"Hairy Root" Technology: An Emerging Arena for Heterologous Expression of Biosynthetic Pathway Genes in Medicinal Plants

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Abstract

Plant-based secondary metabolites play an important role towards the drug development process, but their lower yield in the source plant and uncertainty in supply owing to miscellaneous intervening factors have necessitated biotechnological intervention for devising suitable and economical alternative production systems. The progression of innovative biotechnological tools in tandem with the understanding of the plant metabolic pathways at both biochemical and cellular levels through combining the accumulating knowledge of next-generation sequencing has opened up new avenues for metabolic engineering of biosynthetic pathways. In this context, hairy root (HR) cultures have emerged as a promising platform for tailoring the metabolic flux of a given plant system towards the overproduction of desired metabolites by heterologously or homologously expressing the rate-limiting genes. A rational utilization of such cultures of diverse medicinal plants for heterologous expression of targeted pathway genes has started gaining attention over the years in order to overcome the co-suppression related to normally encountered disadvantages of homologous overexpression. The potential and appropriateness of this approach have gathered the maximum momentum during recent years even though such studies have come into existence for more than two decades ago. The present review summarizes the overall reported advances made in the area of hairy root-mediated heterologous expression of rate-limiting key genes of diverse biosynthetic pathways which remained mainly concentrated on tropane alkaloid, terpene indole alkaloid, and mevalonate and phenylpropanoid pathways. Successful implementation of the entire procedure is also found to be reigned by several other underlying factors, amongst which characteristic/exclusivity of plant families,

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A. rhizogenes strains' specificities, explant types, promoters' specifications and media constituents which are some of the prominent deciding factors that differed amongst the reported observations and have been outlined in this review.

Keywords

Agrobacterium rhizogenes • Biosynthetic pathway • Heterologous plant gene expression • Hairy root culture • Medicinal plants

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1 Introduction

Agrobacterium rhizogenes-mediated "hairy root" (HR) technology has traversed a long way since its judicious utilization as an efficient maneuvering technique for plant transformation by Ackermann in 1970 to reach the central stage of plant-based bioactive molecule production process, meant for sustenance of the whole underlying legacy. Going back to the history of plant-derived drug discovery trend, one can undeniably recount the strength of its progression through a rational drug discovery process since the beginning of the nineteenth century, which gradually thrived by the use of pure compounds instead of primarily used extracts and partly purified natural products [1]. The domineering impact of plant-derived natural compounds in modern drug development succession can very well be gauged by the statistical facts illustrating their indispensability in more than half of the 1073 new chemical entities getting approval between 1981 and 2010 [2]. At the same time, it also cannot be ignored that diverse prevailing complexities have emerged lately which recurrently hindered the access to authentic raw plant materials (with unvarying yield/quality parameters) and consequently discouraged pharmaceutical companies from phytomolecule-based drug development process [1]. In that context, it has recently been eloquently illustrated by Atanasov et al. [3] that most of the existing encumbrances have arose owing to many unavoidable circumstances (such as

demographical, anthropological, environmental, and ecological impediments, coupled with other yield and quality-linked fluctuation hassles), which unquestionably calls for intense attention towards replenishing renewed interest in phytomolecule-based drug development process by overcoming the aforesaid challenges.

Devising an undeviating process of generating alternative production source with tailor-made production profile of plant-based metabolites has progressively turned into a reality all over the world in this decade through the rational advent of the "hairy root" technology [4]. The multifarious beneficial traits of such cultures not only bestow genetic and biochemical stability with rapid growth potential and hormone autotrophy but also offer equivalent or higher biosynthetic potential mimicking that of the parent plant [5]. Hairy root cultures have therefore undeniably fulfilled the prerequisite qualification of cellular differentiation related to sanguinity in terms of higher amount of product accumulation [6] and accordingly have immeasurably intervened in the frontline ventures of medicinal plant research to meet the growing demand of pharmaceutical industries. In this context, the advancements in the modern post-genomic technological insights in elucidating the metabolic pathways and associated regulatory/rate-limiting genes have progressively opened up new avenues for tailoring the metabolic flux of a given plant system towards the production of high-demand, low-yield molecules in greater quantities by pathway engineering in hairy root cultures [4]. In any given scenario, overproduction of a desired metabolite through such organ culture can be achieved by following either of the two realistic ventures, i.e., overexpression of rate-limiting gene for maximizing the metabolic flux towards the desired end product or by diverting the metabolic channel through the incorporation of appropriate participating genes from heterologous system [4, 7]. Although heterologous expression of plant genes in microbes has gathered some attention over the years, several practical impediments in terms of unavailability of the plant-based precursor substrate, complexity in posttranslational modifications, toxicity resulting from the accumulation of plant products, etc. appeared as stumbling block to be solved in the future [8]. On the contrary, the existence of the entire metabolic pathway with physiologically active genetic/enzymatic makeup in hairy root clones makes it extremely lucrative alternative to achieve the maximum reward through heterologous expression of pathway genes towards improved metabolite accumulation [9]. Indisputably, the incorporation of pathway genes from different source plant with the same function has significantly facilitated in rectifying the co-suppression-mediated disadvantage of overexpressing the pathway genes of the same plant system [4]. For the last three decades, the HR cultures of several medicinal and aromatic plants have been widely explored in the arena of heterologous gene expression based on medicinal plant research as depicted in Fig. 1. Literature survey revealed that the maximum number of published reports could be documented in the year 2011–2015 (ten) followed by 2006–2010 (nine), 2001–2005 (eight), 1996–2000 (three), and 1990–1995 (two). More than 85% of the published reports came into existence in the last 15 years showing the significance and potential of such HR-based technology towards the manipulation of any biosynthetic pathway genes for the production of

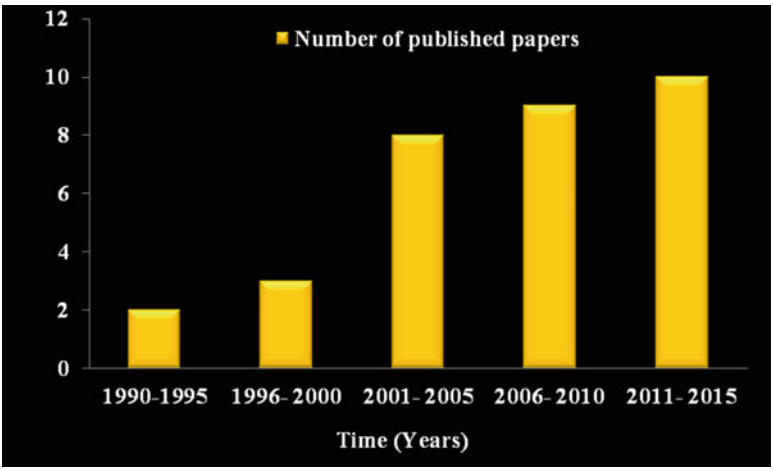

Fig. 1 Representative rate of publications relating to hairy root-mediated heterologous gene expression carried out for tailoring biosynthetic pathways

therapeutically active metabolites through heterologous expression of pathway genes from a separate plant system (Fig. 1).

Accumulating knowledge in this direction needs a comprehensive evaluation for generating future directions in medicinal plant research for tailoring the yield potentials towards a desired biomolecule production in hairy root cultures, to which the present review is committed. The types of metabolites, contributions of the underlying pathways, influence of the rate-liming genes, gross role of plant families and even the hairy root inducing *A. rhizogenes* strains, and culture media's attributes will be figured out in this review as each of these attributes imparts a decisive role towards the final outcome of such endeavor. This review is an attempt to scrutinize each and every aspect in great detail to delineate and mend any misapprehension under the present topic by offering a comprehensive view of global collaborative wisdom.

