremouillaux-Guiller (2003)
Hyoscyamus niger	Tropane alkaloids	Anticholinergic	Jaziri et al. (1988)
Isatis tinctoria	Flavonoids	Antioxidant	Gai et al. (2015)
Linum flavum	Aryltetralin lignans Lignans coniferin	Anticancer	Renouard et al. (2018) and Lin et al. (2003)
Linum usitatissimum	Lignan	Anticancer	Gabr et al. (2016)
Nasturtium officinale	Glucosinolates (gluconasturtiin, glucotropaeolin)	Anticancer, antifungal, antibacterial, antinematode, anti-insect	Wielanek et al. (2009)
Ophiorrhiza pumila	Camptothecin	Antitumor	Saito et al. (2001)
Papaver somniferum	Morphine Sanguinarine Codeine	Sedative, analgesic	Le Flem-Bonhomme et al. (2004)
Polygonum multiflorum Thunb	Anthraquinones	Antifungal, anti-inflammatory, antimicrobial	Thiruvengadam et al. (2014)
Rauvolfia micrantha	Ajmalicine Ajmaline	Antihypertensive	Sudha et al. (2003)

(continued)

Table 10.1 (continued)

Plant species	Secondary metabolite	Biological properties	References
Rauwolfia serpentina	Terpenoid indole alkaloids (reserpine, ajmalicine, ajmaline, serpentine, yohimbine)	Hypertension, high blood pressure, mental illness	Mehrotra et al. (2015)
Solanum chrysotrichum	Saponin	Antifungal	Caspeta et al. (2005)
Stevia rebaudiana	Stevioside glycosides	Antioxidant, anti-inflammatory, antihypertensive	Kumari and Chandra (2017)
Taxus brevifolia	Taxol	Anticancer	Huang et al. (1997)
Valeriana wallichii	Iridoids (valepotriates)	Sedative, spasmolytic	Banerjee et al. (1998)
Withania somnifera	Steroidal lactones (withanolide A)	Anticancer	Murthy et al. (2008)

(Yoshikawa and Furuya 1987). In addition to that, *P. quinquefolium* is another important *Panax* species, and its hairy roots produced 0.2 g g^{-1} dry weight of ginsenoside content within 10 weeks of hairy root culture (Mathur et al. 2010). The hybrid plant was made between *P. ginseng* and *P. quinquefolium* which was more dynamic in ginsenoside production than the parental plant. The hairy roots (8-week-old) derived from the hybrid plant containing equivalent amounts of ginsenosides present in the field-grown parental plant roots revealed the biosynthetic potential of hairy roots maintained in the parent plants (Washida et al. 1998; Tian 2015).

10.3 Role of Bioreactors in Large-Scale Production of Secondary Metabolites

Scaling-up process of commercially important secondary metabolites through bioreactor at the industrial level is the next step after establishing in vitro hairy root cultures (Giri and Narasu 2000; Bourgaud et al. 2001). Bioreactors work as a chemical factory and offer a big hope for the large-scale production of high-quality biologically active compounds from medicinal and aromatic plants cells/tissues. This process is also known as molecular farming (Shanks and Morgan 1999). Large-scale production of secondary metabolites using bioreactor is not an easy process, because designing of the bioreactor and optimization of culture conditions are very difficult. The successful cultivation of hairy roots in bioreactor depends on several requirements, including growth characteristics, morphology, nutrient uptake and availability, oxygen supply, composition of the medium, inoculum concentration, and distribution which can facilitate the growth of inoculum (Giri and Narasu 2000; Roychowdhury et al. 2013; Ho et al. 2017). Also, the productivity in bioreactors depends on several physical and chemical parameters like light, temperature, pH, water, substrate availability, impeller designs, composition of gases, choice of hairy root clone, removal of toxic by-products, reactor operation, etc. (Roychowdhury

et al. 2013; Sharma and Shahzad 2013). There are several types of bioreactor designs that have been reported for hairy root culturing. Generally, three major types of bioreactors are used for hairy root cultivation, namely, liquid-phase reactors, gas-phase reactors, and hybrid reactors (a combination of both liquid-phase and gas-phase reactors) (Srivastava and Srivastava 2007). Liquid-phase reactors are commonly known as submerged reactors, in which roots remain submerged in the culture medium and air is passed or bubbled on culture medium to supply oxygen. The best examples for liquid-phase reactors are air lift, stirred tank, bubble column, liquid-impelled loop, and submerged connective flow reactors. In gas-phase bioreactors, hairy roots were occasionally exposed to air, nutrient liquid, and other gaseous mixtures in the bioreactors. In these reactors, nutrients are provided as either in the form of either spraying liquid nutrients onto the roots or roots getting nutrients in the form of droplets, which significantly depends on the varying sizes. Trickle bed, liquid-dispersed, droplet phase, and nutrient mist reactors are some examples for the gas-phase reactors. In hybrid reactors, hairy roots were first exposed to liquid phase and then grown in a gas phase (Roychowdhury et al. 2013). Bioreactor culture systems are mainly used in the industrial application, and they have several advantages, such as requiring very small amount of the inoculum, controlled environmental conditions, increased working volumes, and standardized growth parameters, viz., pH, light, temperature, nutrient media composition, etc. for inducing metabolite production effectively. In addition, easy separation of the target compounds, reproducible yield of the end product, and simpler and quicker harvesting of the cells are some of the other advantages of using bioreactors (Sharma and Shahzad 2013). Some examples for the production of secondary metabolites through the use of bioreactors are mentioned in Table 10.2. For example, artemisinin and its derivatives are high efficient drugs used for the treatment of *Plasmodium falciparum* (both chloroquine-sensitive and chloroquine-resistant strains) which is the causative agent of cerebral malaria. Traditionally, it is obtained from the plant source *Artemisia annua* which contains low concentrations of artemisinin. Patra and Srivastava (2016) reported that large-scale artemisinin production by *A. annua* hairy roots in nutrient mist bioreactor.

