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REVIEW ARTICLE

Hairy root biotechnology—indicative timeline to understand missing links and future outlook

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Abstract *Agrobacterium rhizogenes*-mediated hairy roots (HR) were developed in the laboratory to mimic the natural phenomenon of bacterial gene transfer and occurrence of disease syndrome. The timeline analysis revealed that during 90 s, the research expanded to the hairy root-based secondary metabolite production and different yield enhancement strategies like media optimization, up-scaling, metabolic engineering etc. An outlook indicates that much emphasis has been given to the strategies that are helpful in making this technology more practical in terms of high productivity at low cost. However, a sequential analysis of literature shows that this technique is upgraded to a biotechnology platform where different intra- and interdisciplinary work areas were established, progressed, and diverged to provide scientific benefits of various hairy root-based applications like phytoremediation, molecular farming, biotransformation, etc. In the present scenario, this biotechnology research platform includes (a) elemental research like hairy root-mediated secondary metabolite production coupled with productivity enhancement strategies and (b) HR-based functional research. The latter comprised of hairy root-based applied aspects such as generation of agro-economical traits in plants, production of high value as well as less hazardous molecules through biotransformation/farming and remediation, respectively. This review presents an indicative timeline portrayal of hairy root research reflected by a chronology of research outputs. The timeline also reveals a progressive trend in the state-of-art global advances in hairy root biotechnology. Furthermore, the review also discusses ideas to explore missing links and to deal with the challenges in future progression and prospects of research in all related fields of this important area of plant biotechnology.

Keywords Hairy root cultures · Chronology · Secondary metabolite production · Bioreactor · Time line analysis · Yield-enhancing strategies

Abbreviations

HRCs	Hairy root cultures
ME	Metabolic engineering
GE	Genetic engineering
SM	Secondary metabolites

Introduction

Hairy roots have been considered as a biological matrix for various biotechnological functions for the last three decades. The neoplastic manifestation or hairy roots ensued from the transfer of *Agrobacterium rhizogenes* transfer DNA (T-DNA) into the plant genome possess a potential for secondary

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metabolite production. In the last century, digging into the molecular insights of crown gall and hairy root diseases of plants, the natural ability of horizontal gene transfer of genus *Agrobacterium* (Rhizobiaceae) came into light. Members of this genus naturally transform host plants into chemical producers of opines, which serve as nitrogen and energy source for bacteria. Four major events orchestrate this parallel gene transfer phenomenon: (a) chemotaxis where phenolic exudates from wounded plant cells cause the bacterium to move towards the roots, (b) generation of single-strand T-DNA in bacterial plasmid and its translocation to host cells, (c) nuclear targeting and integration of T-DNA to host genome, and (d) expression of genes located on the T-DNA resulting in disease (Chandra 2012). Successful gene transfer requires the involvement of both bacterial and host factors, and adequate coordination of these factors determines the success of transformation. The process of transformation results in either tumors or hairy roots at the infection sites of the host plant, depending on the type of plasmid in the bacterial cell. This review focuses on hairy roots which are competent of virtually infinite growth under controlled growth conditions. Owing to various properties such as fast growth potential, ability to produce a range of secondary metabolites, and genetic/biochemical stability, the hairy roots are being exploited in plant biotechnology as a model system to explore biochemical, molecular, and physiological aspects of host–microbe interaction and plant secondary metabolism. The mechanism of *Agrobacterium*-mediated natural transformation has been known for more than 30 years (Chilton et al. 1982). During this period, their enormous potential was perceived, and research focused on their in vitro establishment as hairy root cultures (HRCs) and secondary metabolite production. In the 1990s, there were numerous published reports, and this field entered into an exponential phase where several properties such as high biomass production, high metabolite accumulation, and organogenic potential in hairy roots were targeted (Nilsson and Olsson 1997; Shanks and Morgan 1999). During this decade, metabolite production through hairy roots has got remarkable transition from laboratory bench scale to industrial scale. At present, hairy root culture technology has emerged as a platform for studying numerous aspects of plant behavior under simulated laboratory conditions (Hu and Du 2006; Guillon et al. 2006a; Goel et al. 2011; Ono and Tian 2011; Georgiev et al. 2012). In recent years, efforts to conceptualize the development of hairy root-mediated transgenic plants and their field cultivation are in progress, and this has given "soil–laboratory–soil transition" to this technology. The current review presents a timeline portrayal of hairy root research reflected by the chronology of the evidence and its progressive trend in the current scenario. Moreover, in the light of this trend, the present review also discusses ideas to overcome challenges and emerging trends in future progression of research in this important domain of plant biotechnology.

Soil to laboratory: an exemplar move

The existence of the term 'hairy root' in literature dates back to 1900 when Stewart et al. (1900) firstly coined it for diseased fruit crops. Followed by Hildebrandt (1934) who used the term 'hairy root syndrome' for distinctive disease symptoms, which includes the formation of small, thin hair-like mass of roots appearing as a result of microbial infection. However, it was Riker et al. (1930) who recognized the causal entity, described it, and named it as *A. rhizogenes*. At present, this gram-negative, rod-shaped, pathogenic soil bacterium *A. rhizogenes*, also known as *Rhizobium rhizogenes*, is a definable member of genus *Agrobacterium* of family Rhizobiaceae (Gelvin 2003; Veena and Taylor 2007; http://www.*Agrobacterium*.org). Adequate synchronization of several chromosomal genes (*chvA, chvB*), *vir* (*virD1*, *virD2*, and *virE1*, *virE2*), and T-DNA genes (located on bacterial pRi) are required for the various events of T-DNA transfer to host cell (Chandra 2012). The natural host range of *A. rhizogenes* is limited to a few dicotyledonous plant species, but under laboratory conditions a broad host range including monocotyledonous and gymnosperm plant species can produce hairy roots upon deliberate infection of *A. rhizogenes* (Tepfer 1989). Presently, more than 50 families of angiosperm plants representing > 150 genus with varying number of species are being exploited for their hairy roots (Ono and Tian 2011; Tepfer 1989; SCOPUS; PubMed; Sci-Finder database; Fig. S1). Apart from secondary metabolism, the hairy roots thus produced are the starting point for studying the molecular mechanism of a number of basic phenomena in plant behavior, biochemistry, and physiology. This has also led to the development of in vitro hairy root culture system (HRCs) from various plant species to get the insights of these biological processes. Incessant research in upcoming decades of 1980 and 1990 not only added crucial details to *Agrobacterium* biology but also explored the natural process of genetic transformation on a molecular basis. A gradual progression in hairy root research that describe *Agrobacterium* infection mechanisms and the appearance of disease syndrome can be observed in various published articles par excellence (White et al. 1985; Sinkar et al. 1987; Tepfer 1989; Nilsson and Olsson 1997; Gelvin 2000; Tzfira et al. 2004; Georgiev et al. 2007; Veena and Taylor 2007; Gelvin 2010; Chandra 2012). It was quite unpredictable earlier in what eventual way these details would lead to the current form of hairy root research platform. However, in retrospect, the research work which appeared in succession can be traced to show how time to time different intra- and interdisciplinary work areas were established, progressed, and diverged to provide various applications using hairy root cultures that ultimately lead to the present day's Hairy Root Biotechnology Podium (HRBP) (Fig. 1).

