Review

Genetically transformed roots: from plant disease to biotechnological resource

Milen I. Georgiev[1,2,3*,†], Elizabeth Agostini[4,†], Jutta Ludwig-Müller[5,†] and Jianfeng Xu[6,†]

[1] Department of Applied Biotechnologies, Institute of Microbiology, Bulgarian Academy of Sciences, 139 Ruski Boulevard, 4000 Plovdiv, Bulgaria
[2] Natural Products Laboratory, Institute of Biology, Leiden University, 55 Einsteinweg, 2300 RA Leiden, The Netherlands
[3] Plant Cell Physiology, Institute of Biology, Leiden University, 72 Sylviusweg, 2300 RA Leiden, The Netherlands
[4] Department of Molecular Biology, FCEFQyN, National University of Rio Cuarto, Ruta 36 Km 601, 5800 Rio Cuarto (Cba), Argentina
[5] Institute of Botany, Technische Universitat Dresden, Zellescher Weg 20b, D-01062 Dresden, Germany
[6] Arkansas Biosciences Institute and College of Agriculture and Technology, Arkansas State University, Jonesboro, AR 72401, USA

Hairy root syndrome is a disease that is induced by *Agrobacterium rhizogenes* infection and characterized by a proliferation of excessively branching roots. However, in the past 30 years *A. rhizogenes*-mediated transformation has also provided a valuable platform for studying biosynthesis pathways in plants. Furthermore, the genetically transformed root cultures are becoming increasingly attractive, cost-effective options for mass-producing desired plant metabolites and expressing foreign proteins. Numerous proof-of-concept studies have demonstrated the feasibility of scaling up hairy-root-based processes while maintaining their biosynthetic potential. Recently, hairy roots have also shown immense potential for applications in phytoremediation, that is, plant-based decontamination of polluted environments. This review highlights recent progress and limitations in the field, and outlines future perspectives for the industrial exploitation of hairy roots.

From soil to bench: recent progress in hairy root induction and the underlying molecular mechanisms

A century ago hairy root syndrome, crown, and cane gall diseases, which mainly affect dicotyledonous plants, caused substantial losses in vineyards, orchards, and vegetable nurseries. The losses prompted several studies, which revealed that the causative agents of these neoplastic diseases are phytopathogenic *Agrobacterium* strains carrying tumor-inducing (pTi) or root-inducing (pRi) plasmids [1]. However, during the past 30 years, these systems have been exploited by using *Agrobacterium-rhizogenes*-mediated transformations to produce plant-derived molecules, elucidate biosynthetic pathways and physiological processes, generate recombinant therapeutic proteins, assist molecular breeding, and enhance phytoremediation efforts.

The molecular mechanisms involved in the formation of transformed roots (widely called 'hairy roots'; HRs) are not yet fully understood. Nevertheless, the genetic transformation process (reviewed in [2,3]) can be divided into the following steps: (i) *Agrobacterium*-sensing phenolic compounds released by roots, triggering attachment of the bacteria to root cells; (ii) processing the transferred DNA (T-DNA) into bacterial cells and T complex (T strands and associated proteins) formation; (iii) transfer of T complexes (via the type IV protein secretion system; T4SS) from the bacteria to the plant host genome; (iv) T-DNA integration and expression in the plant genome; and (v) HR formation.

Several loci of the *vir* region of the pRi, T-DNA and chromosomal virulent (*chv*) genes are essential for successful transformation. These include *VirD1* and *VirD2*, which encode proteins that bind to and nick DNA at 25-bp T-DNA border repeat sequences. Proteins encoded by *virE1* and *virE2* genes are also important, because they protect T strands from nuclease attack and facilitate their integration into the plant chromosome, although some *A. rhizogenes* strains lack these genes but still transfer T strands efficiently due to the presence of the pRi *GALLS* gene, which encodes a protein with helicase activity and a nuclear localization signal [1,4]. The T-DNA has two independent sequences, denoted left (T_L) and right (T_R) borders [3]. T_L-DNA and T_R-DNA are generally independently transferred and integrated into the host plant genome, but only the T_L-DNA is essential (and sufficient) to induce HRs. Sequence analysis of T_L-DNA has revealed four open reading frames (among others) that are essential for HR induction (*rolA*, *B*, *C*, and *D*). The products of the *rolABCD* genes all play key roles in HR formation, but the *rolB* gene appears to be the most important in HR induction, because loss-of-function mutation at this locus renders the plasmid avirulent (reviewed in [5]). Recently, *rolB* was shown to suppress reactive oxygen species (ROS), thus it plays a role not only in cell differentiation, but also in ROS metabolism [6].

Laboratory protocols for HR induction involve cultivation of a wounded sterile explant that has been either directly inoculated or co-cultivated with an *A. rhizogenes* suspension, and then treated with antibiotics to eliminate the bacteria (Figure 1a). The resultant neoplastic HRs

Corresponding author: Georgiev, M.I. (milengeorgiev@gbg.bg)
* Current address: Department of Applied Biotechnologies, Institute of Microbiology, Bulgarian Academy of Sciences, 139 Ruski Boulevard, 4000 Plovdiv, Bulgaria.
† All authors contributed equally to this review.