10.4 Advances in Metabolic Engineering of Hairy Roots

A new promising technology known as metabolic engineering or genetic engineering was evolved in the early 1990s (Bourgaud et al. 2001). Metabolic engineering in plants involves the alteration of metabolic pathways to increase the flux toward desired secondary metabolites or to attain better understanding of metabolic pathways and use of cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Chandra and Chandra 2011; Hussain et al. 2012). In other words, metabolic engineering is the alteration or improvement of the cellular activities involving transport and enzymatic and regulatory functions of the cell by using rDNA technology (Bourgaud et al. 2001; Hussain et al. 2012). It is one of the fastest-growing applications for the production of industrially important

Table 10.2 Examples of some important plant secondary metabolites produced through bioreactors

Plant species	Secondary metabolite	Bioreactor type	References
Artemisia annua	Artemisinin	Mist and bubble column reactor; gas- and liquid-phase bioreactors	Kim et al. (2001) and Patra and Srivastava (2016)
Astragalus membranaceus	Astragaloside IV and polysaccharide	Air lift bioreactor	Du et al. (2003)
Artemisia annua	Terpenoids	Mist and bubble column reactor	Souret et al. (2003)
Atropa belladonna	Tropane alkaloids	Stirred bioreactors	Lee et al. (1999)
Atropa belladonna	Tropane alkaloids, atropine	Bubble column bioreactor	Kwok and Doran (1995)
Beta vulgaris	Betalains, peroxidase	Bubble column reactor	Rudrappa et al. (2004, 2005)
Catharanthus roseus	Ajmalicine	Bubble column and rotating drum bioreactor	Thakore et al. (2017)
Datura stramonium	Hyoscyamine	Isolated impeller stirred tank reactor	Hilton and Rhodes (1990)
Eleutherococcus koreanum	Saponins	Air lift bioreactor	Lee et al. (2015a, b)
Genista tinctoria	Phytoestrogens	Prototype basket-bubble bioreactor	Luczkiewicz and Kokotkiewicz (2005)
Hypericum perforatum	Hypericin	Balloon-type bubble bioreactor	Cui et al. (2010)
Hyoscyamus muticus	Tropane alkaloids	Trickle bed bioreactor	Flores and Curtis (1992)
Nicotiana rustica	Nicotine	Air-sparged vessel stirred tank	Rhodes et al. (1987)
Panax ginseng	Ginsenosides	Air bubble bioreactor	Murthy et al. (2017)
Panax ginseng	Saponins	Air lift bioreactor	Yoshikawa and Furuya (1987)
Panax ginseng	Ginsenosides	Wave bioreactor	Palazon et al. (2003)
Polygonum multiflorum Thunb	Anthraquinones, stilbenes, flavonoids, tannins,	Air lift bioreactor	Lee et al. (2015a, b)
Stizolobium hassjoo	Levodopa	Mesh hindrance mist trickling bioreactor	Sung and Huang (2006)
Trigonella foenumgraceum	Diosgenin	Air lift bioreactor	Rodriguez-Mendiola et al. (1991)

bio-active compounds from various plant sources. The main aims of this technique are (1) overproduction of a desired compound which is normally produced in less quantity or increased metabolite production by transferring the pathways to another plant or microorganisms, (2) reducing the production of unwanted compounds, and (3) production of a new compound that is usually produced in nature but not present in the host plant (Verpoorte and Memelink 2002; Capell and Christou 2004; Chandra

and Chandra 2011). This can be achieved by conquering the rate-limiting steps or by jamming competitive pathways and blocking of catabolism successfully.

