Trends in Biotechnology

CellPress REVIEWS

Review

Biotechnological Exploration of Transformed Root Culture for Value-Added Products

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Medicinal plants produce valuable secondary metabolites with anticancer, analgesic, anticholinergic or other activities, but low metabolite levels and limited available tissue restrict metabolite yields. Transformed root cultures, also called hairy roots, provide a feasible approach for producing valuable secondary metabolites. Various strategies have been used to enhance secondary metabolite production in hairy roots, including increasing substrate availability, regulating key biosynthetic genes, multigene engineering, combining genetic engineering and elicitation, using transcription factors (TFs), and introducing new genes. In this review, we focus on recent developments in hairy roots from medicinal plants, techniques to boost production of desired secondary metabolites, and the development of new technologies to study these metabolites. We also discuss recent trends, emerging applications, and future perspectives.

Highlights

Hairy roots are useful tools for studying the biosynthesis of different plant-derived valuable compounds.

Hairy roots could be preferred hosts when the desired compounds mainly accumulate in roots.

Hairy roots are being considered as an alternative system to microbial hosts, including *Escherichia coli* and *Saccharomyces cerevisiae*, for producing plant-derived natural secondary metabolites because they are more similar to the native host plant.

Hairy roots have emerged as valuable tools for the rapid characterization of plant gene function and enzyme activity *in vivo* because hairy roots naturally maintain many cofactors and precusor substrates, and the encoded plant-derived protein is more likely to be properly folded in hairy roots compared with in microbes.

The Biosynthetic Potential of Hairy Roots to Produce Value-Added Molecules: The 'Green Factories' Concept

Medicinal plants produce numerous functionally diverse **secondary metabolites** (see Glossary), including terpenoids, phenylpropanoids, and alkaloids. Many of these compounds are of great pharmaceutical importance. For instance, some alkaloids have anticancer (i.e., camptothecin, taxol, and vinblastine), analgesic (i.e., morphine and codeine), or anticholinergic properties (i.e., atropine and scopolamine) [1–3]. Ginsenosides have antiaging, antioxidative, adaptogenic, and anticancer properties [4]. For instance, two doses of ginseng (1000 or 2000 mg/day) efficiently decreased cancer-related fatigue [4]. **Tanshinones** (diterpenoids) and salvianolic acids (phenylpropanoids) have been used to treat cardiovascular and cerebrovascular diseases due to their anti-inflammatory, antibacterial, antioxidant, cytotoxic, and anticancer properties [5]. Moreover, the sesquiterpene artemisinin is an effective antimalarial drug [6].

Despite their diverse structures and properties, the precursors of all these compounds, such as phosphoenolpyruvate (PEP), shikimate, pyruvate, and acetyl-CoA, are derived from the common glycolytic pathway (Figure 1). Subsequently, a variety of compounds are biosynthesized from different biosynthetic pathways. Terpenoids (otherwise known as isoprenoids), a class of natural compounds with ~50 000 different structures, are produced in plants via the cytosolic mevalonate (MVA) and plastidial methylerythritol phosphate (MEP) pathways [7,8]. Sesquiterpenoids (artemisinin) and triterpenoids (ginsenosides) are mainly derived from the MVA pathway [6,9–11], whereas diterpenoids (i.e., tanshinones and taxol) and monoterpenoids (limonene) are primarily synthesized by the MEP pathway [8,12]. Representative alkaloids, such as vinblastine and camptothecin, are synthesized from tryptophan, derived from the shikimate pathway, and secologanin is a monoterpenoid derived from the MEP pathway [1,13–16]. Phenylpropanoids, such as salvianolic acids, anthocyanin, and catechin, are synthesized from phenylalanine and tyrosine, which are also derived from the shikimate pathway [5,12].

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Many secondary metabolites are present at low levels *in planta*, including plant-derived anticancer compounds [1]. Furthermore, many of these metabolites are synthesized only in specific tissues [9,17]. For instance, tanshinones, ginsenosides, and flavones accumulate primarily in the roots of *Salvia miltiorrhiza*, ginseng, and *Scutellaria baicalensis*, respectively [9,18,19]. Vinblastine and vincristine are produced exclusively in the aerial parts of plants, and catharanthine accumulates in all organs of *Catharanthus roseus* [20]. Glandular secretory trichomes of *Artemisia annua* leaves are 'biofactories' for artemisinin biosynthesis and accumulation [6]. The natural sources of these compounds often grow slowly or produce these metabolites in very small quantities over an extended growth periods (several years) before their roots can be harvested [21]. In addition, medicinal plants grow in various ecological environments and often have bacterial or pesticide residues, leading to degradation of their quality [22,23]. Therefore, it is important to explore other methods for producing these valuable and beneficial compounds.

The production of genetically transformed root cultures (so-called '**hairy roots**') represents a good alternative approach to produce target compounds in medicinal plants. Hairy roots grow faster than the adventitious roots, or even conventional plant cultures [24,25] and accumulate higher levels of certain valuable compounds compared with adventitious roots and native-grown plant roots [18,26,27]. For instance, the total tanshinone content reached up to 15.4 mg/g dry weight (DW) in transgenic *S. miltiorrhiza* hairy roots compared with field-grown plant roots, which had only 1.7–9.7 mg/g DW tanshinone [18,26]. The total wilforine content was significantly higher in hairy roots than in adventitious roots of *Tripterygium wilfordii* Hook.f. [27]. Various novel bioactive compounds can be produced in hairy root cultures and not in adventitious roots (or conventional plant tissues). For instance, novel cadaverine and natural triterpene saponins compounds have been found in *Brugmansia candida* and *Medicago truncatula* hairy roots, respectively, perhaps resulting from transformation or stress, but they were not identified in the leaves or roots of intact plants [28,29].