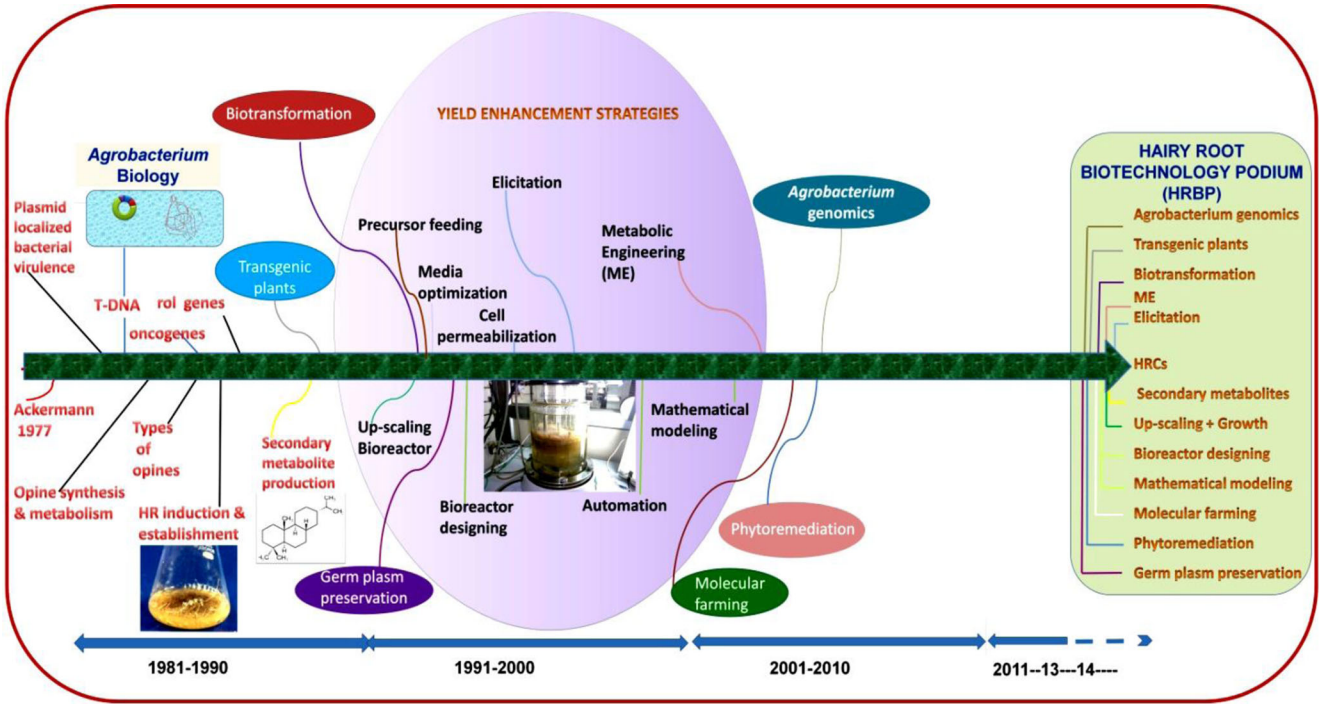

Fig. 1 Timeline portrayal of hairy root research: chronology of research in three decades leading to modern HRBP

In the past decade, with the efforts of whole *Agrobacterium* genome sequencing and transcriptional profiling, *Agrobacterium* research entered in the genomic and post-genomic era. This research has provided large information about *Agrobacterium* gene sequences that direct bacterial activities during gene transfer and host plant defense (Gelvin 2000, 2009, 2010). The *Agrobacterium* proteome analysis under stress (heat, temperature, oxidative, and acidic stress) as well as under simulated plant infection conditions in the laboratory verified the involvement of specific *vir* proteins (*VirE2*, several *VirB* proteins, *VirF*, Tzs, *VirH1*, and *VirK*) during T-DNA formation and its translocation (Tzfira and Citovsky 2006; Lacroix et al. 2008). The current state of knowledge necessitates the involvement of advanced molecular biology techniques with *Agrobacterium* and host plant genomics to unravel the protein–protein interaction during and after the infection. These fundamental knowledge, when coupled with different gene transfer approaches like agrolistic gene transfer, niacinamide treatment, involvement of transposable elements as transgene transporters, etc., may perhaps lead to superior laboratory transformation events in terms of single-copy and site-targeted stable insertions with desired expression levels. In previous years, the role of host plant genes/proteins and their interaction with T-DNA during *Agrobacterium* infection as well as the role of bacterial genes/proteins and their interaction with host safety mechanism during T-DNA translocation through the host cell cytoplasm, chromatin targeting, and T-DNA integration has been studied (Tzfira and Citovsky 2002; Tzfira et al. 2004; Citovsky et al. 2007). Although answered to some extent, still there are issues that justify the need of scientific attention. These include (1) exploration of the exact interplay between various manipulations like enhanced activity of MAPKs, elevated levels of reactive oxygen species, stimulation of specific defense genes, and activated secondary metabolism that occur in host metabolism during bacterial infection that influence host immunity; (2) what is the molecular basis of bacterial survival in such metabolic manipulations and improved host immunity? (3) A multitude of biotic and abiotic factors are supposed to affect the structural and functional diversity of microbial communities in the rhizosphere. How does *Agrobacterium* interact with soil microflora, and does this interaction possess positive/negative influence over the survival or transformation potential of the bacteria? (4) What are the evolutionary facets of this host–bacteria association? (5) Why is natural transformation difficult in plants of lower taxa? (6) Exploring the difficulties encountered in *Agrobacterium*-mediated transformation of monocots, various studies reveal that monocots have differences in cellular structures, wound response, subsequent *vir* gene induction, etc. (Sood et al. 2011). Still the exact molecular phenomenon behind the high susceptibility of dicots over monocot plant species needs the scientific attention; (7) Can there be a possibility to control the natural bacterial host range to avoid loss of productivity simply by knowing the molecular basics of transformation under simulated laboratory conditions? (8) What are the challenges behind the development of genotype-independent transformation procedures for commercially important plant species? Although a large body of research has emerged in the form of excellent reviews and research articles,