 0167-7799/$ – see front matter http://dx.doi.org/10.1016/j.tibtech.2012.07.001

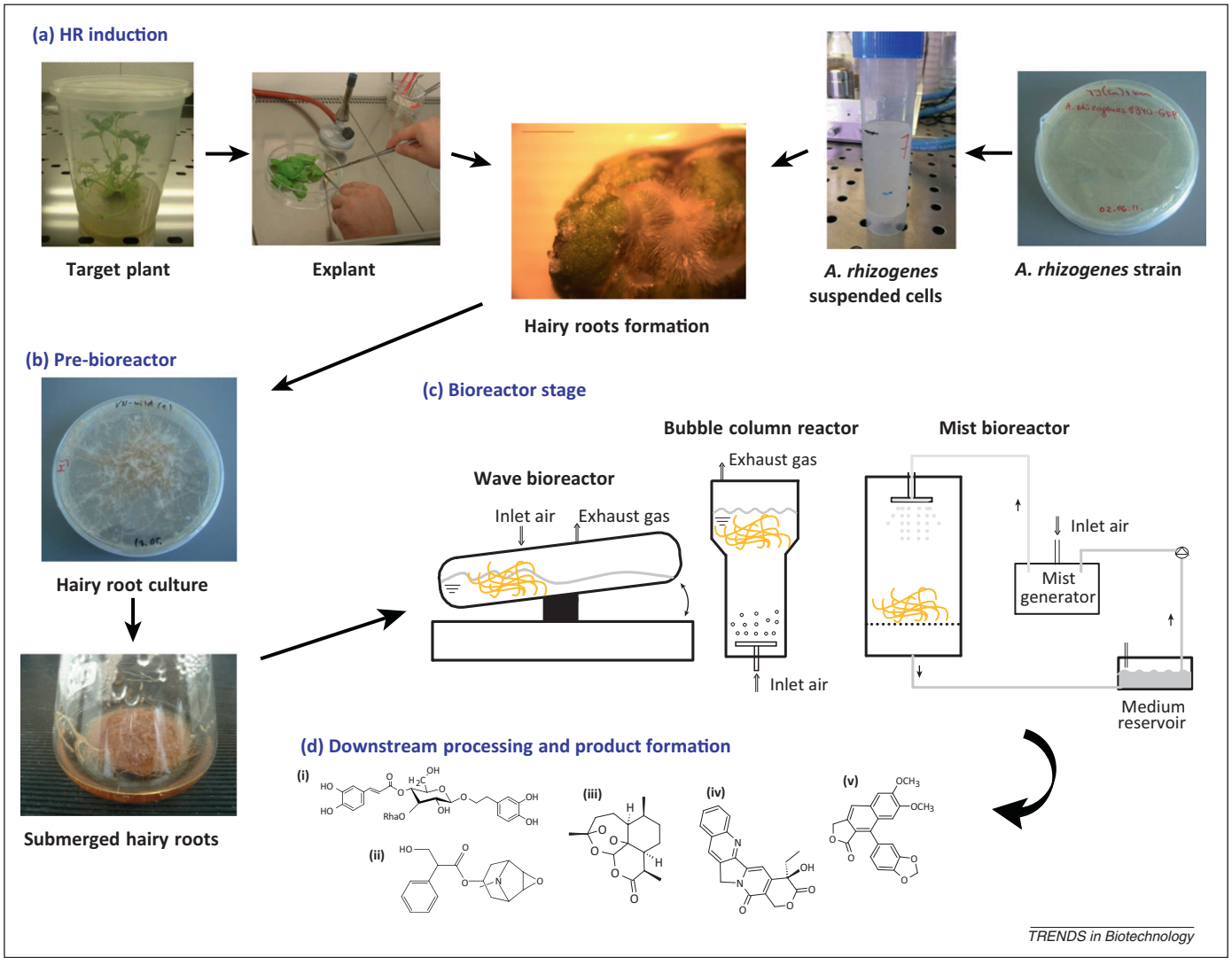

Figure 1. State-of-art technological platform for hairy root (HR) culture. **(a)** HR induction: transformation efficiency depends on many factors, for example, type of explant and age, type and density of *Agrobacterium rhizogenes* strain. Acetosyringone addition promotes HR induction. Of special significance at that stage is the elimination of bacteria with proper antibiotics. **(b)** Pre-bioreactor: several strategies have been applied for exploiting the secondary metabolism of HRs and to boost the yields of desired molecules, for example, selection of high-producing lines, inoculum optimization, metabolic engineering approaches, nutrient medium optimization, elicitation, two-phase systems cultivation, and permeabilization (reviewed in [7,10–12]). **(c)** Bioreactor stage: hardware configurations, bioreactor operation mode (batch, fed-batch) along with culture conditions optimization (e.g., air flow rate, temperature) are crucial for successful scaling up of HR-based bioprocesses (key up-scaling parameters include air flow rate and K_La). Example configurations given are the disposable mechanically driven wave reactor, pneumatically driven bubble column bioreactor and gas-phase bed (so-called 'mist bioreactor') reactor. **(d)** Downstream processing and product formation: examples resulting in valuable molecules are: (i) verbascoside, (ii) scopolamine, (iii) artemisinin, (iv) camptothecin, and (v) justicidine B.

grow in a profusely branched manner, producing many lateral branches, on hormone-free media [5,7]. They are generally subjected to PCR (using primers designed to amplify *rol* and *VirG* genes), southern blot hybridization and/or northern blotting to confirm that the roots have been genetically transformed and the HR-inducing bacteria have been eliminated [7]. Recent advances in HR induction include the introduction of a new technique – sonication-assisted *A. rhizogenes*-mediated transformation (SAArT) – which is particularly suitable for inducing HRs from recalcitrant plant species (e.g., monocotyledonous species). Using SAArT, *Verbascum xanthophoeniceum* HRs have been induced recently (significantly more rapidly than when using other tested procedures) for producing bioactive phenylethanoid and iridoid glycosides [8].

Clearly, having induced HR strains, it is generally desirable to maintain them. There are several ways to do this, and the choice has both practical and financial implications. Monthly subculturing on solid media may be essential for ongoing experiments, but it is expensive and time-consuming, and creates high risks of contamination and eventual losses of original strains [5]. Preservation of HR strains by cryoprotection (e.g., slow cooling, vitrification, encapsulation–dehydration, and encapsulation–vitrification) may avoid these problems [9].

This review summarizes recent advances in the field of HR research with particular emphasis on: utilization of HR culture as green factories for value-added products (e.g., plant-derived metabolites and foreign proteins); metabolic engineering of HRs and elucidation of biosynthetic pathways; and the development of suitable bioreactor configurations for HR-based processes. Recent trends in applications of HRs in phytoremediation are also considered.

HR cultures: green factories for high-value molecules

Currently, the main HR applications include mass production of high-value molecules (e.g., plant-derived metabolites and therapeutic proteins); biotransformation; phytoremediation;

molecular breeding; and elucidation of physiological processes and biosynthetic pathways [5,7,10–12]. The enormous biosynthetic potential of HRs was largely neglected in early research, which mainly focused on the mechanisms underlying the HR syndrome. However, HRs are now considered as green factories for mass producing valuable molecules, because they have several attractive features, including high genetic and biochemical stability, and (relatively) fast growth rates in hormone-free media [7]. Examples of high-value molecules produced by HRs with anticancer (camptothecin and justicidine B), antimalarial (artemisinin), anti-inflammatory (verbascoside) and anticholinergic (scopolamine) properties are presented in Figure 1d. Further examples are discussed below and in other recent reviews [5,11,12]. Another interesting feature of HRs is that they may accumulate metabolites that are not detectable in mother plants [13,14], and thus contribute to the diversity of natural products.

Metabolic engineering of HRs and elucidation of biosynthetic pathways: holistic approaches

The soilborne pathogen *A. rhizogenes* can be used to induce many plant species to form HRs *in vitro* (to date >500 plant species have been successfully transformed), and the resulting organs provide more natural compartments for secondary metabolite synthesis than cell cultures [5,11]. However, before starting experiments to improve yields of desired metabolites, the metabolite profiles of the HRs should be compared to those of the native plants, using appropriate metabolomic approaches (e.g., GC-MS, LC-MS, and especially NMR-based methods) to assess their metabolite complements as comprehensively as possible, and/or more targeted metabolite analysis [15]. In some cases, the production of desired metabolites can be increased simply by changing growth conditions or using appropriate elicitors [7,10–12]. Alternatively, these approaches can be combined with metabolic engineering, using *A. rhizogenes* to transform the host plant with heterologous genes to alter secondary metabolite production, as well as the bacterial genes needed for HR induction. In addition to modifying naturally occurring pathways, the expression of completely foreign genes can lead to the production of novel desired secondary metabolites.

It is essential to know as much as possible about the biosynthetic pathway for a given plant metabolite, including rate-limiting steps, control elements and possible side routes; all of which are prime targets for increasing desired metabolic fluxes (reviewed in [5,16]). A problem can arise from cosuppression phenomena, when genes from the same plant are highly expressed; potentially leading to reductions instead of increases in the production of target metabolites [5,17]. This can often be overcome by using heterologous genes to increase fluxes. Heterologous genes from plants that have been sequenced can also be valuable for metabolically engineering medicinal plants for which genomic knowledge is scarce. Other obstacles are compartmentation, fluxes into side pathways, and competing reactions for a key substrate. However, such diversions can be suppressed by blocking either the first or the most important step in a competing biosynthetic pathway by RNAi approaches [11].