Hairy root cultures are also excellent model systems for identifying novel genes and **TFs** or rapidly characterizing gene function. Moreover, hairy roots can be genetically modified, thereby allowing modulation of metabolite production through genetic engineering or genome editing. Finally, hairy root cultures can be artificially designed to produce unnatural compounds by blocking the biotransformation of an original precursor through RNAi or genome-editing of biosynthetic genes, combined with the feeding of exogenous substrates. For example, several unnatural fluorinated alkaloids, such as fluoro-ajmalicine, fluoro-serpentine, fluoro-catharanthine, and fluoro-tabersonine, were produced in *C. roseus* hairy roots when the tryptamine biosynthesis was suppressed by RNA silencing of tryptophan decarboxylase and feeding with the unnatural starting substrate 5-fluorotryptamine [30]. Hairy root cultures also offer benefits compared with microbes. For instance, they provide an alternative platform that is more similar to that in the native host plant compared with microbes, including *Escherichia coli* and *Saccharomyces cerevisiae*, and the encoded plant-derived protein is more likely to be properly folded in hairy roots than in microbes. Other applications of transformed root cultures include the production of high-value proteins, therapeutic vaccines, and antimicrobial peptides [31–33]. These cultures have also been used for molecular farming, plant regeneration [34,35], and the introduction of new steps and/or pathways to form new compounds [36].

To date, hairy roots have been generated from hundreds of medicinal plants, including *Taxus* × *media*, *C. roseus*, *S. miltiorrhiza*, *Ophiorrhiza pumila*, *Withania somnifera* (L.), *Isatis indigotica*, *Anisodus acutangulus*, *Atropa belladonna*, and *Panax ginseng*, as well as non-medicinal species, such as potato (*Solanum tuberosum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), cotton (*Gossypium hirsutum*), and grape (*Vitis vinifera*; Table 1) for various applications. Early research

Glossary

Clustered and regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9): an RNA-guided gene-editing technique involving the use of a CRISPR-associated endonuclease (Cas9) and an engineered guide RNA to generate site-specific mutations in target genes.
Cytochromes P450s: hemoprotein family enzymes that function as monooxygenases, generally in combination with cytochrome P450 reductase.
Elicitation: strategy involving the application of different elicitors to cells or organisms to increase the production of certain compounds.
Hairy roots: roots that form from the infection sites of explants transformed by *Agrobacterium rhizogenes* harboring an Ri-plasmid. Hairy roots are capable of unlimited growth in liquid medium and produce nearly the same secondary metabolites as natural plants.
Metabolic engineering: a practice used to optimize genetic and regulatory processes through the combined use of gene regulation, metabolic regulation, biochemical engineering, and genetic engineering to increase the production of specific products.
Metabolomics: simultaneous qualitative and quantitative analysis of all low-molecular-weight molecules in an organism or cell during a specific physiological period.
Multigene engineering: simultaneously manipulating two or more genes involved in, or affecting the biosynthesis of valuable compounds to accumulate desired or value-added compounds.
Push–pull: a strategy used to increase the production of target products by simultaneously regulating upstream and downstream genes encoding key enzymes involved in the biosynthesis of valuable compounds.
Secondary metabolites (specialized metabolites): a large group of chemicals that accumulate at low levels in plants, bacteria, and fungi, including terpenoids, phenylpropanoids, and alkaloids.
Synthetic biology: design and assembly of biological components or biological systems that do not exist in nature, or the redesign or construction of existing biological systems.

on hairy roots, including culture methods, conditions, the use of bioreactors, **elicitation**, and **metabolic engineering** to produce valuable secondary metabolites, has been reviewed elsewhere [37–43]. Here, we review new strategies, including **multigene engineering**, the combined use of elicitors and genetic engineering, the use of newly identified key genes or TFs for the metabolic engineering of valuable secondary metabolites, the use of newly emerging **Clustered and regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)** technology, and proteomics and **metabolomics** to explore hairy roots from medicinal plants. We also discuss other applications and functions of hairy roots and address future prospects for using hairy roots as 'green factories' (i.e., plant-based production systems).

Tanshinones: main lipophilic bioactive compounds in the Chinese medicinal plant *Salvia miltiorrhiza* (danshen), comprising abietane-type norditerpenoid naphthoquinones.
Transcription factor (TF): a protein that binds to a specific sequence upstream of a gene to regulate its transcription. TF families include bHLH, WRKY, MYB, bZIP, and AP2/ERF, among others.

Biotechnological Strategies to Improve Secondary Metabolite Production in Hairy Roots from Medicinal Plants

Manipulation of Biosynthetic Pathways or Blocking of Competitive Pathways

Secondary metabolites (or specialized metabolites) are derived from general precursors. Therefore, the availability of a precursor and/or substrate is an important factor affecting target compound production. The engineering of secondary metabolites in hairy roots has been achieved by the genetic manipulation of key genes for the biosynthesis of substrates and/or precursors, intermediate products, and end products (Table 2). For example, the expression of genes encoding geraniol 10-hydroxylase (*G10H*), secologanin synthase (*SLS*), phenyllactate UDP-glycosyltransferase, and littorine synthase for alkaloid production has been manipulated in the hairy roots of various medicinal plants, including *O. pumila* and *A. belladonna* [44–46]. Overexpression of the valerendiene synthase gene *VDS* in *Valeriana officinalis* hairy roots resulted in 1.5–4-fold higher levels of the sesquiterpenoid valerenic acid compared with the control [47]. Co-introduction of the key gene *DXS* (encoding 1-deoxy-D-xylulose-5-phosphate synthase, a key enzyme in the MEP pathway), and *GGPPS* (encoding geranylgeranyl diphosphate synthase, an enzyme in the middle of the pathway, which provides general precursor geranylgeranyl diphosphate for diterpenoid production) in transgenic *S. miltiorrhiza* hairy roots yielded tanshinone levels as high as 12.93 mg/g DW, compared with 0.61 mg/g DW in the controls [48]. This finding suggests that the MEP pathway is more important than the MVA pathway for tanshinone biosynthesis and that crosstalk exists between these pathways. A similar strategy was been used to enhance phenylpropanoid production [49].