2 Biosynthetic Pathways and Genes Involved

The impact of post-genomic technologies on gene discovery and metabolic pathway elucidation can very well be perceived in recent years on the basis of accumulating knowledge in this arena. The crucial steps involved in a successful modulation of biosynthetic pathways through heterologous expression of pathway genes have been impressively elucidated under five headings in a recent review [4]. This information has clearly indicated that a detailed knowledge of the biosynthetic pathways and the role of decisive genes have undoubtedly facilitated the advancement of metabolic engineering as a potential approach to increase the yield of specific metabolites by heterologously or homologously expressing the rate-limiting genes [10]. In this context, the heterologous expression of targeted genes has started gaining attention over the years in order to overcome the disadvantage of overexpression of specific

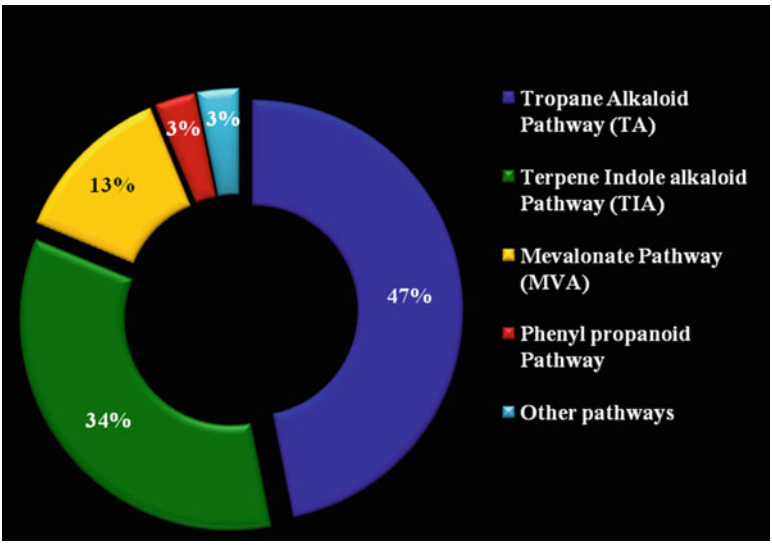

Fig. 2 Prevailing biosynthetic pathways engineered through HR-based heterologous gene expressions – tropane alkaloid (*TA*) pathway, terpene indole alkaloid (*TIA*) pathway, mevalonate (*MVA*) pathway, phenylpropanoid pathway, and other pathways (galacturonate pathway)

genes from the same plant source owing to the phenomenon of co-suppression of genes [11]. Although the prospect of utilizing hairy roots as expression system for modulating the biosynthetic pathways through heterologous gene incorporation was investigated as early as 1993, the expediency of this approach has gathered the maximum momentum during recent years.

It is evident from literature that plant alkaloids (terpene and tropane) constitute the largest group of natural products, offering many pharmacologically active secondary metabolites [10]. Correspondingly the major bulk of information related to successful pathway modulation through heterologous gene expression through hairy roots remained concentrated on these two groups of metabolites (Fig. 2). Out of 32 reported results in this area involving hairy root as expression system, 15 belong to the tropane alkaloids (47%), 11 to the terpene indole alkaloids (34%), and 4 to the mevalonate pathway (13%), while only two were related to other pathways (6%) in which one had covered the phenylpropanoid pathway (Fig. 2).

2.1 Tropane Alkaloid (TA) Pathway (*Putrescine N-methyltransferase* (*PMT*) *and Hyoscyamine- 6β-hydroxylase* (*H6H*) *Genes*)

The tropane alkaloids (TAs), found mostly in the members of the Solanaceae family, constitute a major group of pharmaceutically significant metabolites, amongst which hyoscyamine or atropine and scopolamine are widely reputed for their various pharmacological functions [12]. The supply of these molecules to the

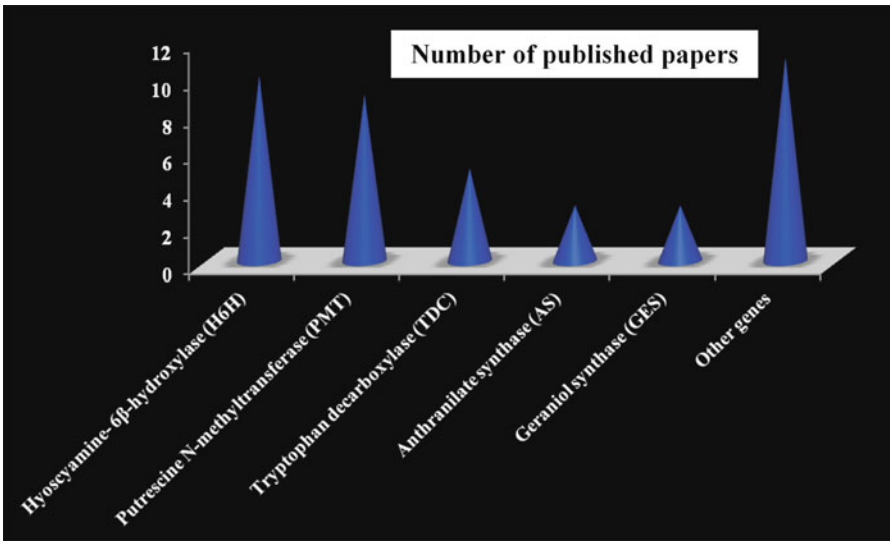

Fig. 3 An assortment of the major biosynthetic pathway genes utilized for the hairy root-mediated heterologous gene expression. [Other pathway genes include deoxy xylulose synthase (*DXS*), deoxy xylulose reductase (*DXR*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*), farnesyl diphosphate synthase (*FPS*), squalene synthase (*SS*), transcription factor (*AtPAP1*), cytochrome P450, and D-galacturonic acid reductase (GalUR)]

pharmaceutical industries is mainly dependent on the plant resources as their chemical synthesis is tedious and not economically feasible [13]. Therefore, to develop an alternative approach for their production, TA biosynthetic pathway has been manipulated through HR-mediated expression of exogenous genes from different medicinal plants. Throughout the TA pathway, there are a number of key enzymes that regulate the flux through the pathway, but the major contributing enzymes were noted to be hyoscyamine-6β-hydroxylase (*H6H*) and putrescine *N*-methyltransferase (*PMT*) genes (Fig. 3). To the best of our knowledge, till date the reported studies relating to these aforementioned genes (*PMT* and *H6H*) contributed about 47% of the total published papers (15 papers). These genes were heterologously expressed (either individually or collectively) in different medicinal plant systems via HR culture for the enhancement in the production of the targeted tropane alkaloids – hyoscyamine and scopolamine (Fig. 3).

The *PMT* gene regulates the N-methylation of putrescine with its further rerouting towards the biosynthesis of alkaloids, while the bifunctional enzyme *H6H* hydroxylates hyoscyamine to 6β-hydroxy hyoscyamine, which subsequently undergoes epoxidation to form scopolamine [12]. Interestingly, the *PMT* and *H6H* genes of the tropane pathway have been the most explored biosynthetic enzymes of the Solanaceae family for the enhancement in the alkaloid productivities through heterologous expression (Table 1). To the best of our knowledge, about 49% of the total published papers reporting heterologous expression of pathway genes are covered by

Table 1 Hairy root-mediated heterologous expression of pathway genes involving different medicinal plants during the last 25 years (arranged in chronological sequence starting from recent reports)

Sl. No.	Engineered plant species	Pathway involved	Gene (s)	Gene source	Promoter	Overall effect	References
1	*Nicotiana tabacum*	Terpene indole alkaloid (TIA)	Geraniol synthase (*GES*)	*Valeriana officinalis*	*CaMV 35S*	Transgenic HR revealed a maximum free geraniol content: (31.3 μg/g DW)	[26]
2	*N. tabacum* *N. benthamiana*	Terpene indole alkaloid (TIA)	Geraniol synthase (*GES*)	*V. officinalis*	*CaMV 35S*	Geraniol production reported in diverse forms of in vitro cultures	[27]
3	*N. tabacum*	Terpene indole alkaloid (TIA)	Geraniol synthase (*GES*) + Geranyl pyrophosphate synthase (*GPPS*)	*V. officinalis* *Arabidopsis thaliana*	*CaMV 35S*	*GES* HR line accumulated significant amounts of geraniol/ geraniol glycosides (151 ± 24 ng/mg DW), while *GES* + *GPPS* expression clones showed lower levels of accumulation than that in the single gene transgenic	[28]
4	*Salvia sclarea*	Mevalonate (MVA)	Deoxy xylulose synthase (*DXS*) + Deoxy xylulose reductase (*DXR*)	*A. thaliana*	*CaMV 35S*	Enhancement in the content of bioactive abietanic diterpenes (aethiopinone, 1-oxoaethiopinone, savipisone, ferruginol, carnosic acid, 1-oxo-ferruginol)	[39]
5	*Platycodon grandiflorum*	Phenylpropanoid (PP)	Transcription factor (*AtPAP1*)	*A. thaliana*	*CaMV 35S*	The best transgenic HR line showed 9.89 times (approx) higher content of chlorogenic acid (CGA) [421.31 μg/100 mg DW]	[43]