Now, multistep metabolic engineering is possible, which overtakes single-step engineering, and it is the best way to produce secondary metabolites in transgenic plants (Capell and Christou 2004). The main advantage of this method is that it is convenient and cost-effectively produces industrially important secondary metabolites continuously (Hussain et al. 2012). Also, this technique is used as a tool for improving crop plants that are resistant to various diseases, plants producing allelopathic compounds to control the weeds, pest-resistant plants to improve the importance of ornamentals and fruits, and enhanced pollination by modifying scent profiles (Chandra and Chandra 2011). Another advantage is the production of valuable secondary metabolites under controlled environment which is free from climate and soil conditions (Hussain et al. 2012). Engineering or structural design of secondary metabolite pathways is quite difficult in plants, because it requires a detailed knowledge of the whole biosynthetic pathways and a detailed perception of its regulatory mechanisms. But, such information is not explored in many medicinal plants known to have vast variety of bio-active metabolites (Oksman-Caldentey and Inze 2004). Recent advances in metabolic engineering have open a new way for the production of secondary metabolites in higher quantities. However, the success of this approach depends on the metabolic pathway elucidation and metabolite pathway mapping and identifying specific restraining enzyme activities. This process can be further improved by using an appropriate genetic transformation procedure. So far, most of the biosynthetic pathway strategies developed for producing secondary metabolites were through various ways which include isolating and expressing of the respective genes in more efficient organisms, construction of promoters to enhance the expression of a target gene, or antisense and co-suppression techniques for knockdown of particular plants for the desired traits (Bourgaud et al. 2001). For example, engineering of the flavonoid pathway in *Saussurea involucrata* by a transgenic approach increased the production of apigenin. The gene responsible for apigenin production in *S. medusa* was found to be chalcone isomerase (*chi*) gene. A complete cDNA sequence of *chi* gene construct was prepared under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The *chi* gene was introduced into the *S. involucrata* genome by *A. rhizogenes*-mediated transformation which resulted in the establishment of transgenic hairy root lines. The enzyme chalcone isomerase converts naringenin chalcone into naringenin, which is the precursor of apigenin. After 5 weeks of incubation, C46 hairy root line accumulated 32.1 mg/l of apigenin with total flavonoids at 647.8 mg/l. The accumulation of apigenin and flavonoid content was found to be 12 and 4 times, respectively, which is superior when compared to the wild-type hairy roots. The enhanced enzyme productivity was obtained due to the superior activity of chalcone isomerase (Li et al. 2006). In addition to that, hairy root metabolic engineering has been widely used to enhance the production of pharmaceutically important secondary metabolites and also the production of certain recombinant proteins. For example, solasodine glycoside harmfully controls its own biosynthesis. A recombinant gene construct, i.e., anti-solamargine (As)-scFv gene, contains single-chain fragment variable (scFv)

antibody region derived from hybridoma cell lines. Transformed hariy root cultures with anti-solamargine (As)-scFv gene controls and enhances the solasodine glycoside concentration up to 2.3-fold more in the transgenic *S. khasianum* than wild-type hairy roots (Putalun et al. 2003). Metabolic engineering of the hairy roots is also used to make the de novo synthesis of secondary metabolites by introducing the specific genes that encode related enzymatic process in other organisms. The transfer of three genes from *Ralstonia eutropha* bacterium into the genome of sugar beet hairy roots directed the accumulation of poly(3-hydroxybutyrate) (Menzel et al. 2003). Recently, Hidalgo et al. (2017) reported the metabolism of tobacco hairy root for the production of stilbenes. In this study, in order to achieve the holistic response in the phenylpropanoid metabolic pathway and also direct the upregulation of multiple metabolic process, transformed tobacco hairy root (HR) cultures carrying the gene stilbene synthase (STS) derived from *Vitis vinifera* and *Arabidopsis thaliana* transcription factor (TF) AtMYB12 were established. In addition to that, the normal flux was arrested through the incorporation of an artificial microRNA responsible for chalcone synthase (amiRNA CHS); otherwise there will be a heavy competition with STS enzyme for precursors. The transgenic tobacco hairy roots were capable to synthesize the target compound, stilbenes.

10.5 Enhancement of Secondary Metabolites Through Elicitation

Elicitation is an efficient and promising method for increasing the production of secondary metabolites using an elicitor which is a substance that when introduced into a living cell system in ideal/little concentrations improves the biosynthesis of secondary metabolites. The mechanism involved in this process is that the addition of elicitors (both biotic and abiotic) into the plant system attacks the plant cell wall and triggers the production of plant-defensive secondary metabolites (Namdeo 2007; Bensaddek et al. 2008).

In general, the plant cells recognize the elicitor compounds through various signaling molecules and interact or bind with specific receptors present on the plasma membrane. These interactions later generate signals and activate genes that are responsible for the defense reactions including systemic acquired responses (SAR) and induced systemic resistance (ISR). This stimulates the biosynthesis of pathogenesis-related (PR) proteins or defense secondary metabolites, and these finally lead to the production of secondary metabolites (Zhao et al. 2005). The mechanism involved in the production of secondary metabolites through elicitors was showed in Fig. 10.2. Elicitors are broadly divided into two types, viz., biotic and abiotic; mostly abiotic elicitors are inorganic salts (minerals) and physical and chemical factors such as pH, temperature, UV light, heavy metal salts (Cu and Cd ions), etc., while biotic elicitors are polysaccharides derived from plant cell wall and microorganisms (pectin, cellulose, chitin, and glucans), glycoproteins (G-protein or intracellular proteins), pathogenic fungi and bacteria, plant hormones (methyl jasmonate and salicylic acid), etc. (Donenburg and Knorr 1995; Bourgaud et al. 2001;