Suppressing and/or blocking a competitive branching pathway has also been tested in efforts to boost the production of plant-derived secondary metabolites. For example, RNAi was used to downregulate the expression of the 4-hydroxyphenylpyruvate dioxygenase gene *SmHPPD* in homogentisate biosynthesis, which competes with rosmarinic acid (RA) biosynthesis, thereby increasing phenolic acid accumulation [49]. However, the levels of the compounds were not high enough to meet commercial requirements, implying that other limiting factors exist. Even if the levels of an enzyme and its product are increased, other steps could become visible and limit the effect of metabolic engineering, highlighting the importance of resolving the entire biosynthetic pathway for each specific compound and completely understanding the regulation of each pathway.

Multigene Engineering

Another approach for increasing secondary metabolite production involves the '**push–pull**' effect of overexpressing several genes simultaneously. For instance, multigene engineering has been used to increase alkaloid accumulation. Co-introduction of *AaPMT* and *AaTRI* in *A. acutangulus* hairy roots resulted in the production of 8.104 mg/g tropane alkaloids, levels 8.7-fold higher than those in the control [50]. Co-introduction of *OpG10H* and *OpSLS* resulted in the production of 3.5 mg/g camptothecin in *O. pumila* hairy roots, whereas 1.38 mg/g was produced in the control [45]. Enhanced total tanshinone levels have also been obtained by overexpressing different combinations of genes (*SmHMGR/SmDXR*, *SmHMGR/SmGGPPS* or *SmDXS2/SmGGPPS*) [18,48,51].