Many authors have described HR-based metabolic engineering of diverse plant species for enhancing and/or altering the production of secondary metabolites (see [5,7,10–12] for reviews and examples). Here, we illustrate possible modifications for enhancing or changing metabolite patterns using *Catharanthus roseus* HRs (Figure 2).

C. roseus produces the powerful anticancer drugs vinblastine and vincristine, via dimerization of the monoterpenoid indole alkaloids (TIAs), vindoline, and catharanthine (reviewed in [18]). Their biosynthesis begins with the generation of indole and terpene precursors by the shikimate and methylerythriol-4-phosphate (MEP) pathways. The biosynthetic pathway of TIAs is especially highly compartmented, various enzymatic steps occurring in the cytosol, plastid, vacuole, and endoplasmic reticulum [11]. Hence,

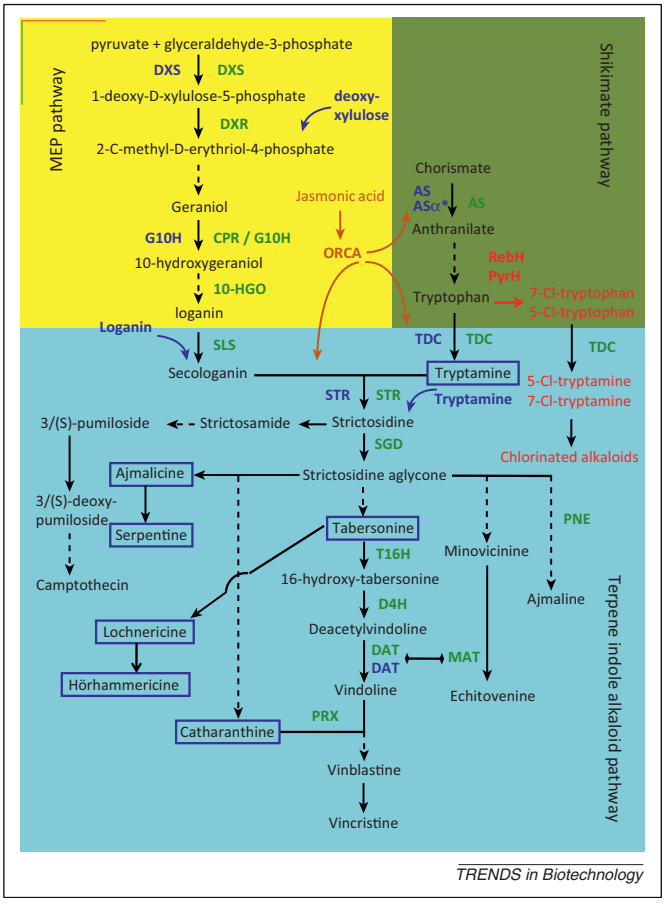

Figure 2. Monoterpene indole alkaloid pathway in *Catharanthus roseus*, showing genetic modifications that have been made (genes encoding native plant enzymes in green; transgenic enzymes from the same plant in blue; heterologous enzymes from another plant in blue, and marked with an asterisk; bacterial transgenes in red), and substrates that have been added to hairy root (HR) systems to increase the flux. Compounds that were increased in genetically modified lines are indicated by the corresponding genetic modification color (when not native to the plant) or blue boxes (if native). Elicitors such as jasmonic acid also increase flux through the pathway by inducing transcription factors (in orange). Unbroken arrows each indicate a single enzymatic step, broken arrows indicate multiple steps. The biosynthesis scheme is modified after [16] with additions (see references in the text). Abbreviations: AS, anthranilate synthase; ASα, anthranilate synthase subunit α; CPR, cytochrome P450 reductase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline acetyltransferase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; G10H, geraniol-10-hydrolase; 10-HGO, 10-hydroxygeraniol oxidoreductase; MAT, minovincinine-19-hydroxy-o-acetyltransferase; MEP, methylerythriol-4-phosphate; ORCA, octadecanoid-derivate responsive *Catharanthus* AP2-domain protein; PNE, polyneuridine aldehyde esterase; PRX, peroxidase; SGD, strictosidine β-D-glucosidase; SLS, secologanin synthase; STR, strictosidine synthase; T16H, tabersonine 16-hydroxylase; TDC, tryptophan decarboxylase.

numerous transporters are also involved. A key enzyme for the pathway is tryptophan decarboxylase (TDC), but several upstream enzymes can also be targets for genetic engineering. *C. roseus* HR cultures expressing various forms of anthranilate synthase, including both the native [19] and feedback inhibition-resistant form from *Arabidopsis thaliana* [20], and/or TDC have been generated, with enhanced fluxes through the tryptophan branch of the monoterpene indole alkaloid pathway, and hence, enhanced levels of tryptamine and serpentine [19]. Overexpressing anthranilate synthase (AS) in combination with feeding the terpenoid precursors 1-deoxy-D-xylulose, loganin, and secologanin results in increased hörhammericine levels, whereas loganin feeding increases catharanthine, ajmalicine, lochnericine, and tabersonine levels [21].

Increasing expression of the gene encoding strictosidine synthase (STR), an enzyme that catalyzes another rate-limiting step for TIA biosynthesis, also enhances the synthesis of downstream metabolites, and their production is further enhanced if a precursor (loganin and/or tryptamine) is added [18]. In addition, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) overexpression reportedly results in significant increases in ajmalicine, serpentine and lochnericine production, accompanied by a significant decrease in tabersonine production, while co-overexpression of DXS and geraniol-10-hydrolase (G10H) (or DXS and ASα) results in significant increases in ajmalicine, lochnericine, and tabersonine levels [22]. Tabersonine is a direct precursor for the anticancer drugs vinblastine and vincristine (Figure 2), therefore, its increase could be beneficial for their production, whereas increased production of side products such as lochnericine could result in less precursor for the desired compounds. However, a potential bioactivity for all compounds that can accumulate in the genetically altered pathway has not been fully elucidated. These findings illustrate the potential utility of modulating the activities of several enzymes simultaneously. Expression of the gene catalyzing the terminal step of vindoline biosynthesis, deacetylvindoline acetyltransferase (DAT), also alters the alkaloid profile of *C. roseus* HRs, leading particularly to horhammericine accumulation, possibly via a negative interaction of DAT with minovincinine-19-hydroxy-o-acetyltransferase (MAT) [23]. Furthermore, RNA-mediated suppression of tryptamine biosynthesis in *C. roseus* HR culture can eliminate all production of monoterpene indole alkaloids, and be used to generate a spectrum of unnatural products by introducing tryptamine analogs to the media of tryptamine-silenced HRs [24].

In addition, bacterial genes have been used to alter the metabolite spectra of HRs. Notably, *C. roseus* HRs have been successfully transformed using bacterial halogenase genes [25]. Transfer of two of these halogenases, PyrH [26] and RebH [27], which chlorinate the indole ring of tryptophan at the five and seven positions, respectively, yields a different spectrum of halogenated metabolites. These approaches can also be exploited to discover novel bioactive compounds and/or improve the biological value of known natural compounds.

Other possible strategies include the modulation of gene expression using transcription factors and/or stresses, because the biosynthesis of TIAs in *C. roseus* (and diverse secondary metabolites in other species) is induced by many stress factors, including fungal elicitors, UV-B light, and jasmonate. For instance, the expression of TDC and STR is coordinately enhanced by various elicitors, with jasmonate acting as a required intermediate (reviewed in [28]). The role of jasmonate has been confirmed by other studies, showing that it increases levels of the transcription factor family ORCA 2 and 3 (octadecanoid-derivate responsive *Catharanthus* AP2-domain protein), thereby enhancing transcription of several genes involved in the TIA pathway, including AS, TDC, and (most importantly) STR [28,29]. In addition, the STR promoter contains more apparent transcription-factor-binding elements, indicating that the pathway is regulated by multiple transcription factors.