yet the information present is either in partial form or with missing links. Several unfolded mysteries are concealed in these missing links, and the questions cited above will likely be answered using various modern biological and interdisciplinary approaches.

Commercially important secondary metabolites are often accumulated in plant roots. These compounds have complex structures and are present in very low volumes, thus difficult to extract. The high cost in organic synthesis and threats of plant annihilation by direct harvesting of roots made their collection more challenging. Thus, the involvement of an alternative method that can meet the commercial requirement along with natural germ plasm maintenance is necessary. Although biotechnological interventions have provided the option of cell suspension cultures for secondary metabolite production, this method has limitations like metabolite production in specialized cells at distinct developmental phases and genetic instability of cells. In contrast, HRCs closely mirror the production potential of intact root systems by producing a range of bioactive molecules. Certain other striking properties like high genetic and biochemical firmness, low doubling time, and very often the ability to produce novel compounds are sufficient to utilize in vitro hairy root cultures for various purposes.

During 1980s, while realizing the enormous biosynthetic potential of HRCs, secondary metabolite production through hairy roots was the main attraction. At present, a wide range of commercially important high-value bioactive molecules (under sub-classes of alkaloids, terpenes, and phenolics) is known to be produced by HRCs from various plant species (Ono and Tian 2011).

The yield enhancement strategies

Media optimization

The primary cause of the lack of success in commercial production of bioactive compounds using HRCs is their low yield. This leads to the high production cost of the molecule. Several strategies have been employed in order to make hairy root-based technology viable at a large scale. In this regard, optimization of growth medium is the preliminary strategy. In general, hairy roots can grow on any of the basal medium used in tissue culture practice, yet the nutritional requirements vary with the plant system as well as with the objective for which the roots are established. For example, variation in nutrient quantities to assess their uptake and product flux or level of elicitor during elicitation experiments requires optimization studies. The conventional method of optimizing the growth medium includes alteration in various components individually and observing their effect on tissue growth. However, the missing link lies in determining the synergistic effects of various culture components on tissue growth at a time. Like other biological entities, the hairy roots possess time variant and non-linear complex growth phenomenon. This complexity of growth can be measured as a product of different physical/chemical culture conditions interacting in a common space (Gallego et al. 2011). To assess the effect of a single condition or their synergistic influence on complete growth and developmental process, the computational scheming is advantageous. It hypothesizes different mathematical algorithms to process and interpret different sets of unpredictable data obtained from the behavior of tissue under variable culture conditions. In HRCs, for the optimization of various growth conditions, the computational modeling is a flexible platform to assimilate the multidimensional biological data. It not only provides clues near to accuracy but also helps to overcome the cost and time deluge of the conventional optimization process. An innovative trend can be observed in recent studies where in silico approaches such as neural network and fuzzy logic-based simulations have been utilized to optimize the chemical conditions of hairy roots and other types of cultured tissue (Gago et al. 2011; Mehrotra et al. 2013a; Gago et al. 2014). In large-scale setups cultivating HRCs for various applications, the use of computational simulations for optimization of culture conditions will definitely lead to high-quality cost-effective protocols.

Bioreactor technology

Bioreactor technology conceptualizes the proportionate production of metabolites with that of mass-scale production. In that sense, bioreactor technology can also be envisioned as yield enhancement strategy. Several reports on growing hairy roots in bioreactor support the idea of designing a culture vessel in order to facilitate O_2/nutrient supply that can lead to subsequent enhancement in tissue growth (Kim et al. 2002; Srivastava and Srivastava 2007; Mishra and Ranjan 2008). The most conventional system used for up-scaling of hairy roots includes air lift bioreactors. In these systems, the oxygen deficiency and concentration gradients are the major challenges which occur due to root clump formation. To deal with such challenges, incorporation of suitable impellers and spargers in reactor configuration needs attention before up-scaling of shear sensitive roots. Rhodes et al. (1986) published an initial report on successful hairy root culture in a bioreactor. However, in early 1990s, the bioreactor technology for hairy root growth and metabolite production has emerged as a promising technology for large-scale setups with companies ready to cultivate hairy root biomass for metabolite production (Curtis 2010; ROOTec bioactives Ltd, Switzerland; http://www.rootec.com; CBN Biotech, South Korea). However, the major challange in commercialization of hairy root technology is the low productivity of cultures and overall cost. An incessant input of ideas can be observed in various