(*continued*)

Table 1 (continued)

Sl. No.	Engineered plant species	Pathway involved	Gene (s)	Gene source	Promoter	Overall effect	References
6	*Rauvolfia serpentina*	Terpene indole alkaloid (TIA)	Tryptophan decarboxylase (*TDC*)	*Catharanthus roseus*	*CaMV 35S*	The best transgenic HR clone revealed better yield potentials with respect to **(i) Reserpine:** (0.1202 ± 0.002% DW) **(ii) Ajmalicine:** (0.0064 ± 0.003% DW)	[30]
7	*P. grandiflorum*	Mevalonate (MVA)	3-Hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*)	*Panax ginseng*	*CaMV 35S*	**Phytosterol levels increased by** 1.1–1.6-fold in the transgenic HR lines	[42]
8	*Scopolia parviflora*	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT1 and PMT2*) + Hyoscyamine-6β-hydroxylase (*H6H*)	*N. sylvestris Hyoscyamus niger*	*CaMV 35S*	Enhancement in the contents of scopolamine and hyoscyamine in the transgenic HR clones	[12]
9	*Atropa belladonna*	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT*) + Hyoscyamine-6β-hydroxylase (*H6H*)	*N. tabacum H. niger*	*CaMV 35S*	**Significant enhancement in the contents of both alkaloids in transgenic HR clones** Compared to non-transgenic and HR normal (intact) root culture **(i) Scopolamine:** five and four times higher, respectively **(ii) Hyoscyamine:** eleven and twenty-four times higher, respectively	[25]

10	*C. roseus*	Terpene indole alkaloid (TIA)	Deoxy xylulose synthase (*DXS*) + geraniol-10-hydroxylase (*G10H*) + anthranilate synthase (*AS*)	*A. thaliana*	*GAL4-UAS*	**DXS expression:** (I) increased: ajmalicine (67%), serpentine (26%), and lochnericine (49%) (II) decreased: tabersonine (66%) and horhammericine (54%) **Co-overexpression of DXS and G10H:** Increased: ajmalicine (16%), lochnericine (31%), and tabersonine (13%) **Co-expression of DXS with AS:** increased: horhammericine (30%), lochnericine (27%), and tabersonine by (34%)	[29]
11	*Withania coagulans*	Mevalonate (MVA)	Squalene synthase (SS)	*A. thaliana*	*CaMV 35S*	The transgenic HR roots accumulated higher content of the total withanolide (ranged from 0.68 to 4.63 μg/gm DW) as compared to the control (0.11 μg/gm DW) after 4 weeks of cultivation	[40]
12	*Centella asiatica*	Terpene indole alkaloid (TIA)	Farnesyl diphosphate synthase (*FPS*)	*P. ginseng*	*CaMV 35S*	Compared to non-transgenic root culture, transgenic HR revealed enhanced **(i)** Squalene content (1.1–1.5-fold**)** **(ii)** Total sterol contents (threefold)	[36]

(*continued*)

Table 1 (continued)

Sl. No.	Engineered plant species	Pathway involved	Gene (s)	Gene source	Promoter	Overall effect	References
13	*Solanum lycopersicum* cv. Money Maker	Galacturonate pathway	D-galacturonic acid reductase (GalUR)	*Fragaria ananassa*	*CaMV 35S*	The total L-ascorbic acid (AsA) content increased by 2.5-fold in a transgenic HR clone treated with D-galacturonic acid (D-GalUA)	[44]
14	*Brassica rapa*	Terpene indole alkaloid (TIA)	Cytochrome P450 (CYP79B2, CYP79B3, CYP83B1)	*A. thaliana*	*CaMV 35S*	Compared to the control HR lines with rol ABC, the incorporation of CYP83B1 in conjunction with CYP79B2 or CYP79B3 enhanced the accumulation of glucobrassicin or 4-methoxy glucobrassicin levels. However, no influence in their accumulation could be noted through individual overexpression of either of these genes	[37]
15	*Nicotiana* cell culture derived from the transgenic HR	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. muticus*	*CaMV 35S*	**Scopolamine content increased in the transgenic HR:** **Shake flask:** 21.6 ± 1.1 mg/L **Bioreactor:** 35.5 ± 0.8 mg/L	[23]
16	*H. niger*	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT*)	*N. tabacum*	*CaMV 35S*	Compared to the control, a fivefold higher PMT activity in the transgenic HR with no increase in alkaloid yield	[14]
17	*Duboisia leichhardtii*	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. niger*	*ParAt*	Enhanced content of scopolamine in the transgenic HR clones	[21]

18	*Atropa baetica*	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. niger*	*CaMV 35S*	Superior alkaloid content in transgenic HR **Scopolamine:** 5.63 mg/g DW **Hyoscyamine:** 0.2 mg/g DW **6β-Hydroxyhyoscyamine:** 1.01 mg/g DW	[24]
19	*C. roseus*	Terpene indole alkaloid (TIA)	Tryptophan decarboxylase (*TDC*) + Anthranilate synthase (*AS*)	*C. roseus* *A. thaliana*	*CaMV 35S*	Enhanced content of tryptamine and tryptophan in transgenic HR	[33]
20	*N. tabacum* *H. muticus*	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. niger*	*CaMV 35S*	Transgenic *N. tabacum* HR showed more conversion of hyoscyamine to scopolamine over that in *H. muticus*	[22]
21	*S. parviflora*	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT1 and 2*)	*N. sylvestris*	*CaMV 35S*	Enhanced content in transgenic HR clones: (i) Hyoscyamine: 3.5-fold (4.2 mg/g DW) (ii) Scopolamine: fivefold (6.4 mg/g DW)	[18]
22	*C. roseus*	Terpene indole alkaloid (TIA)	Tryptophan decarboxylase (*TDC*) + Anthranilate synthase (*AS*)	*C. roseus* *A. thaliana*	*CaMV 35S*	Enhanced content of tryptophan and serpentine	[32]
23	*H. niger*	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*) + Putrescine *N*-methyltransferase (*PMT*)	*H. niger* *N. tabacum*	*CaMV 35S*	Compared to the control, the transgenic HR with both genes showed increase in scopolamine ninefold and twofold higher than only *H6H* expressing HR lines	[10]
24	*Duboisia hybrid* (*D. myoporoides* x *D. leichhardtii*)	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. niger*	*CaMV 35S*	Scopolamine content in transgenic HR: three times (74.50 mg/l) higher than the control	[20]

(*continued*)

Table 1 (continued)

Sl. No.	Engineered plant species	Pathway involved	Gene (s)	Gene source	Promoter	Overall effect	References
25	*Datura metel* *H. muticus*	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT*)	*N. tabacum*	*CaMV 35S*	Only hyoscyamine content increased in *H. muticus* transgenic HR clone while both hyoscyamine and scopolamine contents increased in *D. metel*	[16]
26	*Duboisia hybrid* (*D. myoporoides* x *D. leichhardtii*)	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT*)	*N. tabacum*	*CaMV 35S*	Enhanced N-methylputrescine level: two- to fourfold with no increase in the content of other metabolites	[15]
27	*A. belladonna*	Tropane alkaloid (TA)	Putrescine *N*-methyltransferase (*PMT*)	*N. tabacum*	*CaMV 35S*	Compared to a wild-type HR, the transgenic HR revealed fivefold higher expression of PMT level with no change in the total alkaloid content	[17]
28	*Solanum aviculare*	Mevalonate (MVA)	3-Hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*)	*Artemisia annua*	*CaMV 35S*	Solasodine content enhanced 4.2 times over that in the control HR.	[41]