Molecular farming with HRs

Molecular farming refers to the use of genetically modified plants for producing recombinant proteins; in particular, high-value therapeutic proteins. Compared with other expression systems, such as bacteria, yeasts, and mammalian cells, plants have appealing advantages including low upstream costs, safety (no risk of viral contamination), high scalability (in open field cultivation), and eukaryotic post-translational processing machinery, for example, complex glycosylation [30,31]. Recently, increasing concerns regarding regulatory compliance and product safety have caused a resurgence of interest in molecular farming using *in vitro* or contained culture systems, such as plant cell and organ cultures in bioreactors [30]. These systems provide cGMP-compatible production environments that are more acceptable to the established pharmaceutical industry than open environments [32]. HR systems appear to be among the most attractive *in vitro* culture systems because they combine the merits of both suspension cell culture and whole-plant cultivation. Like suspended cells, HRs propagate rapidly and are grown in sterile, controlled environments, hence they are not affected by the climate, weather or other unpredictable variables and are free from pathogen and herbicide contaminants [33]. However, as more organized organs, HRs present additional benefits, including genotypic and phenotypic stability, with no requirement for exogenous plant hormones [10]. Another important advantage of HR culture systems is the possible extracellular secretion of expressed proteins, rhizosecretion [10,34], which offers a low-cost, convenient method for purifying target proteins from a well-defined, protein-deficient medium (Table 1).

Transgenic HRs expressing certain heterologous protein can be generated either by infecting stably transformed plants (expressing the target protein) with a wild-type *A. rhizogenes* strain or by infecting wild-type plants with a genetically modified *A. rhizogenes* strain harboring binary vectors containing the genes of interest [35]. Since this approach was first used to produce a full-length murine IgG_1 antibody from tobacco HRs in 1997 [36], nearly 20 recombinant proteins, including antibodies [37–39], vaccines [40,41], cytokines [42,43] and other therapeutic proteins and enzymes [32,44], have been expressed in HR systems [45] (Table 2). Examples of proteins

Table 1. Advantages and challenges of HR culture systems compared to other (bio)production platforms

Expression systems	Plant-based platform			Bacteria	Mammalian cells	Refs
	HRs	Suspension cells	Whole-plants			
Advantages	+ High safety + High growth rate + Genotype and phenotype stability + Easy protein separation and purification + Low regulatory concern	+ High safety + High growth rate + Easy protein separation and purification + Low regulatory concern	+ Safety + Unlimited scalability + Low up-front capitalization costs + Options for seed-, fruit-, and/or leaf-based expression + High protein yields in chloroplasts	+ Very high protein yields + High protein quality and authenticity	+ High protein yields + Very high growth rate	[10,30–33,45,47]
Challenges	– Low protein yields – Difficulty in culture scale up	– Low protein yields – Unstable protein expression	– High regulatory concern – Low production cycle – Difficulty in protein separation and purification – Dependence on environmental factors	– High risk of virus contamination – High culture medium costs	– No post-translational modification – Formation of inclusion bodies – Difficulty in protein separation	

Table 2. Summary of recombinant proteins produced by HR cultures, and the systems used

Recombinant proteins	Host plant species	Promoter	Culture approaches	Protein yields	Refs
Antibodies					
Murine IgG_1	*Nicotiana tabacum cv* NT-1	*CaMV35S*	Shake flask Air-lift bioreactor	3.6–18 mg/l 19.8 mg/l	[36,38] [72]
14D9 Murine IgG_1	*N. tabacum*	*CaMV35S*	Shake flask	64.03 mg/l	[37]
Human IgG_1 and IgG_4	*N. tabacum*	*mas2′*	Shake flask	9.7–21.8 μg/g FW/d	[39]
Antigens					
Hepatitis B surface antigens (HBsAg)	*Potato (var. Kufri bahar)*	*$(Aocs)_3$AmasPmas*	Shake flask	97.1 ng/g FW	[40]
Cholera toxin B-surface protective antigen (CTB-spaA)	*Nicotiana plumbaginifolia*	*CaMV35S*	Petri dish	N/A	[41]
Cytokines, growth hormones, and growth factors					
Murine interlukin-12	*N. tabacum* cv Xanthi	*de35S*	Shake flask Mist bioreactor Air-lift bioreactor	0.5% TSP or 434.8 μg/l 5.3 ug/g FW 3.5 μg/g FW	[42,43]
Human epidermal growth factor (hEGF)	*N. tabacum*	*CaMV35S*	Shake flask	2 μg/g FW	[44]
Human growth hormone (hGH)	*Nicotiana benthamiana*	*CaMV35S*	Shake flask	N/A	[73]
Enzymes					
Human acetylcholinesterase	*N. benthamiana*	*de35S*	Shake flask	3.3% TSP	[32]
Human tissue-plasminogen activator (t-PA)	*Cucumis melo* L cv Geumssaragi-euncheon	*CaMV35S*		798 μg/g FW	[74]
Rabbit cytochrome P450 2E1	*Atropa belladonna*	*CaMV35S*	Shake flask	N/A	[75]
Human alkaline phosphatase (SEAP)	*N. tabacum*	*CaMV35S*	Plastic chamber	280 μg/g DW	[34]
β-Glucuronidase (GUS)	*N. tabacum*	*AraHS18.2*	Shake flask	N/A	[51]
Others					
Ricin-B	*N. tabacum* cv Xanthi	*de35S*	Shake flask	N/A	[48]
Thaumatin	*N. tabacum*	*CaMV35S*	Shake flask	2.63 mg/l	[35]
Green fluorescence protein (GFP)	*N. tabacum* cv Xanthi	*CaMV35S*	Shake flask Bioreactor PS	N/A 820 μg/L	[46] [76]
	N. benthamiana	*CaMV35S*	Shake flask	50 μg/g FW	[73]
	Catharanthus roseus	*Gluco-Ind*	Shake flask	N/A	[50]

Abbreviation: *$(Aocs)_3$AmasPmas*, chimeric super-promoter consisting of three copies of the octopine synthase activator (*Aocs*) and one copy of the mannopine synthase activator (*Amas*) located upstream of the mannopine synthase promoter (*Pmas*); *AraHS18.2*, *Arabidopsis* small heat shock protein 18.2 promoter; Bioreactor PS, plastic sleeve bioreactor; *de35S*, double-enhanced cauliflower mosaic virus 35S promoter; DW, dry weight; FW, fresh weight; *Gluco-Ind*, glucocorticoid-inducible promoter; N/A, data not available; TSP, total soluble protein.

expressed at high yields include an acetylcholinesterase produced by *N. benthamiana* HR (3.3% total soluble protein) [32] and a 14D9 murine IgG_1 produced by tobacco HR (64.03 mg/l) [37]. Furthermore, due to the far greater genetic stability of root tissues than suspension cell cultures, production of recombinant proteins can be continued for far longer. For instance, a murine IgG_1 has been reportedly produced at nearly constant levels for 3 years from HRs, while yields sharply declined in suspension cell cultures [38].