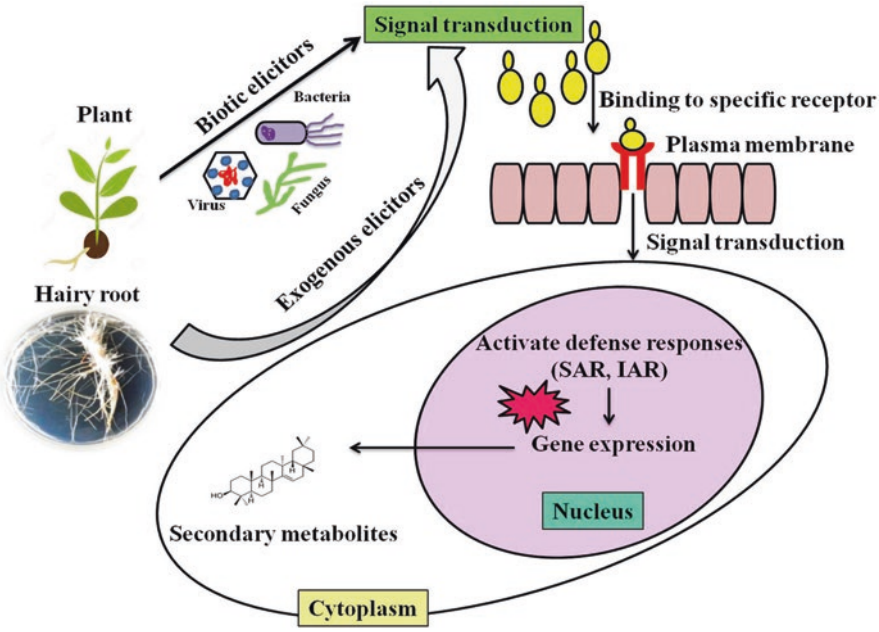

Fig. 10.2 The mechanism of elicitors in secondary metabolite production

Namdeo 2007; Ramirez-Estrada et al. 2016). In addition to that, new types of elicitors have been recently introduced and successfully used in few plant cell cultures. These new elicitors include voliticin, caeliferins, and inceptins. These compounds are derived from plants and insects (which are mostly found in oral secretions of insects). Recently, it was found that they act as an elicitor by activating jasmonates and lead to the production of secondary metabolites, mainly the volatile compounds (Ramirez-Estrada et al. 2016). However, improved production of the metabolites from plant cell cultures through elicitation depends on several parameters, such as selection of suitable elicitor, concentration of elicitor, duration of elicitor treatment, age of the explants, cell line, nutrient composition of the media, growth regulation, etc. (Namdeo 2007). Elicitation method for the plant cell culture system has shown a positive result in secondary metabolite production. However, the study about how plant cells or tissues and their metabolic pathways respond to both abiotic and biotic elicitors is a key route to design the new strategies to enhance the industrially important bio-active compounds in a large scale. For example, a few important bio-active compounds produced through elicitation with biotic and abiotic elicitors are Taxol (Veersham et al. 1995), phytoalexins (Kuroyanagi et al. 1998), saponins (Wu and Lin 2002), tropane alkaloids (Lee et al. 1998), etc. Different types of elicitors used for the production of valuable metabolites are listed in Table 10.3. For example, Largia et al. (2016) reported that the transformed hairy roots plants of *Bacopa monnieri* elicited with 10 mg/L chitosan for 2 weeks enhanced the accumulation of bacoside A (5.83%) content, which is a five- and fourfold increase when compared

Table 10.3 Production of plant secondary metabolites by using different elicitors

Plant species	Secondary metabolite	Elicitors	References
Ammi majus	Coumarine, furocoumarine	BION® *Enterobacter sakazakii*	Staniszewska et al. (2003)
Arachis hypogaea	*Trans*-resveratrol	Sodium acetate	Medina-Bolivar et al. (2007)
Arachis hypogaea	Resveratrol, piceatannol, arachidin-1, and arachidin-3	MeJA and cyclodextrn	Yang et al. (2015)
Astragalus membranaceus	Calycosin and formononetin	*Aspergillus niger*	Jiao et al. (2017)
Artemisia annua	Artemisinin	Chitosan	Putalun et al. (2007)
Azadirachta indica	Azadirachtin	Salicylic acid, jasmonic acid	Satdive et al. (2007)
Catharanthus roseus	Alkaloids (indole)	*Penicillium* sp.	Rijhwani and Shanks (1998)
Centella asiatica	Asiaticoside	Methyl jasmonate	Kim et al. (2007)
Datura metel	Atropine	AgNO3, nanosilver, *Bacillus cereus*, *Staphylococcus aureus*	Shakeran et al. (2015)
Hyoscyamus muticus	Sesquiterpenes	*Rhizoctonia solani*	Singh (1995)
Hyoscyamus niger	Polyamines and tropane alkaloids	Methyl jasmonate	Zhang et al. (2007)
Linum album	Lignan	Coniferaldehyde and methylenedioxycinnamic acid	Ahmadian Chashmi et al. (2016)
Oxalis tuberose	Harmaline, harmine	*Phytophthora cinnamomi*	Bais et al. (2003)
Lotus corniculatus	Isoflavonoids	Glutathione	Robbins et al. (1991)
Papaver orientale	Morphinan alkaloids	MeJA and salicylic acid	Hashemi and Naghavi (2016)
Panax ginseng	Ginseng saponin	Selenium, NiSO4, NaCl	Jeong and Park (2006)
Pharbitis nil	Umbelliferone, scopoletin, skimmin	CuSO4, MeJA	Yaoya et al. (2004)
Salvia miltiorrhiza	Tanshinone	Sorbitol	Shi et al. (2006)
Scopolia parviflora	Scopolamine	*Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*	Jung et al. (2003a, b)
Solanum tuberosum	Sesquiterpene, lypooxygenase	*Rhizoctonia bataticola*, B cyclodextrin, MeJA	Komaraiah et al. (2003)
Tagetes patula	Thiophene	*Furasium conglutanis*, *Aspergillus niger*	Mukundan and Hjortso (1990) and Buitelaar et al. (1993)