29	*Cinchona officinalis "Ladgeriana"*	Terpene indole alkaloid (TIA)	Tryptophan decarboxylase (*TDC*) + Strictosidine synthase (*STR*)	*C. roseus*	*CaMV 35S*	Enhancement in the contents of tryptamine, strictosidine, quinine, and quinidine	[34]
30	*H. muticus*	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. niger*	*CaMV 35S*	Compared to control HR, the best transgenic clone produced 100 times higher content of scopolamine	[13]
31	*A. belladonna*	Tropane alkaloid (TA)	Hyoscyamine-6β-hydroxylase (*H6H*)	*H. niger*	*CaMV 35S*	Compared to control HR, the transgenic clone revealed fivefold enhancement in the scopolamine content	[19]
32	*Peganum harmala*	Terpene indole alkaloid (TIA)	Tryptophan decarboxylase (*TDC*)	*C. roseus*	*CaMV 35S*	The tryptamine level remained unchanged but the indole alkaloid (serotonin) content enhanced by tenfold in the transgenic HR clone. Supplementation with tryptophan further enhanced the serotonin content	[31]

individual or collective expression of the aforementioned genes (*PMT* and *H6H*) in different medicinal plant via HR cultures contributing a major share in the production of the targeted tropane alkaloids – hyoscyamine and scopolamine (Fig. 3).

The exclusive role of *PMT* gene has been studied earlier by different research groups, one of which is through its isolation from *N. tabacum* and successful expression in *H. niger* HR cultures, leading to the fivefold higher PMT activity in the transgenic HR than that in the control, while no increase could be noted in the overall content of the alkaloids [14; Table 1]. Similarly, the same gene (*PMT*) from *N. tabacum* has also been expressed earlier in *Duboisia hybrid* (*D. myoporoides* x *D. leichhardtii*) HR cultures enhancing the *N*-methylputrescine level up to twofold to fourfold, but no enhancement could be observed in the content of other metabolites [15]. In contrast, the production of both hyoscyamine and scopolamine was found to be increased by expressing the *PMT* gene from *N. tabacum* in *Datura metel* HR cultures [16], while the *Hyoscyamus muticus* HR clone with *PMT* gene of the same origin failed to produce the latter, and only the content of the former was augmented [16; Table 1]. Ahead of these results, Sato et al. [17] have fruitfully expressed the *PMT* gene from *N. tabacum* in *Atropa belladonna* HR culture which resulted in a fivefold increase of *PMT* level in transgenic HR as compared to wild-type HR clone but the total alkaloid content remained unchanged (Table 1).

Likewise, the effect of introducing putrescine *N*-methyltransferase (*PMT1* and *PMT2*) under the control of a CaMV 35S promoter from *N. sylvestris* into *Scopolia parviflora* HR cultures has also been investigated by Lee et al. [18]. The transgenic HR line produced over 3.5 and 5 times more hyoscyamine and scopolamine, respectively, than the wild-type HR cultures, substantiating the fact that improvement in the production of such metabolites as the end products could assuredly be achieved through such heterologous expression of the upstream genes of tropane alkaloid pathway [18].

So far as the *H6H* gene is concerned, it was observed that out of the 32 published information under the present topic, ten reports have predominantly demonstrated the efficacy of this specific gene in enhancing the production of scopolamine in different members of Solanaceae family (Fig. 3). The *H6H* gene from *Hyoscyamus niger* has been cloned and introduced into *A. belladonna* HR culture where a fivefold higher scopolamine yield could be documented as compared to the wild-type HR culture, and the content of 6β-hydroxyhyoscyamine was also increased in the transformed roots [19; Table 1]. The *H6H* gene of the same source has also been cloned and introduced into *H. muticus*, where the best transgenic HR clone produced 100 times higher content of scopolamine than that of the control clone. This observation convincingly illustrated the prospect of utilizing hairy root as a promising and reliable tool for the heterologous gene expression system for enhancing the scopolamine productivity [13; Table 1].

In another study, the *H6H* gene from *H. niger* was introduced into the genome of a scopolamine-rich *Duboisia hybrid* (*D. myoporoides* x *D. leichhardtii*) under the regulation of the CaMV35S promoter [20]. The scopolamine levels (74.50 mg/l) in the engineered HR lines improved up to three times as compared to wild-type hairy

roots, but no significant changes could be documented in the HR-regenerated plants [20; Table 1). Another report by Rahman et al. [21] has demonstrated that the incorporation of the *H6H* gene from *H. muticus* under the control of "ParAt" promoter in *Duboisia leichhardtii* HR culture has enhanced the content of scopolamine in the *H6H*-positive HR clones [21; Table 1].

Hakkinen and co-workers [22] have reengineered the *N. tabacum* and *H. muticus* HR cultures by the incorporation of the *H6H* gene from *H. niger* for the enhanced secretion of tropane alkaloids. Both the transgenic hairy roots were examined for their potentials towards the production of scopolamine and other tropane alkaloids after the exogenous supply of the precursor (hyoscyamine) in the culture medium. Amongst them, *N. tabacum* HR clone showed more proficiency towards the uptake of hyoscyamine from the culture medium and a higher bioconversion rate towards the scopolamine production compared to that of the *H. muticus* HR clone (Table 1). Apart from the secretion of maximum scopolamine (85%) into the culture medium, the addition of hyoscyamine also influenced the accumulation of nicotine in the resultant transgenic *N. tabacum* HR line [22].

In another study, the competence of dedifferentiated cell cultures derived from the *N. tabacum* hairy roots carrying the *H. muticus H6H* gene has also been investigated for the bioconversion of hyoscyamine to scopolamine [23]. They have shown that *N. tabacum* cell suspensions, which normally do not produce scopolamine, are able to produce this alkaloid after overexpressing the gene *H6H* in tandem with the precursor feeding of hyoscyamine (Table 1). The transgenic cell suspension cultures showed a higher potential for the conversion of exogenously added hyoscyamine at two different concentrations (100 mg/l and 200 mg/l) to the culture medium [23]. The total scopolamine production (21.6 $\pm$ 1.1 mg/l) in the shake flask could be obtained at the higher concentration. The overall scopolamine accumulation could further be enhanced significantly by upscaling the transgenic cell culture from the shake flask (21.6 $\pm$ 1.1 mg/l) to 5 l turbine-stirred tank bioreactor (35.5 $\pm$ 0.8 mg/l), indicating the commercial prospect of the entire effort [23].

The effect of the *H6H* gene of *H. niger* on the production potentials of *Atropa baetica* HR clone for scopolamine, hyoscyamine, and 6β-hydroxyhyoscyamine has also been investigated by Zarate et al. [24]. The transgenic roots overexpressing *H6H* revealed higher content of scopolamine (5.63 mg/g DW), hyoscyamine (0.2 mg/g DW), and 6β-hydroxyhyoscyamine (1.01 mg/g DW). The scopolamine level increased up to ninefold as compared to the plants [24; Table 1]. The heterologous co-expression of *PMT* and *H6H* genes from *N. tabacum* and *H. niger*, respectively, in *H. niger* HR culture revealed nine times higher content of scopolamine in the transgenic HR clone than that of the wild type (Table 1) and only two times higher than that in the HR lines expressing *H6H* gene exclusively [10]. Subsequently, the cloning and co-expression of the same *PMT* and *H6H* genes from the abovementioned sources in *Atropa belladonna* HR culture showed a distinct effect by producing five and four times higher scopolamine content and 11 and 24 times higher hyoscyamine content than that in the non-transgenic HR and normal (intact) root culture, respectively [25; Table 1].