reactor configurations that have been advised time to time to overcome limitations of low productivity like shear stress, heterogeneity, mass transfer and nutrient uptake (Kim et al. 2002). Concisely, the reactors are categorized in three groups: submerged or liquid phase, gas phase and hybrid reactors. In submerged conditions, the problem of shear stress and mass transfer in and around the growing root tissue remains at high. The bubble column, liquid impelled loop, and air lift reactors are designed to deal with shear stress and to increase the mass transfer between gas–liquid and liquid–solid boundary. These reactors efficiently sparge and mix the inflowing air in the medium, thus facilitating tissue growth (Srivastava and Srivastava 2007). The convective flow, rotating drum, and ebb and flow reactors have been designed to check the bubble entrapping in submerged conditions. These reactors sufficiently reduce the localized O_2 depletion and the nutrient gradient in culture medium. Further, to address the major issue of cost effectiveness, the gas phase and hybrid reactors for example trickle bed, radial flow, droplet phase, and nutrient mist have been introduced. To reduce power input in these reactors, the medium is supplied from the top/sides of the reactor in the form of nutrient mist or spray. As the medium flow is influenced by gravity, the availability of liquid is relatively uniform in the reactor. Among these, the mist reactors are more efficient as mist reduces the thickness of the liquid film deposition on the root surface, thus helpful in overcoming the localized nutrient/gas transfer limitation upto an extent. Simultaneously, the idea of using a reactor configuration having shared advantages of submerged and gas-phase reactors developed the scientific interest. Inoculating the reactor under submerged conditions allows roots to freely circulate and anchor on anywhere in the interior of the vessel, thus distributing evenly in the reactor. When the tissue grows enough such that the submerging appears no longer effective, the reactor operation changes to gas phase to get the benefits of a gas-phase reactor. At present, modified reactors with shared advantages of submerged and gas phases are being used to grow hairy roots (Mishra and Ranjan 2008; Curtis 2010).

To sum up, majority of efforts have been taken to make the hairy root bioreactor technology feasible at a large scale for high biomass production. It is particularly relevant to state that laboratory up-scaling should not only intend to show that the same yields would be obtained during industrial-scale operation, rather, its important purpose should be to test the practical feasibility of existing technology at industrial scale. Although the generalized concept for bioreactor technology lies in the proportionate production of biomass with that of vessel size, the reality is far behind. This is because with the increase in vessel size the homogenous culture ambience remains no longer effective, and various physical factors like gas and liquid flow rates, mass transfer rate, concentration gradients, etc., start affecting the tissue growth simultaneously. The laboratory bench-scale experimentation aimed to explore these issues will result to a protocol with desired productivity and production cost. With this view, nowadays, bioreactor technology utilizes engineering principles and mathematical formulations for mass production. Various research groups are working on issues like optimization of physical, biological, and chemical culture conditions (Prakash et al. 2010; Mehrotra et al. 2013a; Stiles and Liu 2013), offline and/or online measurement of growth (Uozumi 2004), mass transfer behavior (Liu et al. 2011), synergistic effects of various physical and chemical parameters on growth, downstream processing (intracellular/extracellular), and product recovery (Bhagyalakshmi and Thimmaraju 2009; 2012) in the course and/or at the end of scale-up. This may fairly help in filling the gap between capital cost and the benefits of technology at industrial scale (Stiles and Liu 2013). Recently, hairy root cultures are also used for bioremediation of toxic compounds and the production of recombinant proteins at large scale (Liu et al. 2009; Sosa Alderete et al. 2012). Thus, a combination of bioreactor technology with other hairy root-based research applications will definitely extract scientific and technological solutions to the important socioeconomic issues like environment and health. Additionally, use of computational biology and engineering skills to design and operate pilot-scale culture vessels will be advantageous in terms of overcoming limitations like mass transfer and shear stress. This will definitely lead to a completely automated hairy root-based commercially viable low-cost production system.

Growth simulations and modeling

Hairy roots are being used as a model system to understand the correlation between tissue growth and nutrient consumption. A continuous flow of research can be observed in the past few years, which reveals the development of several mathematical models that efficiently describe the nutrient uptake, mass transfer, and gas distribution in different layers and their effect on tissue growth (Mairet et al. 2010; Liu et al. 2011; Palavalli et al. 2012). As known, the tissue attains a nonlinear growth pattern, and one cannot understand the process by focusing only on a single or couple of growth parameters on any one scale. Keeping this in mind, researchers have to focus the temporal changes occurring at different levels like genomic, physiological, and biochemical to completely model the growth process. This leads to the huge influx of data, the exploitation of which requires an interdisciplinary approach of mathematical calculations and computer simulations. An accurate picture of ‘culture in real time’ at any scale, stage, or condition would be immensely helpful in eliminating the possible limitations during the growth. Modeling the tissue growth by measuring osmolality (concentrations of different components) and the conductivity of the medium during culture are the examples of mathematical estimation of root

growth through fresh weight time course data (Leduc et al. 2006; Cloutier et al. 2008). Another relevant example that uses the mathematical calculation to study the biomass increment based on population balance approach has also been proposed (Han et al. 2004). Such examples of inclusion of interdisciplinary approach would definitely provide new dynamism and dimensions to the HRBP.