to wild plants and unelicited transformed plants. Similarly, Shilpha et al. (2016) reported that *Solanum trilobatum* hairy roots (ST-09 clone) elicited for 2 weeks with 4 μM for methyl jasmonate enhanced the solasodine content, which is 1.9- and 6.5-fold higher than unelicited hairy roots and wild roots.

10.6 Biotransformation

Biotransformation is the process in which a substance is transformed from one chemical to another, and it is catalyzed by the effective enzyme structures of biological systems. Plant cell or organ cultures have the capability to convert exogenously added organic compounds into functional analogs (Banerjee et al. 2012; Roychowdhury et al. 2013). This type of protocols has been done by using plant cell/organ cultures which have generated the libraries of analog compounds with limited structural modifications, and it also ensures the sustainable use of the resource under defined culture conditions free from seasonal variations and pathological constraints. The resulted compounds will have the important characteristic potency of a parent molecule and can also attain a superior selectivity, safety, and physicochemical properties with lower toxicity. This can be more appropriate to be used for newer therapeutic applications. The biotransformation method is very useful for the discovery of novel phytochemicals having therapeutic and commercial advantages. Also, this method is attaining more attention toward the green chemistry, because of the reduced usage of hazardous chemicals in the process of chemical modifications. The major reactions involved in biotransformation methods include oxidation, reduction, glycosylation, esterification, methylation, isomerization, and hydroxylation. Hairy root cultures have various advantages as biocatalysts over cell suspension cultures, because of their genetic and biochemical stability, multi-enzyme biosynthetic potential comparable to the parent plant, and cost-effectiveness. Therefore, hairy root cultures also act as an experimental model system in biotransformation studies (Giri et al. 2001; Banerjee et al. 2012). Biotransformation studies were reported in *Ri*-transformed root cultures of several plant species for producing valuable secondary metabolites and are briefly described by Banerjee et al. (2012). For example, the biotransformation ability of *Atropa belladonna* hairy root cultures has been explored by using three carbonyl substrates such as 3,4,5-trimethoxybenzaldehyde, 3,4,5-trimethoxy-acetophenone, and 3,4,5-trimethoxy-benzoic acid. Among the three substrates used, 3,4,5-trimethoxybenzaldehyde and 3,4,5-trimethoxy-acetophenone were biotransformed, but, 3,4,5-trimethoxy-benzoic was not biotransformed. The 3,4,5-trimethoxybenzaldehyde was biotransformed by oxidation and reduction of substrate into 3,4,5-trimethoxy-benzoic acid and 3,4,5-trimethoxy benzyl alcohol, respectively (Srivastava et al. 2012). Overall, the biotransformation using hairy root cultures has got potential to generate new products or to generate already known products very efficiently. The list of reactions involved in biotransformation of hairy roots for metabolites production are shown in Table 10.4.

Table 10.4 Biotransformation of hairy roots for plant secondary metabolite production