To exploit fully the bioproduction potential of transgenic HRs, gene constructs have been strategically designed to include a strong promoter for high-level gene expression and a signal peptide to direct secretion of expressed proteins [46]. The strong constitutive promoter *cauliflower mosaic virus 35S* (*CaMV35S*) has been most commonly

used to drive transgene expression in HRs [47]. Strategies recently developed to enhance transgene expression include use of a double-enhanced *CaMV35S* promoter (*de35S*) [32,48], a chimeric super-promoter (*(Aocs)$_3$AmasPmas*) [40,46] and a 5′-untranslated leader sequence (translational enhancer), such as that from tobacco etch virus (TEV) [43,46] or alfalfa mosaic virus (AMV) [47,49]. In addition, inducible promoters, induced for instance by glucocorticoids [50] or heat [51], have been used to drive controlled gene expression at desired times in HR systems.

Bioprocessing aspects: from bench to plant

The development of an economically viable HR-based commercial process requires detailed knowledge of the physiology of the HR strain, deep understanding of the 'root–culture environment' interactions, and tight control of the whole process [5,7]. Morphological traits of HRs, including their non-homogeneous ('tuft-like') growth and highly branched phenotypes, promote the formation of strong oxygen and nutrient gradients in the tissue and present major challenges to scale-up in bioreactors [7,12]. Monitoring root growth in bioreactors is also challenging. Timely information on the physiological status of the HR is essential for effective control and management of the biosynthetic process, but accurately measuring root growth in bioreactors is difficult, largely due to the impossibility of obtaining homogeneous tissue samples. Measurements of the conductivity, osmolarity and redox potential of the culture medium have been widely used for these purposes, but they only provide indirect estimates of root growth [7].

Bioreactors with diverse configurations (Figure 1c) have been used for cultivating HR cultures, including mechanically driven reactors (e.g., stirred tank, wave and rotating drum reactors), pneumatically driven reactors (e.g., bubble column and airlift reactors), and bed reactors (e.g., trickle bed and mist reactors) [5,7,42,52–54].

Disposable wave bioreactors (which provide wave-induced mixing and aeration), originally developed for highly stress-sensitive animal cell cultures, have been successfully modified in Dr. Eibl's laboratory for hosting HRs from *Hyoscyamus muticus*, *Panax ginseng* and *Harpagophytum procumbens* [52]. The use of disposable reactors, in conjunction with current Good Manufacturing Practices, can minimize complex cleaning, sterilization, validation and overall costs, with consequent reductions in development times and times-to-market for new products [5,52].

Bed reactors also seem to offer suitable environments for hosting HRs. Mist reactors are gas-phase reactors in which thin films of water and nutrients are spayed by ultrasonic systems onto surfaces of HR cultures. Gas-phase reactors can virtually eliminate oxygen deficiency in dense root beds, while providing a low-stress environment [42,53]. Thus, for instance, *Artemisia annua* HRs can accumulate 14.4 g/l biomass in these reactors; transgenic *Nicotiana tabacum* roots accumulate higher amounts of murine interleukin-12 in them than in other tested cultivation systems [42]; and both *A. annua* L. and *Arachis hypogaea* HRs have been successfully up-scaled in 20-l mist reactors [53]. Furthermore, Ramakrishnan and Curtis found that growth of *H. muticus* HRs was substantially higher in a 14-l trickle-bed reactor than in other systems (recording the highest productivity to date, 1.45 g/l/day, and presenting preliminary calculations indicating that scale-up to at least 10 000 l should be feasible [54]).

In contrast to microbial and plant cell suspension-based processes, in which the inoculum is transferred pneumatically, transferring HR inocula from seed reactors to large reactors may be difficult. However, a fed-batch cultivation mode might offer a good solution [5,7]. The recently developed commercial system (10 000 l) for producing ginsenoside from adventitious roots of *P. ginseng* may also provide a solution to the inoculation problem; the inoculation steps prior to the 10 000-l reactors are likely to be readily applicable to HRs, given the high morphological similarities of adventitious root and HR cultures [5].

Applications of HRs in phytoremediation

HRs have proved to be valuable systems for studying key aspects of various phytoremediation strategies, such as phytoextraction, phytostabilization, rhizofiltration and phytotransformation of organic and inorganic pollutants. Phytotransformation, also known as phytodegradation, refers to the take-up and metabolism/transformation of pollutants in plant cells. This involves metabolic cascades, generally including enzymatically catalysed reactions organized in complex networks, that can be subdivided into three distinct phases: transformation (Phase I), conjugation (Phase II) and compartmentation (Phase III), resulting in the detoxification, breakdown and final storage of xenobiotics [55] (Figure 3). In this context, HRs are very useful model systems for studying xenobiotic transformations and the activities of plant pollutant-converting enzymes (e.g., cytochromes P450, peroxidases, and glutathione S-transferases), without interference from the soil matrix or complex interactions with microbial communities in the rhizosphere.

HRs derived from different plant species have been successfully applied for removing phenolic compounds from aqueous solutions, and in some cases for testing the ability of plants to tolerate high levels of pollutants (Table 3). The organized nature of HRs makes them much more amenable than whole plants for cultivation in bioreactors to study removal processes on a large scale, and they can be reused in several consecutive cycles, with high efficiency [56–58]. HRs have provided valuable information about the removal of phenolic compounds, such as the involvement of peroxidase isoenzymes [59]; the nature and compartmentalization of some final metabolic products [57]; the effects of the pollutants and their metabolism on other stress-related physiological processes (e.g., the antioxidative enzymatic machinery and lipid peroxidation); and the influence of rhizosphere interactions, such as mycorrhizal symbiosis, on phenol removal [60]. Recently, the effects of phenol on phospholipid turnover and phospholipase D activity, as a source of phosphatidic acid production, have also been explored using tobacco HRs, thus contributing to our knowledge of the signal transduction pathways triggered by this pollutant [61].

Clearly, it is highly valuable to know the degradation pathway for a given pollutant. Thus, for instance, the ability of various HRs to degrade polychlorinated biphenyls (PCBs) has been studied and some metabolites of a