Elicitation in hairy root cultures

Among various biotechnological strategies that have been hypothesized and applied to the productivity enhancement, elicitation is recognized as the most practical strategy for hairy root-mediated secondary metabolite production. A literature survey of the past 20 years indicates that elicitation is being utilized extensively for increased metabolite production from hairy roots (Guillon et al. 2006a; Georgiev et al. 2007; Fig. 1). Nevertheless, a parallel expansion of research in this domain can be observed during the last decade only to uncover the physiological and molecular aspects of root-specific responses during stress and pathogen challenge (Guillon et al. 2006b; Goel et al. 2011). To visualize the changes in morphology and cytoskeletal dynamics of root hair cells during host–pathogen interaction and in planta functional analysis of receptor-mediated recognition and signal processing within the cell, the HRCs are being used as a model system (Weerasinghe et al. 2005; Madsen et al. 2011). This is only because HRCs are easily manageable and closely mirror the natural molecular behavior of roots under controlled physical and nutritional environment. Hence, revelation of signaling network in HRCs is helpful to define the interaction between signal transducers and functioning/regulation of secondary metabolite biosynthetic genes. In previous years, the transcriptome and metabolome analysis of elicited roots has opened novel and exciting perspectives in identifying rate-limiting steps, transcriptional and post-transcriptional regulation of metabolite synthesis and accumulation. For example, transcriptome analysis of methyl jasmonate-treated and non-treated hairy roots of *Panax ginseng* resulted in the detection of expressed sequence tags (ESTs) and the identification of genes involved in the biosynthetic pathway of ginsenosides and other secondary metabolites (Choi et al. 2005; Sathiyamoorthy et al. 2010; Wang and Wu 2013). Similarly, the molecular analysis of *Salvia miltiorrhiza* hairy roots treated with mycelium pellet of *Streptomyces pactum* Act 12 revealed tanshinone biosynthetic genes that were upregulated (Yan et al. 2014). Thus, through 'omic' analysis of hairy roots under different elicitor treatments, differential expression patterns of plant SM biosynthesis and stress-related genes can be exposed. Such set of genes can be identified through modern techniques and will provide crucial information regarding the function and regulation of gene or a set of genes under specific stress or elicitation challenge. In this regard, microarray-based screening of differentially expressed genes in the fungus, drought-challenged peanut (Oktem et al. 2008), and T- DNA activated and tagged hairy roots of *Arabidopsis*, *Solanum*, and *Nicotiana* can be considered as relevant examples (Seki et al. 2005; Guillon et al. 2006a, b). These examples show a clear transition of hairy root technology towards the new domain of functional characterization of genes related to stress or elicitor challenge in the near future.

Precursor feeding

In precursor feeding, the biosynthetic activity of cells is stimulated through external supplementation of pathway precursor, in the stepwise conversion of which synthesis of desired metabolite takes place. An exogenous supplementation of such precursor to the culture medium may improve the end product (desired metabolite) synthesis/accumulation. However, the process would become costly if the chemical or natural obtainment of the precursor molecule itself is difficult. To circumvent limitations like precursor unavailability or surplus availability that creats negative feedback, designing the metabolic engineering strategies to amend the expression of related genes may optimize the adequate biosynthesis of the precursor molecule (Morgan and Shanks 2000; Goklany et al. 2009). Another judicious alternative and relatively simple method is the co-cultured hairy root systems. In such systems, hairy roots, together with a different in vitro system that produce and secret the compound which can act directly act as a precursor to metabolite synthesis in hairy roots or elicitate the root tissues to produce the metabolite, can be cultured (Lin et al. 2003; Wu et al. 2007). In the same context, precursor feeding in association with elicitation provided significant results in HRCs (Shinde et al. 2009; Verma et al. 2014). An elicitor molecule can also stimulate the gene(s) related to precursor biosynthesis to optimize precursor accumulation or the gene(s) within the biosynthetic pathway, activation of which enable the tissue to efficiently utilize the externally fed precursor. Thus, precursor feeding in HRCs is not only a yield-enhancing strategy, but when coupled with elicitation and engineering approach, it can provide a suitable milieu to study the molecular controls associated with biosynthetic flux and metabolic rate limitations.

Metabolic engineering of root-based secondary metabolic pathways

Metabolic engineering (ME) is known as one of the most important research domains which utilizes engineering principles for desired alterations in metabolic pathways to raise the product flux. Besides simple productivity enhancement strategies like elicitation, modification in culture conditions and medium compositions, etc., often systematic and deliberate amendments in the genetic stature of the cell, is needed to increase secondary metabolite production/accumulation. Utilizing HRCs, such

amendments are being made either through over-expression of a single gene leading to an increase in concerned enzyme activity, thereby resulting in accumulation of the target chemical (single-step engineering), or through over-expression of multiple genes of target biosynthetic pathway (multi-step engineering) (Mehrotra et al. 2010, 2013b; Zhou et al. 2011). These exercises result in overcoming rate-limiting step(s) in the pathway, barring competitive pathways, and/or reducing the catabolism of the desired product. Alternatively, scheming the expression of regulatory genes that control multiple biosynthesis genes is also helpful in regulating product synthesis/accumulation (Peebles et al. 2009; Chandra and Chandra 2011). However, in a root-based multifaceted metabolic network, it is difficult to regulate the whole pathway by altering the function of a single or a set of genes. As a known fact, gene functioning within the cell is not an independent phenomenon; rather, it is linked with and regulated by a number of synchronized molecular events and their products. At this point, a cross examination of hairy root cell under various developmental stages with the help of transcriptome and metabolome analysis can expose the complex interplay between genes and metabolites. At this juncture, the role of RNAi-mediated post-transcriptional gene silencing needs attention. The technology deals with transient RNA silencing using *Agrobacterium*-mediated hairy root transformation (Gherbi et al. 2008). Although the approach is less explored in HRCs yet, it has paved the way for debarring the competitive pathways in a multichannel biosynthetic network and loss of function analysis of related genes. The global 'omic' repositories prepared for related biochemical pathways occurring in different plant species, microbes, and even in animal cells can be used to retrieve information about biosynthetic and regulatory genes and their products. This will be helpful in understanding the crosstalk between gene expression and metabolic flux. Thus, metabolic engineering is the area that provides extensive opportunities to explore molecular insights on metabolite production utilizing the versatility of HRCs. While the integration of bioreactor technology and hairy root-mediated transgenic plant production in genetically engineered roots needs to be materialized, yet it shows strong future perspectives. Reports which show the production of transgenic plants exhibiting desired traits from genetically engineered HRCs are available (Zamanzedeh and Ehsanpour 2011).

Hairy root-based value-added applications

On the whole, HRCs are used to investigate secondary metabolite production, growth, physiology, and molecular communications within and/or outside the cell under different environmental conditions. Nevertheless, with time, this system has also emerged as a suitable matrix to explore a broad range of biotechnological applications (Guillon et al. 2006a; b; Georgiev et al. 2010; Ono and Tian 2011).