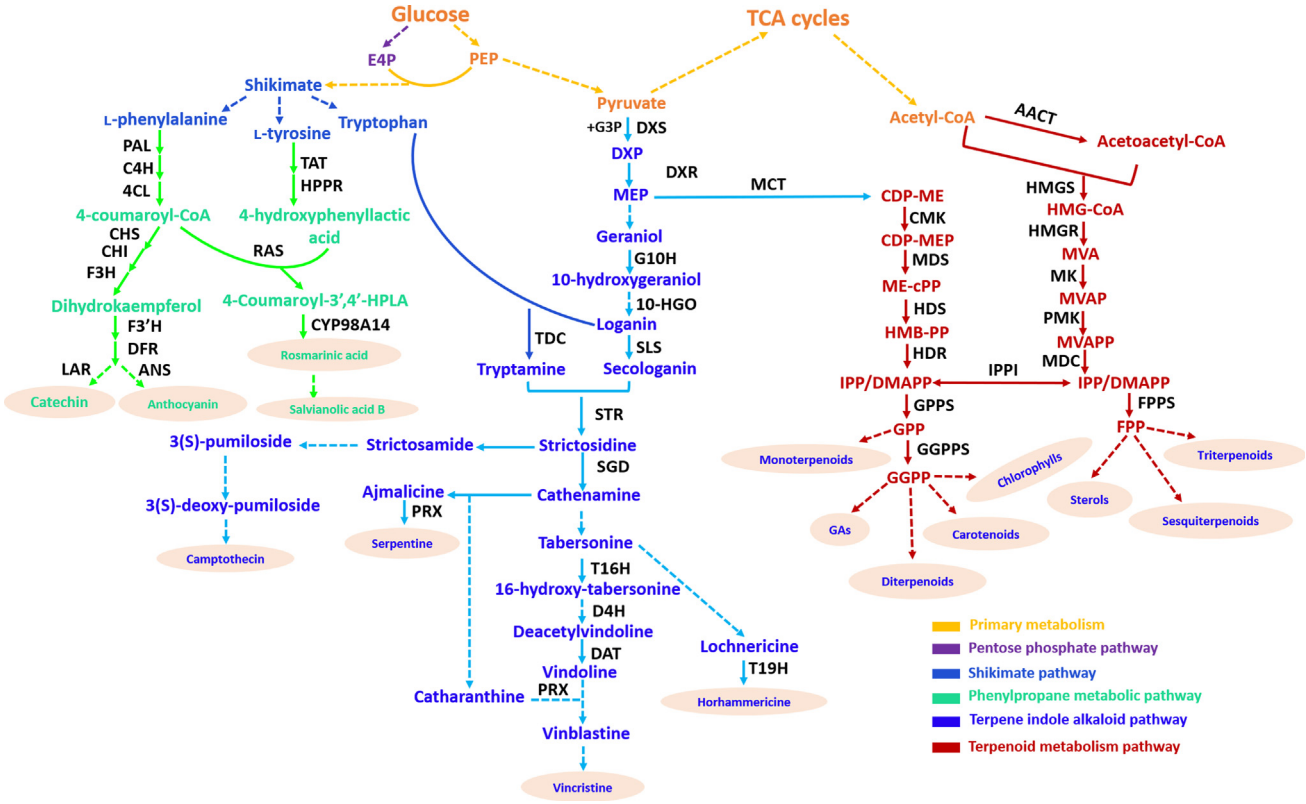

Figure 1. Major Biosynthetic Pathways for the Production of Secondary Metabolites in Hairy Roots from Medicinal Plants. Abbreviations: 4CL, 4-coumaric acid:CoA ligase; AACT, acetoacetyl-CoA thiolase; ANS, anthocyanidin synthase; C4H, cinnamic acid 4-hydroxylase; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; CHS, chalcone synthase; CHI, chalcone isomerase; CMK, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; CYP98A14, cytochrome P450-dependent hydroxylase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline 4-O-acetyltransferase; DFR, dihydroflavanol 4-reductase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; E4P, Erythrose 4-phosphate; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FPP, farnesyl diphosphate; FPPS, FPP synthase; G10H, geraniol 10-hydroxylase; G3P, glyceraldehyde 3-phosphate; GAs, gibberellins; GPP, geranyl diphosphate; GPPS, GPP synthase; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; 10-HGO, 10-hydroxygeraniol oxidoreductase; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HPPR, 14-hydroxyphenylpyruvate reductase; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LAR, leucoanthocyanidin reductase; MK, mevalonate kinase; MCT, 2*C*-methyl-D-erythritol 4-phosphate cytidyl transferase; MDC, mevalonate pyrophosphate decarboxylase; MDS, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ME-cPP, 2-*C*-methyl-D-erythritol-2,4-cyclopyrophosphate; MEP, 2*C*-methyl-D-erythritol 4-phosphate; MVA, mevalonate; MVAP, mevalonate 5-phosphate; MVAPP, mevalonate 5-diphosphate; PAL, phenylalanine ammonia-lyase; PEP, phosphoenolpyruvate; PMK, phosphomevalonate kinase; PRX, peroxidase; RAS, rosmarinic acid synthase; SGD, strictosidine-*O*-beta-D-glucosidase; SLS, secologanin synthase; STR, strictosidine synthase; T16H, tabersonine 16-hydroxylase; T19H, tabersonine 19-hydroxylase; TAT, tyrosine aminotransferase; TDC, tryptophan decarboxylase.

These findings suggest that multigene engineering is a more effective strategy than overexpressing a single gene in hairy roots, most likely due to the complexity of plant biosynthetic pathways. Furthermore, the combined overexpression of *SmDXS2* and *SmGGPPS* was more effective than the combined expression of *SmHMGR* (the key gene in the MVA pathway) with *SmGGPPS*, highlighting the crucial role of the MEP pathway in tanshinone biosynthesis.

Similar to approaches directly overexpressing several important biosynthetic genes, targeting key TF genes individually or in combination can also have global effects on biosynthetic pathways. Indeed, key TFs have been overexpressed in hairy roots; for example, simultaneously overexpressing the TF gene *CrBIS1* (a new bHLH TF gene from *C. roseus*), *CrORCA3*, and *CrMYC2a* significantly

Table 1. Selected Secondary Metabolites Produced in Hairy Root Cultures from Various Plant Species[a]

Metabolite or function	Plants species	Strain	Explant	Culture medium (liquid)	Refs
Camptothecin	*Ophiorrhiza pumila*	C58C1	Stem	B5	[44]
Tanshinones	*Salvia miltiorrhiza*	C58C1	Leaf	1/2MS	[18]
Phenolic acids	*S. miltiorrhiza*	C58C1	Leaf	1/2MS	[17]
Lariciresinol	*Isatis indigotica*	C58C1	Leaf	1/2MS	[59]
Hyoscyamine	*Anisodus acutangulus*	C58C1	Leaf	1/2MS	[40]
Scopolamine	*Atropa belladonna*	C58C1	Leaf	MS	[46]
Withanoloide A	*Withania somnifera*	R1000; ATCC15834	Leaf; cotyledon	MS	[34]
Flavones	*Scutellaria baicalensis*	A4	Leaf	B5	[19]
Taxol	*Taxus media*	LBA9402; C58C1	Leaf; stem	B5	[92]
Rhein	*Polygonum multiflorum*	R1601	Leaf	MS	[93]
Scutellarin	*Erigeron breviscapus*	C58C1	Leaf	B5	[94]
Salidroside	*Rhodiola crenulata*	C58C1	Leaf	1/2MS	[95]
Ginsenoside	*Panax ginseng*	A4	Root	1/2MS	[96]
Parthenolide	*Tanacetum parthenium*	ATCC15834	Leaf	MS	[97]
Chicoric acid	*Echinacea purpurea*	R15834	Leaf	WPM	[98]
Farnesiferol B	*Ferula pseudalliacea*	ATCC15834	Leaf	1/2MS	[99]
Rishitin	*Solanum tuberosum*	ATCC15834	Tuber	MS	[100]
Flavonoids/Isoflavonoids	*Glycine max*	ARqual1	Cotyledon, hypocotyls	B5	[101]
Nicotine	*Nicotiana tabacum*	ATCC15834	Leaf	B5	[102]
Gossypol	*Gossypium hirsutum*	A4	Leaf	B5	[103]
Resistance to pathogen infection	*Vitis vinifera*	A4	Stem	MS	[104]

[a]Abbreviations: 1/2 B5, half-strength B5 solid medium; 1/2MS, half-strength Murashige and Skoog medium; B5, Gamborg B5 medium; MS, Murashige and Skoog medium; WPM, Woody-plant medium.

increased the yields of at least 23 indole alkaloids in *C. roseus* [20]. Overall, many studies have demonstrated the potential of this approach for enhancing the yields of target compounds. However, the level of compounds produced remain too low to meet commercial requirements. The challenges encountered in these approaches indicates the complexity and difficulties of metabolic engineering of specific compounds.