In another contemporary investigation, the genes for the two key enzymes of tropane alkaloid pathway (i.e., *PMT1* and *PMT2*) from *Nicotiana sylvestris* and *H6H* from *Hyoscyamus niger* were expressed in the metabolically engineered hairy root cultures of *Scopolia parviflora* [12]. The morphology of the *S. parviflora* transformed roots with *PMT1*, *PMT2*, and *H6H* genes was noted to be distinct, and the overall effects of these genes led to the enhancement in the content of scopolamine and hyoscyamine in the transformed roots [12; Table 1]. Supplementation with different phytohormones further enhanced the accumulation of scopolamine and hyoscyamine in these transgenic HR lines [12].

2.2 Terpenoid Indole Alkaloid (TIA) Pathway

TIAs are one of the major classes of phytomolecules renowned for their various applications in the pharmaceutical, fragrance, and cosmetic industries [26]. There are several TIAs found in nature governed by numerous pathway genes, but very few have been explored in terms of HR-mediated heterologous gene expression for improving the productivity of targeted metabolites involving distinct medicinal plants (Fig. 2). The success stories are discussed below.

2.2.1 Geraniol Synthase (*GES*) Gene

Geraniol is synthesized from the monoterpenoid precursor geranyl diphosphate by the geraniol synthase (*GES*) gene, which is an intermediate metabolite in the monoterpenoid pathway, interconnecting with the indole pathway to form pharmaceutical products of TIAs [27]. Geraniol possesses several pharmacological importances by suppressing different types of cancer such as colon, pancreatic, hepatic, and prostate tumors and also reduces serum cholesterol levels [27]. The low yield from the natural plant sources and intricate chemical structure of geraniol has attracted considerable recent interest towards developing biotechnology-based production alternatives to meet the escalating demand of this valuable phytomolecule. Literature survey revealed that out of the total 32 reported observations on hairy root-mediated heterologous expression of different pathway genes, three reports are available concerning the expression of geraniol synthase (*GES*) gene till date (Fig. 3).

The heterologous expression of *GES* gene under the control of cauliflower mosaic virus (CaMV 35S) promoter from *Valeriana officinalis* has been achieved in *Nicotiana tabacum* and *N. benthamiana* HR cultures which resulted in the production of geraniol in different in vitro cultures [27; Table 1]. The maximum geraniol content could be noted in the stable transgenic plants grown in vitro (48 μg/g FW), and the least could be noted in the hairy root culture (9 μg/g FW), while the other expression systems showed intermediary results in reducing the order as follows: transient expression system (27 μg/g FW), transgenic plants under hydroponic conditions in the greenhouse, and cell suspension cultures (16 μg/g FW) [27].

Similarly, Ritala et al. [26] have successfully expressed the *GES* gene from *V. officinalis* in the HR clone of another species of *Nicotiana*, i.e., *N. tabacum* which resulted in the production of twenty transgenic HR clones having a maximum

free geraniol content of 31.3 μg/g DW [26; Table 1]. In another study by Masakapalli et al., [28], enhancement in the biosynthesis of geraniol in the HR cultures of *N. tabacum* through metabolic engineering has also been accomplished by expressing (i) either *GES* alone from *V. officinalis* or (ii) by combining *GES* with geranyl pyrophosphate synthase (*GPPS*) gene from *Arabidopsis thaliana* (*GES* + *GPPS*). The transgenic HR line expressing *GES* accumulated significant amounts of geraniol and geraniol glycosides (151 ± 24 ng/mg DW), while HR clones with *GES* + *GPPS* accumulated lower levels of geraniol/geraniol glycosides compared to that in the former [28; Table 1). With regard to the growth and biomass accumulation potentials of both the transgenic HR lines, no significant differences were observed corroborating the possibility of increasing the accumulation of a useful secondary metabolite through such heterologous gene expression approach. The main conclusion of this study was that the simultaneous manipulation of the precursors of the TIA pathway through metabolic engineering can lead to a superior result in terms of enhancement in the production of the geraniol and geraniol glycosides [28].

2.2.2 Tryptophan Decarboxylase (*TDC*) and Other Related Genes

The terpene indole alkaloids (TIAs) are formed by the condensation of the indole moiety – tryptamine and the monoterpenoid – secologanin by strictosidine synthase (*STR*) to form strictosidine, the precursor to a wide variety of TIAs. Tryptamine is synthesized by tryptophan through tryptophan decarboxylase (*TDC*) activity, and anthranilate synthase (*AS*) catalyzes the first committed step in the synthesis of tryptophan [29]. Literature survey revealed that out of the total 32 reported observations on hairy root-mediated heterologous expression of different pathway genes, so far six reports are available concerning the expression of *TDC* gene (Fig. 3).

In order to manipulate the reserpine and ajmalicine flux, an attempt has been carried out to heterologously express the single *TDC* gene from *Catharanthus roseus* in *Rauvolfia serpentina* HR cultures for enhancing the production of such important alkaloids [30]. The best transgenic HR clone (RT4) bearing the exogenous *TDC* gene, cultivated in Gamborg's B_5 medium, accumulated higher reserpine (0.1202 ± 0.002% DW) and ajmalicine (0.0064 ± 0.003% DW) content as compared to the control non-transgenic HR clone after 10 weeks of cultivation [30; Table 1].

The same *TDC* gene from *C. roseus* has also been expressed in the hairy root culture of *Peganum harmala* by Berlin and co-workers [31]. The tryptamine and β-carboline alkaloid levels (other tryptamine-derived alkaloids) in the transgenic lines did not change, while the content of indole alkaloid serotonin was enhanced up to tenfolds as compared to that in the non-transgenic root. Supplementation of tryptophan to the cultures further enhanced the serotonin content in the transgenic HR clones [31].

In another study, the *TDC* from *C. roseus* and *AS* from *A. thaliana* were concurrently co-expressed in *C. roseus* HR cultures, resulting in the enhancement in the content of tryptophan and serpentine [32]. Similarly, Hong et al. [33] also successfully expressed both these genes of the same abovementioned sources in

C. roseus HR culture for the production of tryptamine and tryptophan. Geerlings and co-workers [34] isolated the two key enzymes *TDC* and strictosidine synthase (*STR*) from *C. roseus*, cloned under the control of constitutive CaMV35S promoter and successfully co-expressed both genes in the HR cultures of a woody plant – *Cinchona officinalis "Ladgeriana"* (Table 1). This resulted in improving the yields of tryptamine and strictosidine in the transgenic HR lines (1200 and 1950 μg/g DW, respectively). Concurrently, the levels of quinine and quinidine were also enhanced (500 and 1000 μg/g DW, respectively) in the same transgenic HR clone [34]. It is however disappointing to note that after one year of culture establishment, the transgenic *C. officinalis* HR clones completely lost their capacities of accumulating these alkaloids even though the presence of the *TDC* and *STR* genes was observed with the total loss of activity of the *TDC* gene [34]. On the contrary, another study has demonstrated the long-term stability of a transgenic *C. roseus* HR clone containing the inducible expression of a feedback-insensitive *Arabidopsis* anthranilate synthase (*AS*) gene after several years of culture establishment [35]. Stabilized production of an array of metabolites in these transgenic hairy root cultures was obtained after 5 years, which indicated that an initial transient stage might be mandatory to reap the maximum benefit out of such cultures before the metabolite profile reaches a stabilized state [35].

The regulatory mechanism behind the overexpression of several key genes in the upstream of the TIA pathway has also been investigated through heterologous approach in order to increase the flux towards the secondary metabolite production within hairy root cultures of *C. roseus* [29]. The inducible overexpression of *DXS* alone or together with "Anthranilate-synthase A" (*ASA*) subunit or *DXS* with geraniol-10-hydroxylase (*G10H*) under the control of *GAL4-UAS* promoter in the *C. roseus* HR cultures demonstrated variable outcomes. The transgenic HR clone with *DXS* gene alone revealed increment in the content of ajmalicine (67%), serpentine (26%), and lochnericine (49%), while the tabersonine and horhammericine contents were decreased by 66% and 54%, respectively [29]. On the other hand, co-expression of *DXS* with *G10H* resulted in the enhancement of the overall productivity of ajmalicine (16%), lochnericine (31%), and tabersonine (13%) [29; Table 1], while co-expression of *DXS* and *AS* demonstrated improved contents of horhammericine (30%), lochnericine (27%), and tabersonine (34%) (Table 1). These results clearly exemplified that the modulation of the complete range of metabolites could be achieved through the functional expression of single gene or through multiple genes within the pathway depending upon the targeted flux towards the final products [29].