Biotransformation

HRC-based biotransformation is the structural and/or conformational alteration of molecules (natural or synthetic) into its analogs through the enzymatic machinery of root tissue. Such modifications lead to the required changes in physiochemical and therapeutic properties of parent molecules. Because of in vitro conditions, these modifications are independent of seasonal and pathological constraints. In spite of an initial report on ginseng hairy root-mediated conversion of digitoxigenin in 1990 (Kawaguchi et al. 1990; Fig. 1), major research in hairy root-mediated biotransformation occurred in the previous decade. HRCs are advantageous over other in vitro systems due to ingrained characteristics of genetic/biochemical stability, hormonal independence, efficient enzyme paraphernalia, and low-cost cultural requirements. Additionally, avoiding hazardous chemicals and reagents, hairy root-mediated biotransformation ensures the sustainable use of resources under defined culture conditions. Recently, seven distinct kinds of biotransformation reactions like glycosylation, glucosylation, reduction, condensation, etc., were classified as major reaction types that have been reported (Banerjee et al. 2012). Besides, HRCs of some plants like *Pharbitis nil*, *Brassica napus*, and *Atropa belladonna* bear the potential of more than one type of chemical conversion simultaneously (Banerjee et al. 2012; Srivastava et al. 2012, 2013). These abilities actually strengthen the use of HRCs as a model system for in vitro biotransformation of exogenous substrates. In a recent study, HRCs of *Rhodiola kirilowii* were found to be more potent in synthesizing rosavin when compared to the wild-type non-transformed roots (Grech-Baran et al. 2014). However, the current requirement involves systematic discovery of the enzymatic machinery of hairy roots involved in biological transformations. Identification and characterization of enzymes and related genes involved in biotransformation will lead to their isolation and their further utilization through engineering approaches. Accordingly, in future line of action involving biotransformation and HRCs, efforts would be focused to enhance the built-in potential of transforming an exogenous molecule in a transgenic root line through over-expression of the concerned enzyme. Moreover, culturing hairy roots in bioreactors for the production of structurally modified molecules of commercial importance would be an ideal alternative to root-based biochemical production at commercial-scale setups.

Remediation

Genetically unwavering and fast growing nature of HRCs made them superior choice over intact plants and other

isolated organ cultures for studying plant interaction with various organic and inorganic contaminants. As an isolated in vitro systems, HRCs evade the interference of translocation of compounds to other tissues, soil matrix, and also interactions with rhizosphere microbial communities. A facsimile of whole plant, these hairy roots would be an ideal system to study phytostabilization or rhizofilteration (accumulation of pollutants on root surface followed by their precipitation and complexation), rhizo/phytodegradation, phytoaccumulation, or phytoextraction (uptake and accumulation of pollutant), phytotransformation (uptake and metabolism or transformation of pollutant utilizing cellular enzymatic flow followed by their compartmentalization either simply or in conjugated form), and phytovolatilization (uptake and volatilization of pollutants via transpiration mechanism). However, to date, only phytoaccumulation and phytodegradation of toxic compounds have largely been focused. The literature survey reveal that during accumulation and degradation of toxic compounds, HRCs use their inherent enzyme system. HRCs of *Daucus*, *Lycopersicon*, *Tagetes*, and *Brassica* have shown the involvement of endogenous peroxidases, laccase, and H_2O_2 in uptake and transformation of hazardous compounds like phenols and dyes (Zhou et al. 2013). Besides organic pollutants, HRCs also show accumulation of metals and thus offer their use for in vitro analysis of biological mechanisms responsible for the high tolerance of hyper-accumulator plant species. For example, HRCs of Cd hyper-accumulator plants *Thlaspi caerulescens* and *Hyptis capitata* and Ni hyper-accumulator plant *Alyssum bertolonii* provide apparent information regarding Cd and Ni uptake and tolerance, respectively, through antioxidative defense and the use of organic acids present in the cells (Boominathan and Doran 2003a, b; Needlekoska and Doran 2000). Nevertheless, HRC-mediated phytoremediation is a less-explored area and restricted to the laboratory only. The production of transgenic plants and wider expansion of this technology to real contaminated sites is still a big dream. In this context, thorough knowledge base is required with regard to various detoxification mechanisms used by the plants to survive with xenobiotics in the environment. To strengthen this knowledge and to answer the various mysteries of plant–xenobiotic interaction, HRC-based phytoremediation studies can provide a suitable milieu. To understand plant-based remediation of toxic compounds, numerous important links ask for thorough experimentation and need to be completely explored: (1) most of the studies in which HRCs are used to study remediation generally revolve around externally supplied doses and leftover quantities of chemical in question after certain culture duration. The rationalization of root behavior in terms of cellular activities in the presence of a particular chemical is a missing link; (2) transgenic HRCs expressing candidate genes required in xenobiotic metabolism have been less attempted so far; (3) although HRCs of highly tolerant and hyper-accumulator plant species have been generated, still the physiological and molecular mechanism of pollutant uptake, metabolism, and in planta fate remains a subject of study; (4) retrieval of information from genomic data banks regarding the molecular mechanism of remediation in lower plants, algae, bacteria, etc., and their use in reference to higher plants; and (5) most of the toxic elements are present in the soil in different forms (mono, di, trivalent or conjugated) and their availability to the plant tissue varies. Do the plants have a differential/preferential mechanism to accumulate/degrade different forms of toxic elements? At this stage, considering the arbuscular mycorrhizal fungi (AMF)-assisted hairy root remediation needs attention. In an example, lowering of Cd induced growth inhibition of carrot hairy roots was observed in cultures inoculated with AMF *Glomus intraradices* (Janoušková and Vosátka 2005). Moreover, over-expression of tpx1 (encoding peroxidase) in HRCs of tobacco co-cultured with AMF showed increased protections of tissues against phenol-induced oxidative damage (Ibáñez et al. 2011). This, plant–fungus relationship justifies attention to bring new outcomes in HRC-based remediation. Additionally, the use of integrated approaches like genetic engineering and up-scale culture can also show the way to big success. However, dearth of knowledge of plant detoxification mechanisms is a major technical constraint for focused engineering approach. Solving some of the aforesaid questions will bring well-versed decisions on which genes to engineer and provide new directions for manipulating a suitable in vitro culture system with superior remediation potential.