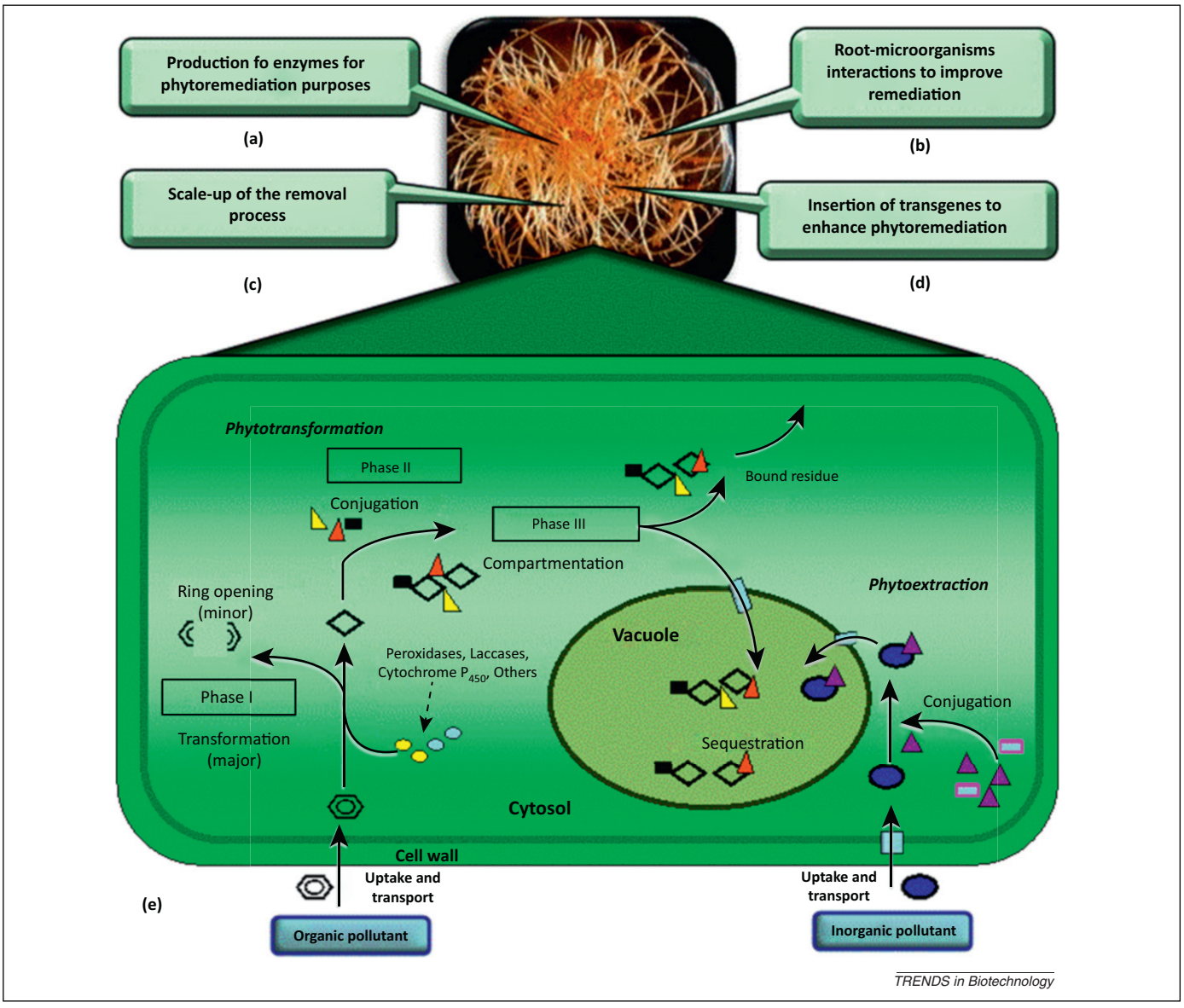

Figure 3. Valuable applications of hairy roots (HRs) in phytoremediation research. HRs allow: the production of enzymes for removing xenobiotics **(a)**; analysis of plant–microorganism interactions that offer new opportunities to improve phytoremediation **(b)**; study of pollutant removal on a large scale **(c)**; and the introduction of foreign genes into plant genomes to increase removal efficiency **(d)**. They also provide interesting information related to biochemical, physiological, and molecular mechanisms involved in pollutant metabolism, toxicity, and tolerance in plant cells **(e)**. Phytotransformation of organic compounds generally involves breakdown of pollutants by plant enzymes (*inter alia* peroxidases, laccases, and cytochromes P_{450}), followed by their conjugation with glucose, glutathione, or peptides (represented by triangles and boxes) and active sequestration in the vacuole and/or cell wall and apoplast. Inorganic pollutants are frequently phytoextracted and conjugated with phytochelatins, metallothioneins, nicotinamine, organic acids, or other complexing agents (represented by triangles and boxes) and actively sequestered in the vacuole. Active transporters are shown as boxes with arrows.

Table 3. Phytoremediation of organic and inorganic environmental pollutants by HR cultures

Plant species	Pollutant	Remediation strategy/Effect studied	Refs
Solanum lycopersicon, Brassica napus, Nicotiana tabacum	Phenol	Phytotransformation – tolerance and removal/physiological studies	[56,59–61,71,77]
B. napus, N. tabacum	2,4-DCP	Phytotransformation/scale up	[57,58]
Cichorium intybus, Brassica juncea	DDT	Phytotransformation	[78]
Solanum nigrum	PCBs	Phytotransformation/metabolic studies	[62]
Tagetes patula L., B. juncea L.	Textile dyes	Phytotransformation	[64,65]
Armoracia rusticana L.	N-acetyl-4-aminophenol	Conjugation/detoxification	[63]
Thlaspi caerulescens, Alyssum bertoloni, Alyssum murale	Cadmiun, Nickel	Phytoextraction	[66,79]
Daucus carota, A. rusticana	Uranium	Rhizofiltration	[68,69]
S. nigrum	Zinc	Phytoextraction	[80]

Abbreviations: 2,4-DCP, 2, 4 dichlorophenol; DDT, 1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane; PCBs, polychlorinated biphenyls.

wide range of individual PCB congeners have been identified using *Solanum nigrum* HRs [62]. In addition, *Armoracia rusticana* L. HRs have demonstrated ability to take up, conjugate, and detoxify N-acetyl-4-aminophenol (paracetamol), and several HRs can degrade textile dyes [63–65].

HRs can also be used to explore responses of metals/metalloids, which cannot be degraded, hence HRs accumulate them, facilitating analysis of biological mechanisms responsible for the high tolerance of hyperaccumulator species [66]. They also have proven utility for screening the capacity of diverse plant species to extract and sequester metals and radionucleides, as well as for potential practical applications, such as phytomining [66,67]. As a result of their highly branched nature, HRs have large surface areas in comparison with control roots, and can also be used for rhizofiltration purposes. For example, *Arm. rusticana* HRs accumulate uranium in the presence of phosphate, which has a stimulating effect on the HR growth and pollutant accumulation, whereas carrot HRs seem to be suitable for studying uranium toxicity under optimal conditions [68,69]. There is now a vast literature describing a wide variety of organic and inorganic compounds that can be removed by HRs (Table 3).

A direct method for improving phytoremediation, is to transfer genes involved in the uptake, transport, multistep metabolic pathways, and sequestration of specific pollutants to plant tissues. Furthermore, root-specific expression of transgenes may enhance the rhizodegradation of highly recalcitrant compounds. For instance, transgenic tomato HRs overexpressing *tpx1*, a native peroxidase, reportedly remove phenol more efficiently than wild-type HRs [70], whereas transgenic tobacco HRs can degrade phenol at high concentrations, and are valuable for studying oxidative stress and antioxidant responses to phenol treatment [71].

Concluding remarks and perspectives

Today, HRs can be induced from practically any plants. Hence, they could be generated from valuable, rare or threatened medicinal species, thereby helping efforts to preserve biodiversity. In addition, HRs have become valuable tools for studying the biosynthesis of plant-derived molecules and they are attractive (bio)production systems, with several advantages over field-grown plants and cell suspensions. They are also convenient, close to optimal model systems for elucidating the complex interactions involved in phytoremediation. Several challenges remain to be addressed (e.g., better understanding of the biochemical machinery of roots and more sophisticated techniques for managing the biosynthetic processes are required). However the progress made to date, and anticipated advances towards identified goals (e.g., designing and establishing efficient, cost-effective bioreactor configurations), should lead to wide commercialization of HR cultures, given their high and increasing applicability for diverse purposes.