Kim et al. [36] have demonstrated that the cloning and expression of farnesyl diphosphate synthase (*FPS*) from *P. ginseng* in *Centella asiatica* have played a significant role towards enhancing the production of total sterols in its HR cultures (Table 1). The content of squalene and total sterol in the best transgenic HR lines of *C. asiatica* was increased to 1.1–1.5-fold and three times, respectively, as compared to that in the control HR line [36].

Indole glucosinolates (IGs) are found widely in the Brassicales, which play an important role as a defense compounds in plants. Zang and co-workers [37] attempted to produce IG by heterologously expressing three cytochrome P450 (CYP79B2, CYP79B3, and CYP83B1) genes from *A. thaliana* under the control of CaMV35S promoter in the hairy root clone of *Brassica rapa.* The incorporation of CYP83B1 along with CYP79B2 or CYP79B3 enhanced the accumulation of glucobrassicin or 4-methoxy glucobrassicin than the control HR line with *rol* ABC. However, overexpression of either of these genes alone did not influence the accumulation level [37; Table 1].

2.3 Mevalonate (MVA) Pathway

The biosynthesis of isoprenoid in the plant systems initiates from the common precursor prenyl diphosphate (prenyl-PP) and is synthesized through two different pathways, i.e., 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway and the mevalonate (MVA) pathway, occurring in plastids and cytoplasm, respectively [38]. Diterpenoids are the important phytomolecules which not only are required for the survival of the plant but also bear desirable pharmacological significance. In *Salvia sclarea*, the abietanic diterpenes are normally synthesized in the roots, but at a very lower level [39]. Recently, the two upstream genes of the MVA pathway – deoxy xylulose synthase (*DXS*) and deoxy xylulose reductase (*DXR*) – have been isolated from a Brassicaceae family member (*A. thaliana*) and have been successfully co-expressed in the HR culture of a Lamiaceae plant – *Salvia sclarea* [39; Table 1]. This has led to the increment in the content of all the targeted bioactive abietanic diterpenes (i.e., aethiopinone, 1-oxoaethiopinone, savipisone, ferruginol, carnosic acid, and 1-oxo-ferruginol), which has clearly exhibited that the heterologous expression of MVA pathway genes can play a decisive role towards enhancing the production of pharmaceutically important diterpenes [39].

The squalene synthase (SS) from *A. thaliana* has been cloned and successfully expressed in the HR of *Withania coagulans* resulted in the enhancement of the total withanolides (ranged from 0.68 to 4.63 μg/g DW) as compared to the control HR (0.11 μg/g DW) after 4 weeks [40].

In another study, the gene for the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*), which catalyzes the rate-limiting step in the MVA pathway, has been isolated from *Artemisia annua* and cloned under the control of CaMV 35S promoter in *Solanum aviculare* HR lines [41]. The expression of this construct in the resultant *S. aviculare* HR lines resulted in the increment of the solasodine content by 4.2 times higher as compared to the control HR line [41]. On the other hand, heterologous expression of the same *HMGR* gene from *Panax ginseng* in *Platycodon grandiflorum* HR clones has demonstrated only 1.1–1.6-fold higher phytosterol levels in transgenic HR lines [42].

2.4 Phenylpropanoid Pathway

The phenylpropanoid pathway serves as a starting point for the production of many important compounds, such as the flavonoids, coumarins, and lignans, and is required for the biosynthesis of lignin. However, only a single published report is available till date under the current topic (Fig. 2). Tuan and co-workers (2014) have made a sole attempt for the production of chlorogenic acid (CGA) in the *Platycodon grandiflorum* HR culture by expressing *A. thaliana* transcription factor (*AtPAP1*) cloned under the control of CaMV 35S promoter (Table 1). The best HR line produced ten times higher CGA (421.31 μg/100 mg DW) as compared to that of the control HR line, which emphasized that HR culture of *P. grandiflorum* can be a promising alternative route for the production of CGA through heterologous expression of the *AtPAP1* gene [43].

2.5 Other Pathways

A sole report was documented with regard to the galacturonate pathway, where D-galacturonic acid reductase (GalUR) gene from *Fragaria ananassa* under the control of *CaMV 35S* has been heterologously expressed in the HR culture of *Solanum lycopersicum* cv. Money Maker [44; Table 1). As compared to the wild-type HR clones, the content of the total L-ascorbic acid (AsA) improved up to 2.5-fold in the transgenic HR clone upon treatment with D-galacturonic acid (D-GalUA) [44].

3 Other Contributing Factors

The successful implementation of the entire procedure is reigned by several underlying factors, which play a crucial role in implementing the positive gene transfer events and also substantially influence the overall expression mechanism of heterologously transferred plant genes. The characteristic and exclusivity of plants families (depending upon their degrees of susceptibilities), the types of *A. rhizogenes* strains, explant types, promoters' specifications, and media constituents are some of the prominent deciding factors that differed amongst the reported observations and have been outlined below.

4 Involvement of Plant Families

It is evident from literature survey that certain plant families played crucial role as the front-runner with respect to improving the production of targeted metabolites through hairy root (HR)-based heterologous gene expression of pathway genes. In this context, the major contribution could be noted on account of the Solanaceae family (60%) followed by Apocynaceae and Brassicaceae (Fig. 4).

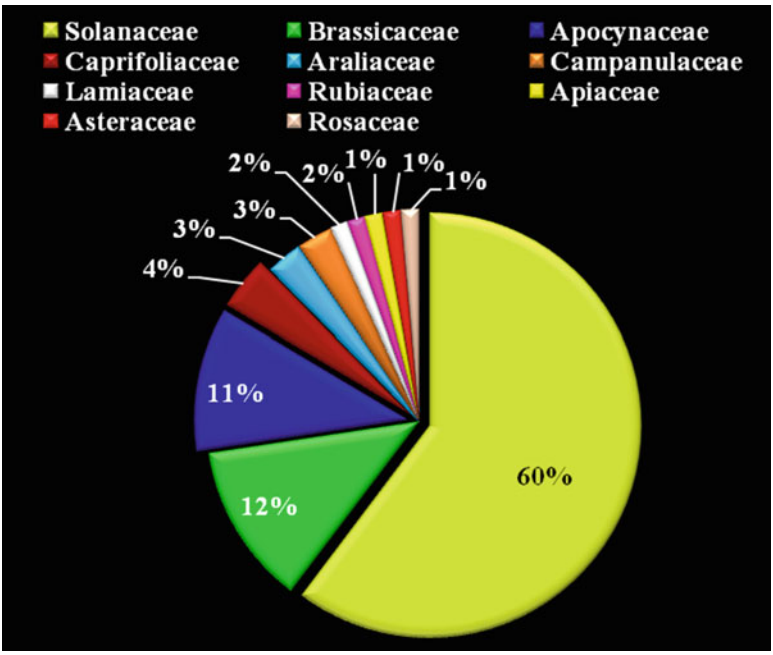

Fig. 4 Preferential involvement of plant families in HR-mediated heterologous gene expression studies

Different species of *Nicotiana* (i.e., *N. tabacum*, *N. benthamiana*, *N. sylvestris*), *Atropa* (i.e., *A. belladonna*, *A. baetica*), *Duboisia* (i.e., *D. leichhardtii*, *D. hybrid*, *D. myoporoides* x *D. leichhardtii*, *Hyoscyamus* (i.e., *H. niger*, *H. muticus*), and *Solanum* (i.e., *S. aviculare*, *S. lycopersicum*) along with *Scopolia parviflora*, *Withania coagulans*, and *Datura metel* constituted the major plant species of Solanaceae family which dominated the entire HR-based heterologous gene expression progression (60%) relating to tropane alkaloid biosynthesis (Fig. 4). The second most explored family is Brassicaceae, in which the major attention remained mostly focused on *Arabidopsis thaliana* and *Brassica rapa* HR cultures, which contributed about 12% of the total reported literatures for heterologous expression (Fig. 4).