Molecular farming

Expression and production of recombinant protein in hairy roots have gotten attention during the last decade (Fig. 1). At present, different categories of recombinant proteins like antibodies, cytokines, vaccines, and enzymes are known to be expressed and produced by HRCs of various plants (Obembe et al. 2011; Georgiev et al. 2012). The first ever report of HRC-based pharmaceutical protein production was of full-length murine IgG1 antibody from tobacco hairy roots (Wongsamuth and Doran 1997). In comparison to other (bio) production platforms, the HRCs provide (1) assurance of climate- and contamination-free production of pharmacologically active proteins and (2) easy acetylation, phosphorylation, glycosylation, and other post-translational modifications because of their eukaryotic molecular organization. These post-translational modifications are required for the stability and activity of synthesized protein. HRC-based foreign protein production requires two major issues to be focused: first, the high-quality, stable gene expression, and second, the safe downstream processing, out of which the latter exercise is tedious and expensive due to low accumulation level and structural instability of the synthesized protein. Continuous

efforts for inclusion of strong constitutive promoters such as double-enhanced CaMV35S promoter (de35S), inducible promoters, a chimeric super-promoter ($(Aocs)_3A$*mas*P*mas*), and translational enhancers in order to stabilize the desired expression of the transgene are in progress (Nopo et al. 2012; Georgiev et al. 2012). On the other hand, constant supervision of the culture conditions for growth of transgenic roots is a prime necessity as culture environment significantly influences transgene expression. Additionally, development of secretion-based HRCs for easy processing have also been attempted (Gaume et al. 2003; Komarnytsky et al. 2006; Pham et al. 2012). To improve the feasibility of the process, addition of stabilizing agents like polyvinylpyrrolidone and NaCl to the growth medium can also be considered, which will support the stability of secreted protein, thus making the extraction easy. Provision of an integrated metabolic trap strategy will also definitely facilitate the yield and downstream processing of recombinant protein synthesized in hairy root tissues. At the same time, a combined approach of using bioreactors for HRCs producing recombinant proteins will certainly give a new strength to the industrial-scale utilization of this research. Furthermore, the problems of high cost, harmful contamination, and storage of recombinant products that generally occur in microbes and animal-based expression systems will get a solution through transgenic hairy root-based regenerants.

Ri-mediated non-transgenic and transgenic plant production

The hairy root-mediated regenerants possess normal and/or altered phenotype, and this phenotypic alteration occurs due to the presence of TL-DNA (Christey and Braun 2005; Makhzoum et al. 2013; Mehrotra et al. 2013c). Out of 18 ORFs of TL-DNA, four, viz., ORF 10, 11, 12, and ORF 15 (corresponding to four rol gene loci rol A, B, C, and D, respectively), are responsible for hairy root induction. Individual and/or combined expression of these rol genes contribute to the phenotypic alterations in regenerants. The Ri-mediated regenerants can be non-transgenic (contain rol genes) or transgenic plants (plant carrying foreign gene coding for specific trait along with rol genes). Plants regenerated from hairy root explants can be beneficial in various applications (Fig. 2). Introduction of agro-economically useful morphological traits such as improved flowering and robust branching in ornamentals or in plants having economic importance of aerial parts, improved nutritional and biochemical value, molecular farming, superior physiological traits such as abiotic/biotic stress tolerance, and hyper-accumulation and/or metabolism of organic and metal pollutants is the current area of research using hairy root-mediated regenerants. However, notwithstanding the advantages of transgenic plants as a production system for high metabolite or recombinant proteins, several issues are yet to be addressed in order to make this technology platform widely accepted as a mainstream alternative to traditional production systems. Although transgenic plants with improved traits are of great importance, yet the large-scale commercial release of a transgenic crop requires authoritarian supervision that greatly varies from one country to another. The risk analysis of releasing a transgenic variety should deal on first priority with the scientific assessment of the probability of specific harmful effects followed by decision making which will suggest that the risk is acceptable. However, the acceptability must depend upon the social, economic, and ethical policies of the place along with the ability to manage the risk. Although success in obtaining HRCs mediated transgenic plants for commercial cultivation is in its nacent phase yet, in spite of all such scruples, research is continued and trials are being made for the development of HRC-based transgenic plants bestowed with fine characters.

Conclusive remarks: direction for future research

A new area of biotechnology-based research was evolved with the establishment of hairy root culture system during early 1980s. Paradigm transition has been observed in using plant underground resources as such to exploit them for unearthing various plant-based interactions and physiological, biochemical, and molecular phenomena. The hairy roots were developed in the laboratory to mimic the natural phenomenon of bacterial gene transfer and occurrence of disease syndrome. However, hairy root cultures have now become a most-sought-after technique and are being exploited around the globe for basic as well as applied research purposes. The technique is commercially applicable not only for secondary metabolite production but also for certain value-added purposes such as remediation of toxic elements and recombinant protein production. For better understanding, the vast area of ongoing research in HRCs can be hypothetically divided into (a) elemental research domain where HRC-mediated secondary metabolite production and various productivity enhancement strategies are explored and (b) functional research domain that deals with HRC-based applied research (Fig. 2). Further, the HRC-based functional research can have two branches, out of which one includes the exercises related to the introduction of agroeconomical traits in plants, whereas the second branch includes different prolific research such as phytoremediation, biotransformation, and molecular farming using HRCs (Fig. 2). The timeline representation of hairy root research reveals that much emphasis has been given to the strategies that are helpful in making this technique practically feasible in terms of high productivity at low cost as well as for other value-added applications. In this regard, bioreactor technology has an important role. Be it the area of hairy root-based elemental research or functional research, bioreactor