Acknowledgments

Financial support from the Marie Curie Programme of the European Community (grant PIEF-GA-2009-252558 to M.I.G.) and the National Science Fund of Bulgaria (grant DO-02-261/2008 to M.I.G.) is greatly appreciated. E.A. is member of the research career from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

References

1 Gelvin, S.B. (2009) *Agrobacterium* in the genomics age. *Plant Physiol.* 150, 1665–1676
2 Lee, L-Y. and Gelvin, S.B. (2008) T-DNA binary vectors and systems. *Plant Physiol.* 146, 325–332
3 Chandra, S. (2012) Natural plant genetic engineer *Agrobacterium rhizogenes*: role of T-DNA in plant secondary metabolism. *Biotechnol. Lett.* 34, 407–415
4 Hoges, L.D. *et al.* (2009) The *Agrobacterium rhizogenes* GALLS gene encodes two secreted proteins required for genetic transformation of plants. *J. Bacteriol.* 191, 355–364
5 Georgiev, M.I. *et al.* (2010) Hairy root culture: copying nature in new bioprocesses. In *Medicinal Plant Biotechnology* (Arora, R., ed.), pp. 156–175, CAB International
6 Bulgakov, V.P. *et al.* (2012) The *rolB* gene suppresses reactive oxygen species in transformed plant cells through the sustained activation of antioxidant defense. *Plant Physiol.* 158, 1371–1381
7 Georgiev, M.I. *et al.* (2007) Hairy root type plant *in vitro* systems as sources of bioactive substances. *Appl. Microbiol. Biotechnol.* 74, 1175–1185
8 Georgiev, M.I. *et al.* (2011) Sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum* Griseb. for bioactive metabolite accumulation. *Plant Cell Rep.* 30, 859–866
9 Lambert, E. *et al.* (2009) Cryopreservation of hairy root cultures of *Maesa lanceolata* and *Medicago truncatula*. *Plant Cell Tissue Organ Cult.* 96, 289–296
10 Guillon, S. *et al.* (2006) Harnessing the potential of hairy roots: dawn of a new era. *Trends Biotechnol.* 24, 403–409
11 Chandra, S. and Chandra, R. (2011) Engineering secondary metabolite production in hairy roots. *Phytochem. Rev.* 10, 371–395
12 Ono, N.N. and Tian, L. (2011) The multiplicity of hairy root cultures: prolific possibilities. *Plant Sci.* 180, 439–446
13 Pollier, J. *et al.* (2011) Metabolite profiling of triterpene saponins of *Medicago truncatula* hairy roots by liquid chromatography fourier transform ion cyclotron resonance mass spectrometry. *J. Nat. Prod.* 74, 1462–1476
14 Gyurkovska, V. *et al.* (2011) Anti-inflammatory activity of devil's claw *in vitro* systems and their active constituents. *Food Chem.* 125, 171–178
15 Kim, H.K. *et al.* (2011) NMR-based metabolomics: where do we stand, where do we go? *Trends Biotechnol.* 29, 267–275
16 Gomez-Galera, S. *et al.* (2007) The genetic manipulation of medicinal and aromatic plants. *Plant Cell Rep.* 26, 1689–1715
17 Pollier, J. *et al.* (2011) Combinatorial biosynthesis in plants: a (p)review on its potential and future exploitation. *Nat. Prod. Rep.* 28, 1897–1916
18 El-Sayed, M. and Verpoorte, R. *et al.* (2007) *Catharanthus* terpenoid indole alkaloids: biosynthesis and regultation. *Phytochem. Rev.* 6, 277–305
19 Hughes, E.H. *et al.* (2004) Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. *Metabol. Eng.* 6, 268–276
20 Hong, S-B. *et al.* (2006) Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. *J. Biotechnol.* 122, 28–38
21 Peebles, C.A.M. *et al.* (2006) Effects of terpenoid precursor feeding on *Catharanthus roseus* hairy roots over-expressing the alpha or the alpha and beta subunits of anthranilate synthase. *Biotechnol. Bioeng.* 93, 534–540
22 Peebles, C.A.M. *et al.* (2011) The expression of 1-deoxy-d-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots. *Metabol. Eng.* 13, 234–240
23 Magnotta, M. *et al.* (2007) Expression of deacetylvindoline-4-O-acetyltransferase in *Catharanthus roseus* hairy roots. *Phytochemistry* 68, 1922–1931
24 Runguphan, W. *et al.* (2009) Silencing of tryptamine biosynthesis for production of nonnatural alkaloids in plant culture. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13673–13678