Notably, in most of the studies summarized earlier, only two or three genes were co-expressed in transformed root cultures. By contrast, many genes have been used to engineer microbes, such

Table 2. Examples of Metabolically Engineered Hairy Root Cultures for the Production of Valuable Secondary Metabolites[a,b]

Genes	Origin	Engineered species	Strategy	Promoter	Metabolite produced	Metabolite level	Yield increase	Refs
GGPPS-DXS2	*Salvia miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Tanshinones	12.93 mg/g DW	21-fold	[48]
HMGR-DXR	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Tanshinones	3.25 mg/g DW	5.15-fold	[51]
HMGR-GGPPS	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Tanshinones	2.727 mg/g DW	4.74-fold	[18]
MYB98	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Tanshinones	15.4 mg/g DW	3-fold	[26]
MYB98	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Phenolic acids	20.9 mg/g DW	1.5-fold	[26]
WRKY1	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Tanshinones	13.7 mg/g DW	6.3-folds	[60]
WRKY2	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Tanshinones	3.985 mg/g DW	1.83-fold	[61]
ERF115	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Phenolic acids	/	1.43-fold	[17]
HPPD	*S. miltiorrhiza*	*S. miltiorrhiza*	Suppression	CaMV 35S	Phenolic acids	RA 542 mg/l; LAB334 mg/l	/	[49]
C4H	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Phenolic acids	RA 201 mg/l; LAB 584 mg/l	RA 3.6-fold; LAB 11.1-fold	[49]
HPPR	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Phenolic acids	RA 616 mg/l; LAB 669 mg/l	/	[49]
TAT-HPPR	*S. miltiorrhiza*	*S. miltiorrhiza*	Overexpression	CaMV35S	Phenolic acids	RA 906 mg/l; LAB 992 mg/l	RA 16.1-fold; LAB 16.8-fold	[49]
VDS	*Valeriana officinalis*	*V. officinalis*	Overexpression	CaMV35S	Valerenic acid	/	1.5–4-fold	[47]
ORCA4	*Catharanthus roseus*	*C. roseus*	Overexpression	CaMV 35S	Terpenoid indole alkaloids	/	Tabersonine 40-fold	[58]
li049	*Isatis indigotica*	*I. indigotica*	Overexpression	CaMV35S	Lariciresinol	425.60 μg/g DW	8.3-fold	[59]
MYB1	*Ophiorrhiza pumila*	*O. pumila*	Overexpression	CaMV35S	Camptothecin	Reduced	/	[62]
G10H-STR	*C. roseus*	*O. pumila*	Overexpression	CaMV35S	Camptothecin	1.54-1.77 mg/g DW	56%	[44]
G10H	*O. pumila*	*O. pumila*	Overexpression	CaMV 35S	Camptothecin	2.40 mg/g DW	/	[45]
SLS	*O. pumila*	*O. pumila*	Overexpression	CaMV 35S	Camptothecin	3.28 mg/g DW	/	[45]
G10H-SLS	*O. pumila*	*O. pumila*	Overexpression	CaMV 35S	Camptothecin	3.5 mg/g DW	/	[45]
TRI-PMT	*Anisodus acutangulus*	*A. acutangulus*	Overexpression	CaMV 35S	Hyoscyamine	1.914 ± 0.021 mg/g DW	2.3-fold	[50]

[a]Abbreviations: C4H, cinnamic acid 4-hydroxylase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS2, 1-deoxy-D-xylulose-5-phosphate synthase; ERF, AP2/ERF transcription factors; GGPPS, geranylgeranyl diphosphate synthase; G10H, geraniol 10-hydroxylase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPPR, 4-hydroxyphenylpyruvate reductase; H6H, hyoscyamine 6 β-hydroxylase; li049, a APETALA2/ethylene response factor (AP2/ERF) family member; MYB, MYB transcription factors; ORCA, octadecanoid-responsive *Catharanthus* AP2-domain proteins; PMT, putrescine *N*-methyltransferase; SGD, strictosidine β-glucosidase; SLS, secologanin synthase; STR, strictosidine synthase; TAT, tyrosine aminotransferase; TRI, tropinone reductase I; VDS, valerendiene synthase; WRKY, WRKY transcription factors.
[b]'/' indicates that information about metabolite production or yield increase is not available.

as *E. coli* and *S. cerevisiae* and plants including rice (*Oryza sativa*), Brassica, and tobacco, to produce carotenoids, vitamin E, polyunsaturated fatty acids, artemisinin, glucosinolates, and tanshinone precursors [8,52,53]. Therefore, to further increase the accumulation of target compounds in hairy roots, the co-overexpression of many target genes (**synthetic biology**) must be considered. However, the biosynthetic pathways of many important secondary metabolites have not yet been completely resolved. For example, the complete biosynthetic pathway of

camptothecin remains unclear [15]. The entire biosynthetic pathway of tropane alkaloids (TAs) in many Solanaceae plants is also not yet fully understood [46]. The **cytochrome P450** (CYP) enzyme CYP76AH1 in *S. miltiorrhiza* catalyzes the conversion of miltiradiene to ferruginol, but the exact steps for the conversion of ferruginol to individual tanshinones remain unknown [54]. Given that other rate-limiting steps that function downstream of the biosynthesis of target compounds might be present but remain unknown, a more complete understanding of these biosynthetic pathways is needed to formulate better methods for the metabolic engineering of desired compounds.