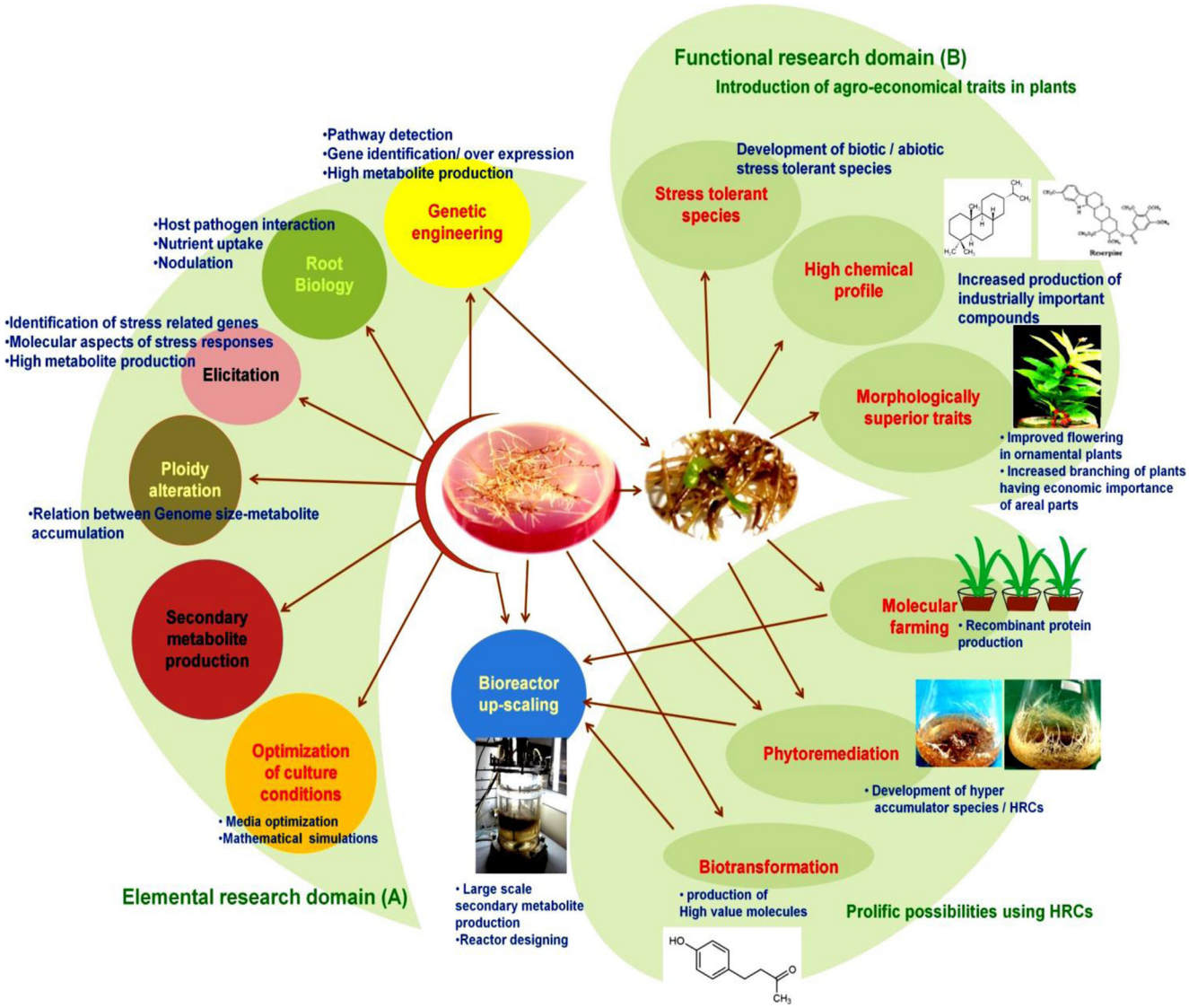

Fig. 2 Research network in elemental and functional research areas of hairy root technology. Elemental research domain (*A*) comprised of hairy root culture-based secondary metabolite production, yield enhancement strategies, and study of root biology (indicated by *arrows*). Functional research domain (*B*) where hairy root cultures can be used to generate transgenic plants with desired agro-economical traits or can be used to pursue prolific research directly or via transgenic plants (indicated by *arrows*). *Arrows* also indicate that the bioreactor technology can play an important role in elemental as well as prolific research through up-scaling of normal/transgenic hairy roots

technology offers ceaseless opportunities (Fig. 2). Recently, hairy roots with different ploidy levels have been cultivated in bioreactors for secondary metabolite production (Marchev et al. 2012). This indicates that merging of different approaches with large-scale culture using bioreactors will give successful outputs. Thus, the need is to develop techniques that can envisage the growth kinetics of HRCs and thermodynamics of culture conditions in such a reliable way that can significantly influence the complex developmental pattern of HRCs at mass scale. For this purpose, involvement of interdisciplinary platforms of computational biology and mathematical simulations would be the future talk.

It is pertinent to discuss the cryopreservation of HRCs as another striking line of work that can be explored in the future. Currently, when companies (e.g., CBN Biotech, South Korea; ROOTec bioactives, Switzerland) are showing interest in commercial-scale production of hairy root biomass, the cryopreserved root tips provide solution not only for the contamination-free maintenance of explants but also for storing the germ plasm of important plant species. Moreover, hairy roots re-grown after preservation can be used as plant material for exploring the plant responses during freezing cold temperature. However, this area will be in need of more attention.

The major understanding of the molecular and physiological phenomenon behind natural gene transfer needs an incorporation of recent technologies such as RNA interference, T-DNA tagging and knock out, fusion protein constructs,

genome specific microarrays, etc. The information obtained with the help of these techniques will provide a clear picture of genes and their functioning. Thus, an integrative methodology is critical for the continued advancement of research. Besides, exploring HRCs as a promising tool for studying plant-based complex interactions during various natural events such as salt, drought, cold/heat, and pathological stress, a much better understanding of the biochemical and molecular machinery of roots is required. For this purpose, new ideas and incorporation of newer techniques to explore the plant behavior is obligatory. The timeline analysis of hairy root research reveals that in the upcoming years, although the HRC-based value-added applications will be given preferential advantage, yet traditional research areas such as hairy root induction, secondary metabolite production, elicitation, genetic engineering, etc., will be explored as well in order to make this technology more commercially feasible. However, major emphasis will be given to the research which will provide a holistic picture based on the molecular basis of this natural gene transfer phenomenon. Besides, serious efforts will be made to achieve more scientific and technical solutions to the major issues related to environment, health, and agriculture.

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