On the other hand, Apocynaceae family occupied the third position in this list (11%) in which *Rauvolfia serpentina* and *Catharanthus roseus* HR cultures have been explored as the expression system for the pathway modulation (Fig. 4). The HR cultures of several other plant families too contributed towards the HR-mediated heterologous gene expression (17%) in which Caprifoliaceae, *Valeriana officinalis* (4%); Araliaceae, *Panax ginseng* (3%); Campanulaceae, *Platycodon grandiflorum* (3%); Lamiaceae, *Salvia sclarea* (2%); Rubiaceae, *Cinchona officinalis* (2%); Apiaceae, *Centella asiatica* (1%); Asteraceae, *Artemisia annua* (1%); and Rosaceae, *Fragaria ananassa* (1%) were investigated towards improving the yield potentials relating to targeted metabolites (Fig. 4).

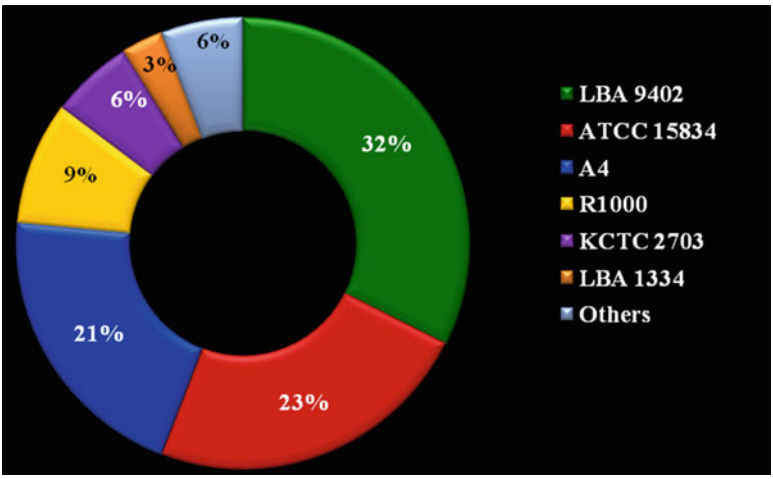

Fig. 5 Influence of *Agrobacterium rhizogenes* strains on the frequency of affirmative results relating to heterologous gene expression

5 Preference of *A. rhizogenes* Strain

An in-depth analysis concerning the use of different strains of *A. rhizogenes* has clearly indicated the preferential utilization of six different strains with varying degrees of success through their gainful exploitation in heterologous expression of pathway genes in HR cultures (Fig. 5). In this context, the LBA 9402 strain attained maximum deliberations (32%) followed by the ATCC 15834 (23%), A4 (21%), R1000 (9%), KCTC 2703 (6%), and LBA 1334 (3%) strains (Fig. 5). Exclusive susceptibilities of the targeted medicinal plant systems towards the specific bacterial strain might be the underlying reason for such diversity.

6 Popularity of Explant Types

A review of the published literature has clearly indicated that preference of explant types has played an implicit role in all the successful reports of the HR-mediated heterologous pathway gene expression studies. Noticeably, leaf has most prevalently been used as the universally preferred explants with 71% reported frequency of uses (Fig. 6). The use of seedlings attained the second preference with 11% reported cases, while the utilization of either stem or both stem and leaf attained equal preference (total 7% reported usages). It is however worth mentioning that Rahman and co-workers have observed better performance of leaf explants over that of stem explants in their heterologous gene expression study where both have been tested. The use of cotyledon has gained minimum application in similar studies with 4% of use amongst the reported cases (Fig. 6).

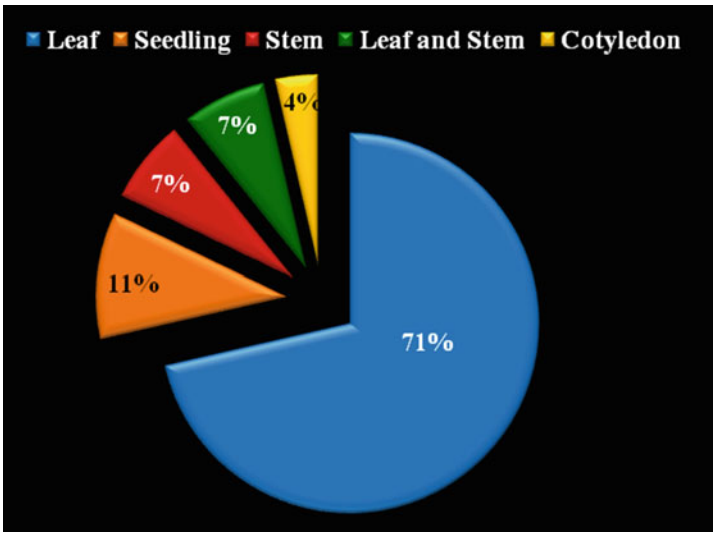

Fig. 6 Preferential utilization of different explants for HR-mediated heterologous gene expression

7 Choices of Promoters' Specifications

Literature survey indicates that although promoters play a crucial role in such heterologous gene expression studies, a single constitutive promoter (i.e., cauliflower mosaic virus 35S promoter – CaMV 35S) has fulfilled the underlying requirement of most the presently reviewed studies and showed 94% of prevalence amongst the reported HR-mediated heterologous plant gene expression studies (Fig. 7). Two individual reports are available that deviate from such usually accepted practice and have used ParAt and GAL4-UAS as the preferred promoters [21, 29].

8 Media Choices

Careful analysis of the available published data relating to the preferential selection of media composition for successful execution of the entire heterologous gene expression procedure evidently revolved around the two most reputed media formulations with distinct divergence in their utilization (Fig. 8). The Gamborg's B5 medium has been used in majority (69%) of the studies, while the Murashige and Skoog's medium has been utilized in 31% of the reported investigations which indicates that the vitamin and other additives of the former medium might be having some influence on the overall success of the entire procedure.

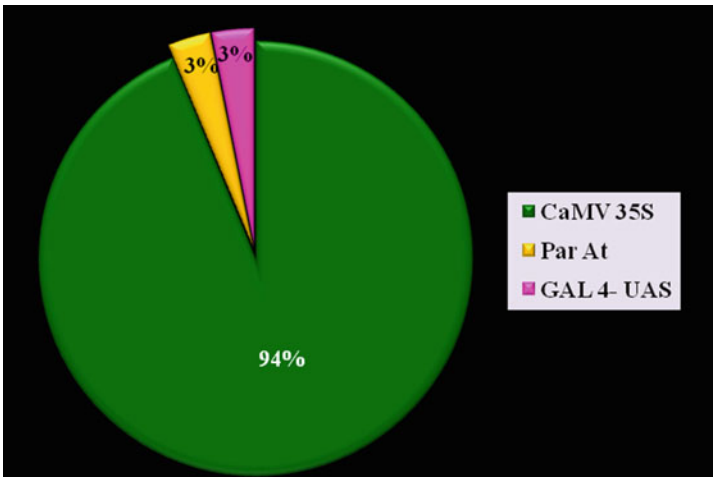

Fig. 7 Options of different promoters for HR-mediated heterologous gene expression