Combined Use of Genetic Engineering and Elicitation

Elicitors have been used to further enhance the accumulation of high-value compounds in the hairy roots of medicinal plants. For instance, treatment with yeast extract (YE) alone, silver ions (Ag^+) alone, or YE-Ag^+ increased the total tanshinone content in *S. miltiorrhiza* hairy roots overexpressing *SmHMGR* and *SmDXR* [51]. Treatment with methyl jasmonate (MeJA) and salicylic acid (SA) significantly increased the total tanshinone content in transgenic hairy roots overexpressing *SmGGPPS* by upregulating downstream genes in this pathway [55]. Therefore, the use of elicitors combined with other strategies, such as genetic engineering, could further enhance the accumulation of target compounds. Unfortunately, the yields of the compounds produced in these studies were too low to meet commercial requirements.

The Use of Transcription Factor Genes

Targeting TFs that coordinately regulate multiple biosynthetic pathway genes has immense potential for increasing secondary metabolite accumulation via metabolic engineering. Various TFs have recently been identified in different medicinal plants. For instance, in contrast to CrORCA3, which positively regulates some genes downstream of loganic acid [56], CrBIS1 transactivates the expression of genes upstream of loganic acid [57]. Although *CrBIS1*-overexpressing *C. roseus* hairy roots did not produce higher levels of indole alkaloids compared with the wild-type, perhaps due to the downregulation of *CrORCA3* target genes, silencing *CrBIS1* decreased indole alkaloid accumulation [57], supporting a role for BIS1 in regulating the iridoid pathway. Therefore, a better understanding of the complex regulatory mechanisms for indole alkaloid biosynthesis is needed to enable precise metabolic engineering.

CrORCA3 forms a cluster with the AP2/ERF TF genes *CrORCA4* and *CrORCA5*. Overexpressing *CrORCA4* increased tabersonine production by more than 40-fold in *C. roseus* hairy roots [58]. The AP2/ERF TF *li049* positively regulates lariciresinol in *I. indigotica*. Transgenic hairy roots overexpressing *li049* produced 425.60 μg/g lariciresinol, levels that are 8.3-fold higher than the wild-type [59]. The R2R3-MYB TF SmMYB98 positively regulates tanshinone and salvianolic acid biosynthesis in *S. miltiorrhiza* hairy roots [26]. SmWRKY1 and SmWRKY2 positively regulated tanshinone biosynthesis by targeting *SmDXR* and *SmCPS*, respectively [60,61]. Overexpressing the MeJA-responsive AP2/ERF TF gene *SmERF115* in *S. miltiorrhiza* hairy roots increased salvianolic acid B levels 1.43-fold, but reduced tanshinone production to 8% of control levels [17]. Overexpressing *OpMYB1* reduced camptothecin content in transgenic *O. pumila* hairy roots compared with the control [62]. Thus, these TF genes could be targeted for secondary metabolite engineering, but a suitable target should be selected in keeping with the objectives of the engineering process. The genetic engineering of new TF genes combined with the use of elicitors could further enhance metabolite production.

Introducing Heterologous Genes and Pathways

The metabolic engineering of target compounds from medicinal plants has also been achieved through the introduction of new genes and/or pathways. For instance, six enzymes for etoposide

aglycone biosynthesis were identified from *Podophyllum hexandrum* and the pathway was reconstituted in *Nicotiana benthamiana* via the co-expression of ten pathway genes [63]. In addition, two unknown enzymes essential for vinblastine biosynthesis were recently identified and heterologously expressed in *N. benthamiana* for production of stemmadenine-derived bioactive compounds, such as catharanthine and tabersonine [2]. Similar strategies were used to generate new triterpenes [64]. Although these studies were not conducted in transformed root cultures, similar approaches could be used to produce valuable compounds in hairy roots of medicinal plants. Moreover, these studies highlight the importance of completely understanding the biosynthetic pathways of valuable compounds.

Applications of Biotechnology to Dissect Metabolite Biosynthesis Pathways for Hairy Root Engineering

CRISPR/Cas9 to Characterize Gene Function

CRISPR/Cas9 is a powerful genome-editing tool that has been extensively used to engineer many plants, including various medicinal plant species [65–67]. For instance, CRISPR/Cas9 was used to eliminate *OpG10H* and *OpSLS* in *O. pumila* hairy roots, thereby reducing camptothecin levels by >90% [45]. Knocking out *SmMYB98* in *S. miltiorrhiza*, using CRISPR/Cas9, resulted in a decrease in tanshinone and phenolic acid levels, suggesting that *SmMYB98* has positive roles in tanshinone and phenolic acid biosynthesis [26].

CRISPR/Cas9 has many advantages over other genetic modification techniques, including RNAi and other targeted nucleases. For example, CRISPR/Cas9 is simple and less expensive than these techniques and can target multiple genes [68]. Also, a gene edit will not be distinguished by a 'naturally occurring' mutation once the genomic-editing agents have been segregated out. Moreover, emerging CRISPR applications, such as prime editors [CRISPR-Cas9 nickase (H840A)-reverse transcriptase fusions programmed with prime editing-guide RNAs] have recently been used to generate point insertions, mutations, and deletions in rice and wheat protoplasts [69]. Therefore, CRISPR/Cas9 has great potential for use in identifying gene function, for large-scale, precise genome editing, and for characterizing the enzymes and pathways involved in regulating secondary metabolite production to increase yields in medicinal plants.