25 Runguphan, W. *et al.* (2010) Integrating carbon–halogen bond formation into medicinal plant metabolism. *Nature* 468, 461–464
26 Zehner, S. *et al.* (2005) A regioselective tryptophan 5-halogenase is involved in pyrroindomycin biosynthesis in *Streptomyces rugosporus* LL-42D005. *Chem. Biol.* 12, 445–452
27 Yeh, E. *et al.* (2005) Robust *in vitro* activity of RebF and RebH, a two component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3960–3965
28 Memelink, J. and Gantet, P. (2007) Transcription factors involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *Phytochem. Rev.* 6, 353–362
29 Peebles, C.A.M. *et al.* (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. *Metabol. Eng.* 11, 76–86
30 Xu, J.F. *et al.* (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol. Adv.* 29, 278–299
31 Obembe, O.O. *et al.* (2011) Advances in plant molecular farming. *Biotechnol. Adv.* 29, 210–222
32 Woods, R.R. *et al.* (2008) Hairy-root organ cultures for the production of human acetylcholinesterase. *BMC Biotechnol.* 8, 95
33 Boitel-Conti, M. *et al.* (2011) Universite De Picardie Jules Verne. Method for producing recombinant proteins from plant hairy roots, EP2385130
34 Gaume, A. *et al.* (2003) Rhizosecretion of recombinant proteins from plant hairy roots. *Plant Cell Rep.* 21, 1188–1193
35 Pham, N.B. *et al.* (2012) Production and secretion of recombinant thaumatin in tobacco hairy root cultures. *Biotechnol. J.* 7, 537–545
36 Wongsamuth, R. and Doran, P.M. (1997) Production of monoclonal antibodies by tobacco hairy roots. *Biotechnol. Bioeng.* 54, 401–415
37 Martinez, C. *et al.* (2005) Expression of the antibody 14D9 in *Nicotiana tabacum* hairy roots. *Electron. J. Biotechol.* 8, 170–176
38 Sharp, J.M. and Doran, P.M. (2001) Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. *Biotechnol. Prog.* 17, 979–992
39 Komarnytsky, S. *et al.* (2006) Cosecretion of protease inhibitor stabilizes antibodies produced by plant roots. *Plant Physiol.* 141, 1185–1193
40 Kumar, G.B.S. *et al.* (2006) Expression of hepatitis B surface antigen in potato hairy roots. *Plant Sci.* 170, 918–925
41 Ko, S. *et al.* (2006) Expression of the protective antigen (SpaA) in transgenic hairy roots of tobacco. *Plant Mol. Biol. Rep.* 24, 251a–251g
42 Liu, C.Z. *et al.* (2009) Production of mouse interleukin-12 is greater in tobacco hairy roots grown in a mist reactor than in an airlift reactor. *Biotechnol. Bioeng.* 102, 1074–1086
43 Liu, J. *et al.* (2008) Expression of bioactive single-chain murine IL-12 in transgenic plants. *J. Interferon Cytokine Res.* 28, 381–392
44 Parsons, J. *et al.* (2010) Production of human epidermal growth factor (hegf) by in vitro cultures of *nicotiana tabacum*: effect of tissue differentiation and sodium nitroprusside addition. *Int. J. Biotechnol. Biochem.* 6, 131–138
45 Hood, E. *et al.* (2012) Protein targeting: strategic planning for optimizing protein products through plant biotechnology. In *Plant Biotechnology and Agriculture: Prospects for the 21st Century* (Altman, A. and Hasegawa, P.M., eds), pp. 35–54, Elsevier
46 Nopo, L. *et al.* (2012) Super-promoter:TEV, a powerful gene expression system for tobacco hairy roots. *Methods Mol. Biol.* 824, 501–526
47 Anuar, M.R. *et al.* (2011) Expression analysis of the 35S CaMV promoter and its derivatives in transgenic hairy root cultures of cucumber (*Cucumis sativus*) generated by *Agrobacterium rhizogenes* infection. *Afr. J. Biotechnol.* 10, 8236–8244
48 Medina-Bolivar, F. *et al.* (2003) A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. *Vaccine* 21, 997–1005
49 Gallie, D.R. *et al.* (1987) The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. *Nucleic Acids Res.* 15, 3257–3273
50 Hughes, E.H. *et al.* (2002) Characterization of an inducible promoter system in *Catharanthus roseus* hairy roots. *Biotechnol. Prog.* 18, 1183–1186
51 Lee, K.T. *et al.* (2007) Heat-inducible production of beta-glucuronidase in tobacco hairy root cultures. *Appl. Microbiol. Biotechnol.* 73, 1047–1053
52 Eibl, R. *et al.* (2009) Disposable bioreactors for plant liquid cultures at litre-scale: review. *Eng. Life Sci.* 9, 156–164
53 Sivakumar, G. *et al.* (2010) Biomass production of hairy roots of *Artemisia annua* and *Arachis hypogaea* in a scaled-up mist bioreactor. *Biotechnol. Bioeng.* 107, 802–813
54 Ramakrishnan, D. and Curtis, W.R. (2004) Trickle-bed root culture bioreactor design and scale-up: growth, fluid-dynamics and oxygen mass transfer. *Biotechnol. Bioeng.* 88, 248–260
55 Schroder, P. *et al.* (2008) Phytoremediation of organic xenobiotics – glutathione dependent detoxification in *Phragmites* plants from European treatment sites. *Bioresour. Technol.* 99, 7183–7191
56 Coniglio, M.S. *et al.* (2008) Application of *Brassica napus* hairy root cultures for phenol removal from aqueous solutions. *Chemosphere* 72, 1035–1042
57 Talano, M.A. *et al.* (2010) Removal of 2,4-diclorophenol from aqueous solutions using tobacco hairy root cultures. *J. Hazard. Mater.* 176, 784–791
58 Angelini, V.A. *et al.* (2011) Scale up of 2,4-dichlorophenol removal from aqueous solutions using *Brassica napus* hairy roots. *J. Hazard. Mater.* 185, 269–274
59 González, P.S. *et al.* (2008) Comparison of the removal of 2,4-dichlorophenol and phenol from polluted waters by peroxidases from tomato hairy roots, and protective effect of polyethylene glycol. *Chemosphere* 70, 982–989
60 Ibañez, S.G. *et al.* (2011) Phenol tolerance, changes of antioxidative enzymes and cellular damage in transgenic tobacco hairy roots colonized by arbuscular mycorrhizal fungi. *Chemosphere* 83, 700–705
61 Sosa-Alderete, L.G. *et al.* (2011) Antioxidant response of tobacco (*Nicotiana tabacum*) hairy roots after phenol treatment. *Plant Physiol. Biochem.* 49, 1020–1028
62 Rezek, J. *et al.* (2007) Plant metabolites of polychlorinated biphenyls in hairy root culture of black nightshade *Solanum nigrum* SNC-9O. *Chemosphere* 69, 1221–1227
63 Huber, C. *et al.* (2009) Metabolism of acetaminophen (paracetamol) in plants-two independent pathways result in the formation of a glutathione and a glucose conjugate. *Environ. Sci. Pollut. Res. Int.* 16, 206–213
64 Patil, P. *et al.* (2009) Degradation analysis of Reactive Red 198 by hairy roots of *Tagetes patula* L. (Marigold). *Planta* 230, 725–735
65 Telke, A.A. *et al.* (2011) Biochemical characterization of laccase from hairy root culture of *Brassica juncea* L. and role of redox mediators to enhance its potential for the decolorization of textile dyes. *Planta* 234, 1137–1149
66 Doran, P.M. (2011) Hairy root studies in phytoremediation and phytomining. In *Handbook of Phytoremediation* (Golubev, I.A., ed.), pp. 591–612, Nova Science Publishers
67 Soudek, P. *et al.* (2006) ^{137}Cs and ^{90}Sr uptake by sunflower cultivated under hydroponic conditions. *J. Environ. Radioact.* 88, 236–250
68 Soudek, P. *et al.* (2011) Uranium uptake and stress responses of *in vitro* cultivated hairy root culture of *Armoracia rusticana*. *Agrochimica* 1, 15–28
69 Straczek, A. *et al.* (2009) Tolerance of hairy roots of carrots to U chronic exposure in a standardized in vitro device. *Environ. Exp. Bot.* 65, 82–89
70 Wevar-Oller, A.L. *et al.* (2005) Overexpression of a basic peroxidase in transgenic tomato (*L. esculentum* Mill. Cv. Pera) hairy roots increases phytoremediation of phenol. *Plant Sci.* 169, 1102–1111
71 Sosa-Alderete, L.G. *et al.* (2012) Phospholipid turnover and phospholipase D activity in tobacco hairy roots exposed to phenol. *Environ. Exp. Bot.* 77, 141–145
72 Sharp, J.M. and Doran, P.M. (2001) Characterization of monoclonal antibody fragments produced by plant cells. *Biotechnol. Bioeng.* 73, 338–346
73 Skarjinskaia, M. *et al.* (2008) Production of recombinant proteins in clonal root cultures using episomal expression vectors. *Biotechnol. Bioeng.* 100, 814–819
74 Kim, S.R. *et al.* (2012) Expression and large-scale production of the biochemically active human tissue-plasminogen activator in hairy roots of oriental melon (*Cucumis melo*). *J. Biosci. Bioeng.* 113, 106–111
75 Banerjee, S. *et al.* (2002) Expression of functional mammalian P450 2E1 in hairy root cultures. *Biotechnol. Bioeng.* 77, 462–466
76 Medina-Bolivar, F. and Cramer, C. (2004) Production of recombinant proteins by hairy roots cultured in plastic sleeve bioreactors. *Methods Mol. Biol.* 267, 351–363

77 Agostini, E. *et al.* (2011) Phytoremediation of phenolic compounds: recent advances and perspectives. In *Handbook of Phytoremediation* (Golubev, I.A., ed.), pp. 1–50, Nova Science Publishers
78 Suresh, B. *et al.* (2005) Uptake and degradation of DDT by hairy root cultures of *Cichorium intybus* and *Brassica juncea*. *Chemosphere* 61, 1288–1292
79 Vinterhalter, B. *et al.* (2008) Nickel tolerance and hyperaccumulation in shoot cultures regenerated from hairy root cultures of *Alyssum murale*. *Plant Cell Tissue Organ Cult.* 94, 299–303
80 Subroto, M.A. *et al.* (2007) Accumulation of zinc by hairy root cultures of *Solanum nigrum*. *Biotechnology* 6, 344–348