Plant species	Types of reaction	Product	References
Anethum graveolens	Acetylation, reduction	Menthyl acetate linalool, α -terpineol, citronellol	Faria et al. (2009)
Anisodus tanguticus	Oxidation	Androst-4-ene-3,17-dione 6 α-hydroxy androst-4-ene-3	Liu et al. (2004)
Astragalus membranaceus	Deglycosylation	Calycosin Formononetin	Jiao et al. (2017)
Atropa belladonna	Reduction	Scopolamine	Subroto et al. (1996)
Brassica napus	Reduction, glycosylation	6-(1(S)-hydroxyethyl)-2,2-dimethyl-2,3-dihydro-4H-chromen-4-one	Orden et al. (2006)
Brugmansia candida	Glucosylation	4-Hydroxyphenyl β-D-glucopyranoside (arbutin)	Casas et al. (1998)
Coleus furskohlii	Glycosylation	Methyl β-D-glucopyranosides, methyl β-D-ribo-hex-3-ulopyranosides	Li et al. (2003)
Cyanotis arachnoidea	Reduction	Deoxyartemisinin	Zhou et al. (1998) and Ligang et al. (1998)
Daucus carota	Reduction	(S)-1-phenyl ethanol)	Caron et al. (2005)
Lobelia sessilifolia	Glucosylation	Protocatechuic acid 3-O-β-D-glucopyranoside	Ishimaru et al. (1996)
Lobelia sessilifolia	Glucosylation	(+)-catechin 7-O-β-D-glucopyranoside	Yamanaka et al. (1995)
		Protocatechuic acid, protocatechuic acid 3-O-β-D-glucopyranoside	
		(–)-epicatechin 7-O-β-D-glucopyranoside	
		(–)-epiafzelechin 7-O-β-D-glucopyranoside	
Levisticum officinale	Isomerization	Linalool, nerol	Nunes et al. (2009)
Panax ginseng	Esterification	Digitoxigenin stearate	Kawaguchi et al. (1990)
		Digitoxigenin palmitate	
		Digitoxigenin myristate	
		Digitoxigenin laurate	
Panax ginseng	Glycosylation	(RS)-2-phenylpropionyl β-D-glucopyranoside	Yoshikawa et al. (1993)
		(2RS)-2-0-(2-phenylpropionyl) D-glucose	
		(2RS)-2-phenylpropionyl) 6-0-β-D-xylopyranosyl β-D-glycopyranoside	

(continued)

Table 10.4 (continued)

Plant species	Types of reaction	Product	References
		Myoinositol ester of (R)-2-phenylpropionic acid	
Panax ginseng	Glycosylation	30-O-[β-D-glucopyranosyl (1→2) β-D-glucopyranosyl]	Asada et al. (1993)
		18 β-Glycyrrhetinic acid	
		30-O-[β-D-glucopyranosyl] 18 β-glycyrrhetinic acid	
		3-O-[β-D-glucopyranosyl -(1→2) β-D- glucopyranosyl] 18 β-glycyrrhetinic acid	
		3-0-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl] -30-0-(β-D-glucopyranosyl) 18 β-glycyrrhetinic acid	
Panax ginseng	Glycosylation	p-carboxyphenyl β-D-glucopyranoside	Chen et al. (2008)
		p-hydroxybenzoic acid	
		β-D-glucopyranosyl ester	
		m-carboxyphenyl β-D-glucopyranoside	
Pharbatis nil	Glucosylation	Skimmin	Kanho et al. (2004, 2005)
		4-Methylskimmin	
		Scopoline	
		3,4,8-Tri methylskimmin	
		Scopolin, aesculin, eichoriin, vanillin-4-O-β-glucopyranoside	
		Vanillyl alcohol-4-O-β-D-glucopyranoside	
Physalis ixocarpa	Glucosylation	Arbutin	Bergier et al. (2008)
Plantago lanceolata	Glucosylation	(E)-p-coumaroyl-1-O-β-D-glucopyranoside	Fons et al. (1999)
Polygonum multiflorum	Glycosylation	3-oxo-eremophila 1,7(11)-dien-12,8-olide	Yan et al. (2008)
		3-oxo-8-hydroxy-eremophila 1,7(11)-dien-12,8-olide	
Polygonum multiflorum	Glucosylation	4-Hydroxybenzene derivatives: 1-4-benzendiol	Yan et al. (2007)
		4-Hydroxybenzaldehyde	
		4-Hydroxybenzyl alcohol	
		4-Hydroxybenzoic acid	
Polygonum multiflorum	Glucosylation	5-Methyl-2-(1-methylethyl) phenyl-β-D-glucopyranoside	Dong et al. (2009)

10.7 Hairy Root Applications in Environmental Protection (Phytoremediation)

Environmental pollution is a universal problem that adversely affects both the developed and developing countries. The major reason for environmental pollution is due to human activities and natural hazards. Contaminants are usually classified into two types: organic and inorganic. Due to the human activities including oil spills, agriculture wastage, military explosives, fuel production, and wood treatment, organic contaminants are released into the environment. Some of important organic pollutants such as trichloroethylene (TCE), atrazine, trinitrotoluene, polycyclic aromatic hydrocarbons, benzene, toluene, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons, and methyl tert-butyl ether contaminating the soil and water are a challenge to the world. Generally, inorganic contaminants are originated from either human activities or natural processes. The most dangerous inorganic contaminants include heavy metals such as copper, zinc, manganese, lead, molybdenum, mercury, and nickel which are released into the environment by natural and human activities causing a health threat to humans and livestock (Suza et al. 2008). The removal of these contaminants from the environment is not an easy task, and decontamination is a very expensive process. Phytoremediation, as an emerging alternative technology, is highly appreciated in recent times for its effectiveness in cleaning up of the contaminated environment. Phytoremediation is defined as the ability of plants to uptake contaminants from the polluted environment (soil, water, or air) and convert the toxic chemical molecules to harmless forms enzymatically (Roychowdhury et al. 2013; Guillon et al. 2006). The key advantage of phytoremediation technique is that it is about ten times less expensive than conventional environmental cleanup methods, and it is a safe method. Generally, plants act as natural soil stabilizers, reduce the amount of contaminants, and maintain the surroundings free from pollutants. Phytoremediation is better than bioremediation methods that uses microbes in terms of easy monitoring. This is because, in phytoremediation, the plants' condition is visible, and the presence of pollutants in plant tissues can be easily tested (Doty 2008). The major phytoremediation strategies involved in the removal of contaminants include phytoextraction, phytostabilization, and rhizofiltration of organic and inorganic pollutants (Gonzalez et al. 2006). In this regard, hairy root technology also plays an important role in the process of phytoremediation. Some of the advantages offered by hairy roots for this purpose include fast growth and high branching of hairy roots allowing increase absorption of contaminants, high biochemical and genetic stability, easy maintenance, scaling-up in bioreactors being easy, and provision of a huge surface area of contact with the contaminants. Moreover, hairy roots contain essential enzymes and metal chelating agents to detoxify the harmful compounds (Gonzalez et al. 2006; Roychowdhury et al. 2013). In recent years, hairy roots are serving as a potential tool to decontaminate the environment and are being highly appreciated by environmental biologists for its effectiveness. A wide variety of environmental pollutants that can be removed by hairy roots derived from different plant species are shown in Table 10.5. However, it is required to completely understand the enzymatic machineries involved in the