'Omics Technologies

'Omics-related techniques, including genomics, transcriptomics, proteomics, and metabolomics, have been used to investigate non-medicinal plants, medicinal plants such as *C. roseus* and *Podophyllum hexandrum*, and hairy roots of medicinal plants, including *S. miltiorrhiza*, *A. belladonna*, *A. acutangulus*, *Datura stramonium*, and *S. baicalensis*, to facilitate the identification of novel genes, pathways, and compounds [1,46,63,70–72]. Genomics and transcriptomics have been used more frequently than proteomics and metabolomics to analyze medicinal plants. For example, many novel genes involved in tanshinone biosynthesis and new TFs that regulate the tanshinone and/or salvianolic acid biosynthesis have been isolated by transcriptomic analysis of different tissues, elicitor-treated samples, or hairy roots [73]. Proteomics analysis of *S. miltiorrhiza* hairy roots treated with silver ions and yeast extract revealed the presence of five novel cytochromes P450s and five berberine bridge enzyme-like enzymes thought to function in tanshinone biosynthesis, and indicated that carbohydrate metabolism in these plants was redirected toward secondary metabolites, such as tanshinone, lignin, phenolic acids, and other antimicrobial compounds [74,75].

Metabolomics could be useful for elucidating pathways and obtaining an overview of the metabolites in a plant [76]. For example, metabolomics analysis of *SmCPS1*-RNAi plant roots identified 21 potential intermediates [77], providing useful data for the clarification of tanshinone

biosynthesis. Nuclear magnetic resonance-based metabolomics of transformed roots derived from *Verbascum nigrum* revealed an abundance of glutamine in hairy roots but not in mother plant tissues [78]. Furthermore, a combination of metabolomic and transcriptomic analyses revealed genes possibly involved in the specialized metabolism in *O. pumila* and *S. miltiorrhiza* [79,80]. These studies demonstrated the value of 'omics techniques to analyze the hairy root and/or roots of medicinal plants and to discover new genes and pathways.

Rapidly Identifying Gene Function *In vivo*

Hairy roots can also be used for the rapid characterization of gene function *in vivo*. This approach is especially beneficial for medicinal plants because many of these plants take several years to accumulate valuable compounds, while hairy roots can accumulate these compounds in only 3–4 months. Hairy roots from medicinal plants, such as *C. roseus* [57], *S. baicalensis* [19,72], and *A. belladonna* [46,81], and other plants, including tobacco, tomato, *M. truncatula*, and *Brassica rapa*, were recently used to rapidly characterize the functions of unknown genes. For example, RNAi silencing of flavone synthase II (*FNSII*) and cinnamic acid-specific coenzyme A ligase (*CLL-7*) in hairy roots of *S. baicalensis* provided direct evidence for the roles of these enzymes in root-specific flavones biosynthesis in *S. baicalensis* roots [19]. Silencing of *SbCYP82D2* (encoding flavone 8-hydroxylase) in hairy roots of *S. baicalensis* decreased wogonoside levels from 4.7 mg/g DW in the vector control to 1.1 mg/g DW, and decreased wogonin levels from 1.9 mg/g DW in the control to 1.1 mg/g DW [72]. The transcriptional repressor MYB2 abolished proanthocyanidin and anthocyanin accumulation in *M. truncatula* hairy roots [82]. These findings demonstrated that hairy roots are efficient tools for identifying gene function *in vivo*.

Challenges to the Use of Hairy Root Cultures

Despite great progress so far, no valuable metabolites generated from hairy roots have been commercially produced, primarily due to insufficient production and value of the target compounds. Most increases in the levels of target compounds in hairy roots have been small and could have been achieved using normal root cultures, suggesting that better experimental designs are needed for the metabolic engineering of hairy roots. For example, proper controls should be used, more than one wild-type line, empty vector-transformed control, and normal root cultures (adventitious roots). Several other factors must also be considered, such as the stability of the compounds produced in hairy roots, as well as their toxicity due to the large amounts of target compounds produced and the cost involved in generating these target compounds from hairy root cultures. The practical use of hairy roots to produce valuable compounds requires that the hairy roots stably produce large quantities of target metabolites, and to produce similar amounts of target compounds in each subculture. Indeed, the genetic and metabolic activities of engineered hairy roots from *Hyoscyamus muticus* and *C. roseus* were genetically and metabolically stable after many years of continuous subculture (16 and 11 years, respectively) [83,84]. While hairy roots appear to be stable, more studies are needed to examine the stability of other hairy root systems.

Many valuable compounds produced in hairy roots are secondary metabolites and can negatively affect root growth when they accumulate to a certain level; this toxicity poses another challenge that limits the practical application of hairy roots. This problem could be solved using transporters of target compounds, *in situ* via adsorption, textile dye adsorption, advanced bioreactors, or the combined use of these methods. Although much is known about transporters in model plants [85], more information is urgently needed to identify potential transporters of secondary metabolites, particularly in medicinal plants.

Given that hairy roots must be cultured in the dark, the liquid culture medium must contain sugars as the principal carbon source, and the cultures must be continuously shaken at a constant

temperature. The cost of producing target compounds includes the cost of sugars, a shaking incubator with a constant temperature, the electricity required for shakers, and other devices needed to collect target metabolites and/or a system for medium recycling, which are also considered to be limiting factors. The need to design a suitable bioreactor is another limiting factor for large-scale cultivation of transformed root culture [41]. For example, to date, *P. ginseng* hairy roots have only been tested in a 19-L airlift bioreactor [86].

Compared with microbes, which require only one to several days to complete one generation, hairy roots grow slowly, requiring 1–2 months. The relatively longer growth period of hairy roots also makes them more easily prone to contamination. In addition, resupplying the culture medium poses another impediment to hairy roots culture. Furthermore, the morphology of hairy roots differs among plant species, and cell growth and metabolite production in hairy roots are also nonhomogeneous [86], suggesting that more factors must be considered when optimizing bioreactors. Therefore, a suitable bioreactor design is needed for hairy roots cultivation [38,41,87,88].

Compared with hairy roots, greater progress has been made in the large-scale culture of adventitious roots. For example, 20-l to 10 000-l bioreactors have been successfully used to scale-up adventitious root cultures from *P. ginseng*, *Morinda citrifolia*, *Hypericum perforatum*, *Echinacea purpurea*, and *E. angustifolia* [24,87–89]. Various approaches used to design bioreactors for culturing adventitious roots could be used as guidelines for the large-scale culture of hairy roots. Finally, single-use bioreactors have recently emerged as an alternative for plant cell culture, including hairy roots culture, given the reduced time and cost of culture, low contamination rate, and reduced environmental impact [88,90,91]. Several single-use bioreactors with different working volumes (up to 2000-l) and mixing principles are commercially available [91]. The use of single-use bioreactors to produce valuable compounds in hairy roots should be further explored. Indeed, all of the discussed challenges must be properly addressed before the industrial production of valuable metabolites from hairy roots can be achieved.

Concluding Remarks and Future Perspectives

In summary, we have described recent developments made with hairy roots from medicinal plants, including strategies to increase the concentrations of desired metabolites. Various strategies have been used to enhance secondary metabolite production in hairy roots, including new approaches incorporating 'omics, CRISPR/Cas9, and synthetic biology. We also discussed the use of hairy roots for new gene identification and the rapid characterization of gene function. Significant progress has been made in the engineering of secondary metabolites in hairy roots from medicinal plants. New strategies have emerged, and new genes and TFs involved in metabolite biosynthesis have been identified. However, the biochemical pathways and regulatory mechanisms for the production of many secondary metabolites are still not fully understood. Much less is known about the transporters of valuable compounds in medicinal plants. The quantity of valuable compounds produced in the hairy roots of medicinal plants remains insufficient for practical applications. Several challenges remain, such as the low yields of high-value target compounds, the instability of hairy roots, the toxicity of target compounds, and the need for suitable bioreactors. These challenges, together with a lack of information about biosynthetic pathways and complex regulatory mechanisms for target compound biosynthesis, explain why metabolites generated from hairy roots have yet to be commercially produced.

All of these challenges must be addressed. The toxicity problem could be solved by using transporters for target compounds, *in situ* via adsorption, textile dye adsorption, or advanced bioreactors. In particular, approaches used to design bioreactors for the adventitious roots of medicinal plants could be used as guidelines for the large-scale culture of hairy roots from medicinal plants.

Outstanding Questions

Can a transient expression system, which would be particularly useful for testing root-derived gene function, be established in hairy roots?

Can single-cell RNA sequencing be used to better understand biosynthesis and genetic regulation mechanism of metabolites produced natively in hairy roots?

Could hairy roots be used in synthetic biology to reconstruct new biosynthetic pathways to obtain valuable compounds, especially those that mainly accumulate in roots or are used to generate non-natural compounds, by modifying biosynthetic pathways?

Can we use hairy roots as an alternative tool to investigate symbiotic interactions between roots (especially for medicinal plants that grow slowly) and beneficial microorganisms (i.e., nitrogen fixation)?

Could hairy roots be utilized as a platform to explore stress resistance for healthy plant production and biotic/abiotic stress management?

Can hairy roots be applied as a model system for studying root growth and development?

Are transproters that can transfer valuable compounds from cells to an outside medium useful to decrease toxicity caused by metabolite overaccumulation?

How can we integrate multi-omics in hairy roots?

How can we further improve fermentation systems to enhance the production of desired metabolites?

Synthetic biology has been successfully applied for the commercial production of valuable compounds, such as artemisinin, from microbes. Advances in 'omics-related techniques and newly developed genome-editing techniques, such as CRISPR/Cas9, should soon lead to the identification of biosynthetic pathways, principal steps, and regulatory mechanisms for target compound production. For example, in addition to using CRISPR/Cas9 to generate targeted multiplex mutant hairy roots (loss function of several isoforms of the same gene family) to avoid gene redundancy, which would facilitate gene identification and biosynthetic pathway discovery, this technique could be used to introduce multiple biosynthetic genes into the genomes of hairy roots in a single step with few markers, which would be valuable for the metabolic engineering of target compounds [1]. Subsequently, metabolic engineering of valuable compounds utilizing synthetic biology designed based on the improved understanding of biochemical pathways and regulatory mechanisms of specific metabolites will be helpful to further increase the quantities of target compounds produced in hairy roots of medicinal plants. Finally, the availability of a suitable bioreactor for hairy roots should lead to the production of the first commercialized compounds from hairy roots, such as high-value compounds (e.g., vinblastine and camptothecin) or compounds that primarily accumulate in roots.

Notably, hairy roots are less useful than other systems for producing photosynthesis-dependent compounds because they are cultured in the dark. Other organisms, including *N. benthamiana* or algae, might be better hosts. However, it would be worth further exploring the use of hairy roots as tools, platforms, model systems, or synthetic biology systems to answer various important scientific questions (see Outstanding Questions), which might lead to a better understanding of hairy roots to help realize their full potential.

Acknowledgments

This work was funded by National Natural Science Fund of China (81522049, 1571735, 31270007), Zhejiang Provincial Ten Thousands Program for Leading Talents of Science and Technology Innovation (2018R52050), Zhejiang Provincial Program for the Cultivation of High-level Innovative Health talents, Shanghai Science and Technology Committee Project (17JC1404300, 15430502700) and the 'Dawn' Program of Shanghai Education Commission (16SG38). M.I.G. acknowledges the financial support of the European's Union Horizon 2020 Research and Innovation Programme, project PlantaSYST (SGA-CSA No. 739582 under FPA No. 664620).

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