Table 10.5 Phytoremediation of environmental pollutants by hairy root cultures

Plant species	Pollutant	Reference
Solanum nigrum	PCBs (polychlorinated biphenyls) and zinc	Macková et al. (1997a, b) and Subroto et al. (2007)
Thlaspi caerulescens	Cadmium	Nedelkoska and Doran (2000) and Boominathan and Doran (2003)
Alyssum sp. *A. bertolinii*, *A. tenium*, and *A. troodi*	Nickel	Nedelkoska and Doran (2001) and Suresh et al. (2005)
Catharanthus roseus	RDX (hexahydro-1,3-5-trinitro-1,3-5-triazine) and HMX (oxtahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)	Bhadra et al. (2001)
Daucus carota	Phenol and chloroderivatives	De Araujo et al. (2002)
A. bertolonii and *Thlaspi caerulescens*	Nickel, and cadmium	Boominathan and Doran (2002)
Atropa belladonna	TCE (trichloroethylene)	Banerjee et al. (2002)
Brassica napus	2,4-Dichlorophenol, Phenol	Agostini et al. (2003) and Coniglio et al. (2008)
B. juncea and *Chenopodium amaranticolor*	Uranium	Eapen et al. (2003)
B. juncea and *Cichorium intybus*	DDT (Dichloro-diphenyl-trichloroethane)	Suresh et al. (2005)
Helianthus annuus	Tetracycline and oxytetracycline	Gujarathi et al. (2005)
Lycopersicon esculentum	Phenols	Wevar-Oller et al. (2005)
Daucus carota, *Ipomoea batata*, and *Solanum aviculare*	Guaiacol, catechol, phenol, 2-chlorophenol, and 2,6-dichlorophenol	De Araujo et al. (2004, 2006)
Brassica juncea	Phenol	Singh et al. (2006)
Lycopersicon esculentum	Phenol	Wevar-Oller et al. (2005) and González et al. (2006)
Alyssum murale	Nickel	Vinterhalter et al. (2008)
Solanum lycopersicon	Phenol	Wevar-Oller et al. (2005) and González et al. (2006)
Nicotiana tabacum	Phenol, 2,4-DCP	Alderete et al. (2009) and Talano et al. (2010)
Armoracia rusticana	Uranium	Soudek et al. (2011)

bioconversion of toxic contaminants to nontoxic complexes and also the mechanisms involved in the hyperaccumulation and metal tolerance (Roychowdhury et al. 2013). In the future, the application of genetic engineering to insert specific detoxifying genes in hairy roots enhances their capacity to effectively clean up the contaminant.

10.8 Germplasm Conservation

Germplasm conservation is one of the prominent techniques to preserve/restore the plant biodiversity, because most of the plants do not produce viable seeds and propagate vegetatively, while some plants produce recalcitrant seeds, and the storage of seeds is affected by pests or other pathogens. So, the conservation of wild, rare, and endangered medicinal plant species for future use has become a big problem, and more efforts are initiated in this direction. Biotechnological tools such as plant tissue culture micropropagation and cryopreservation have certainly benefited in protecting plant germplasms including vegetatively propagated plant species, genetic resources of recalcitrant seeds, rare and endangered plant species, cell lines with special attributes, genetically transformed plant material, and clones obtained from elite genotypes (Engelmann 2011). Based on the storage duration, in vitro conservation methods are classified into three types, namely, short-, medium-, and long-term storage. Among them, cryopreservation is the most efficient technique for long-term conservation of the germplasm of a valuable plant, because of its cost-effectiveness and safety. Three types of cryopreservation methods are highly employed for the biodiversity conservation. They include freeze-induced dehydration, encapsulation-dehydration, and encapsulation-vitrification (Shibli et al. 2006). Hairy root cultures can be used for the germplasm conservation, because hairy root cultures are significantly a good resource for the production of several secondary metabolites and, in recent times, they are obtained in many medicinal plants for commercial applications. Hence, conserving such hairy roots will be more useful for future applications. However, there are only very few reports available on the conservation of hairy roots of medicinal plants. Hairy roots in the form of artificial seeds are a reliable delivery system for the clonal propagation of elite plants with genetic uniformity, high yield, and low production cost. Cryopreservation method for root tips was first developed by Benson and Hamill (1991) from hairy root cultures of *Beta vulgaris*, and the same technique was implemented in *Nicotiana rustica*. Yoshimatsu et al. (1996) reported the cryopreservation of *Panax ginseng* hairy roots. In addition to that, cryopreservation of hairy roots was reported in some more medicinal plants like *Artemisia annua* (Teoh et al. 1996), *Armoracia rusticana* (horseradish) (Phunchindawan et al. 1997; Hirata et al. 1998), *Atropa belladonna* (Touno et al. 2006), *Eruca sativa*, *Astragalus membranaceus* and *Gentiana macrophylla* (Xue et al. 2008), *Maesa lanceolata* and *Medicago truncatula* (Lambert et al. 2009), and *Rubia akane* (nakai) (Kim et al. 2010, 2012; Salma et al. 2014).

10.9 Omics Approaches in Secondary Metabolite Production

The omics approaches, namely, genomics, transcriptomics, proteomics, and metabolomics, have been majorly utilized in hairy root-based secondary metabolite production. As transcriptomic tools the microarrays and expressed sequence tags (EST) were useful in measuring the gene expression studies in large scale. Expression of target genes in a plant cell can be modified through various methods such as

precursor feeding, elicitor treatment, overexpression or silencing of transgenes, etc. Generation of cDNA microarrays and EST database provides the information about the changes at mRNA level and also briefs the functions of genes and its regulation in secondary metabolism of hairy root cultures. Transcriptome analysis of hairy root cultures has been done in several plants including *P. ginseng* (ginsenoside), *C. roseus* (indole alkaloids), *Medicago truncatula* (anthocyanin), *S. miltiorrhiza* (tanshinones), etc. (Jung et al. 2003a, b; Murataa et al. 2006; Pang et al. 2008; Gao et al. 2009; Wang et al. 2010). In studying the tanshinone biosynthesis, *S. miltiorrhiza* hairy root cultures were used as a model system. The combined analysis of metabolite profiling and cDNA-AFLP identified the candidate genes which are potentially involved in the biosynthetic pathway (Yang et al. 2012). Proteomics is an important, powerful, and under-explored omics technology for the secondary metabolite elucidation in hairy root cultures. Proteomic approach for hairy root cultures has been initiated in *P. ginseng* and opium poppy (Kim et al. 2003; Zulak et al. 2009). Metabolomics is an emerging approach which is highly useful in secondary metabolite production (Yang et al. 2012). The systems biology approaches with a combination of omics approaches will offer a great opportunity for high-throughput secondary metabolite elucidation in various plant species.

10.10 Conclusions and Future Prospects

In the modern era, humankind is facing the problem of high demand for several potent plant secondary metabolites possessing many bio-pharmacological activities. Previously, in vitro dedifferentiated plant tissue cultures were used for obtaining plant metabolites. As the years passed, cell suspension and adventitious root cultures were widely adopted for the same. However, to elucidate such metabolites, there is a need to develop an efficient and reliable, fast-growing in vitro tissue culture model to overcome the problem of wild plant availability. In this regard, hairy root cultures offer a great value to the continuous production of several precious secondary metabolites, because of their unique characteristics discussed above. Since the emergence of hairy root technology, a lot of improvements have been made day by day especially the use of bioreactors, application of elicitation strategy, and biotransformations. Overall, hairy root technology has shown its wide utility in many medicinal plants. Moreover, the production of plant secondary metabolites in the hairy root culture system has delivered very encouraging findings, for example, illuminating the sites of biosynthesis or rate-regulating stages, precursor's requirements, role of regulatory genes, transcription factors, and putative metabolite intermediates relating to secondary metabolite biosynthesis. Also, it offers the possibility of recognizing a suitable gene candidate required for metabolic engineering of specific plant traits and to improve their secondary metabolite secretion. However, more efforts are to be encouraged to better understand the biosynthetic pathways and regulatory cascades involved in secondary metabolite synthesis. Therefore, it is crucial to make use of genetic engineering approaches in order to fully realize the biosynthetic prospective of hairy roots. Plant biotechnologists are required to work

closely with bioengineers to overcome the challenges faced during the scaling-up of hairy root cultures in bioreactors. In the future, research efforts should be encouraged toward making use of hairy root culture technology for producing high-value secondary metabolites commercially from many unexplored medicinal plant species.

Acknowledgments The author A. Sagina Rency gratefully acknowledges the Department of Science and Technology for financial support in the form of DST INSPIRE Fellowship (DST/INSPIRE Fellowship/03/2014/004363). Also the authors sincerely thank the Bioinformatics Infrastructure Facility of Department of Biotechnology, Alagappa University (funded by Department of Biotechnology, Government of India: Grant No. BT/BI/25/015/2012), for providing the computational facility.

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