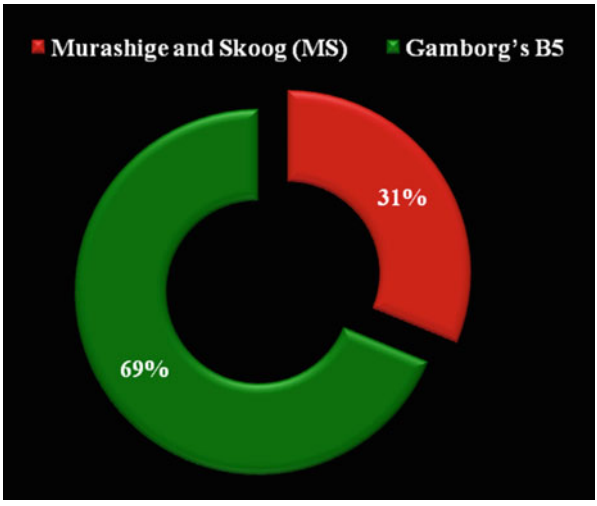

Fig. 8 Documented media preferences for HR-mediated heterologous gene expression

9 Conclusions

The advancement of next-generation sequencing and innovative biotechnological tools along with the understanding of the plant metabolic pathways at both the biochemical and cellular levels has opened up a new avenue for metabolic engineering of any biosynthetic pathways, which is quite evident through the increasing number of reported observations in the most recent years. The higher commercial demand with lower supply of plant-based secondary metabolites has necessitated the

search for appropriate and inexpensive expression systems other than the host plant, where hairy root cultures have emerged as a promising alternate approach for the production of metabolites. This review summarizes the HR-mediated heterologous expression of several rate-limiting key genes of TIA, TA, and MVA biosynthetic pathway. The maximum utilization of HR cultures of Solanaceae family has been explored for enhancing the productivity of tropane alkaloids by expressing *H6H* and *PMT* genes. The detailed literature survey indicated that several factors including the targeted genes and their sources as well as recipient plant systems, promoter types, explant types, bacterial strain specificity, and media choices may ultimately decide the fate of such efforts which undoubtedly can bridge the gap between the demand and supply of several plant-based vital bioactive molecules. Further refinement of this HR technology for heterologous expression of biosynthetic pathway genes in medicinal plants can become a powerful approach in the coming years through focused efforts towards translating this technology into industrial applications for the large-scale production of therapeutically important phytomolecules, which lacks any other alternative production source other than the resource mother plant.

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References

1. David B, Wolfender JL, Dias DA (2015) The pharmaceutical industry and natural products: historical status and new trends. Phytochem Rev 14:299–315
2. Newman DJ, Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 75:311–335
3. Atanasov AG, Waltenberger B, Wenzig EMP, Linder T, Wawrosch C, Uhrin P, Temml V, Wang L, Schwaiger S, Heiss EH, Rollinger JM, Schuster D, Breuss JM, Bochkov V, Mihovilovic MD, Kopp B, Bauer R, Dirscha VM, Stuppner H (2015) Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol Adv 33:1582–1614
4. Muller JL, Jahn L, Lippert A, Puschel J, Walter A (2014) Improvement of hairy root cultures and plants by changing biosynthetic pathways leading to pharmaceutical metabolites: strategies and applications. Biotechnol Adv 32:1168–1179
5. Banerjee S, Singh S, Rahman LU (2012) Biotransformation studies using hairy root cultures – a review. Biotechnol Adv 30:461–468
6. Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1:13–25
7. Zhou ML, Zhu XM, Shao JR, Tang YX, Wu YM (2011) Production and metabolic engineering of bioactive substances in plant hairy root culture. Appl Microbiol Biotechnol 90:1229–1239
8. Miralpeix B, Rischer H, Hakkinen ST, Ritala A, Tuulikki SL, Oksman-Caldentey KM, Capell T, Christou P (2013) Metabolic engineering of plant secondary products: which way forward? Curr Pharmaceu Design 19:5622–5639

9. Ikram NKBK, Zhan X, Pan XW, King BC, Simonsen HT (2015) Stable heterologous expression of biologically active terpenoids in green plant cells. Front Plant Sci 6:129–139
10. Zhang L, Ding R, Chai Y, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu T, Pi Y, Wang Z, Zhang H, Kai G, Liao Z, Sun X, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci U S A 101:6786–6791
11. Matzke MA, Matzke AJM (1995) How and why do plants inactivate homologous (Trans) genes? Plant Physiol 107:679–685
12. Kang YM, Park DJ, Min JY, Song HJ, Jeong MJ, Kim YD, Kang SM, Karigar CS, Choi MS (2011) Enhanced production of tropane alkaloids in transgenic *Scopolia parviflora* hairy root cultures over-expressing putrescine *N*-methyl transferase (PMT) and hyoscyamine-6-β-hydroxylase (H6H). In Vitro Cell Dev Biol Plant 47:516–524
13. Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey KM (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551
14. Zhang L, Yang B, Lu B, Kai G, Wang Z, Xia Y, Ding R, Zhang H, Sun X, Chen W, Tang K (2007) Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing *Putrescine N-methyltransferase* is methyl jasmonate-dependent. Planta 225:887–896
15. Moyano E, Fornale S, Palazon J, Cusido RM, Bagni N, Pinol MT (2002) Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the pmt gene. Phytochemistry 59:697–702
16. Moyano E, Jouhikainen K, Tammela P, Palazon J, Cusido RM, Pinol MT, Teeri TH, Oksman-Caldentey KM (2003) Effect of pmt gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. J Exp Bot 54:203–211
17. Sato F, Hashimoto T, Hachiya A, Tamura K, Choi KB, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci U S A 98:367–372
18. Lee OS, Kang YM, Jung HY, Min JY, Kang SM, Karigar SC, Prasad DT, Bahk JD, Choi MS (2005) Enhanced production of tropane alkaloids in *Scopolia parviflora* by introducing the PMT (putrescine *N*-methyltransferase) gene. In Vitro Cell Dev Biol Plant 41:167–172
19. Hashimoto T, Yun DJ, Yamada Y (1993) Products of tropane alkaloids in genetically engineered root cultures. Phytochemistry 32:713–718
20. Palazon J, Moyano E, Cusido RM, Bonfilla M, Bagni N, Oksman-Caldentey KM, Pinol MT (2003) Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the h6h gene. Plant Sci 165:1289–1295
21. Rahman LU, Kitamura Y, Yamaguchi J, Mukaia M, Akiyamaa K, Yamamotoa H, Muranaka T, Ikenaga T (2006) Exogenous plant *H6H* but not bacterial *HCHL* gene is expressed in *Duboisia leichhardtii* hairy roots and affects tropane alkaloid production. Enz Microbial Technol 39:1183–1189
22. Hakkinen ST, Moyano E, Cusido RM, Palazon J, Pinol MT, Oksman-Caldentey KM (2005) Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous hyoscyamine-6b-hydroxylase. J Exp Bot 56:2611–2618
23. Moyano E, Palazon J, Bonfill M, Osuna L, Cusido RM, Oksman-Caldentey KM, Pinol MT (2007) Biotransformation of hyoscyamine into scopolamine in transgenic *tobacco* cell cultures. J Plant Physiol 164:521–524
24. Zarate R, Jaber-Vazdekis NE, Medina B, Ravelo AG (2006) Tailoring tropane alkaloid accumulation in transgenic hairy roots of *Atropa baetica* by over-expressing the gene encoding hyoscyamine 6β-hydroxylase. Biotechnol Lett 28:1271–1277
25. Yang C, Chen M, Zeng L, Zhang L, Liu X, Lan X, Tang K, Liao Z (2011) Improvement of tropane alkaloids production in hairy root cultures of *Atropa belladonna* by overexpressing pmt and h6h genes. Plant Omics J 4:29–33
26. Ritala A, Dong L, Imseng N, Seppanen-Laakso T, Vasilev N, van der Krol S, Rischer H, Maaheimo H, Virkki A, Brandli J, Schillberg S, Eibl R, Bouwmeester H, Oksman-Caldentey KM (2014) Evaluation of tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) hairy roots for

the production of geraniol, the first committed step in terpenoid indole alkaloid pathway. J Biotechnol 176:20–28
27. Vasilev N, Schmitz C, Dong L, Ritala A, Imseng N, Hakkinen ST, van der Krol S, Eibl R, Oksman-Caldentey KM, Bouwmeester H, Fischer R, Schillberg S (2014) Comparison of plant-based expression platforms for the heterologous production of geraniol. Plant Cell Tiss Org Cult 117:373–380
28. Masakapalli SK, Ritala A, Dong L, van der Krol AR, Oksman-Caldentey KM, Ratcliffe RG, Sweetlove LJ (2014) Metabolic flux phenotype of tobacco hairy roots engineered for increased geraniol production. Phytochemistry 99:73–85
29. Peebles CAM, Sander GW, Hughes EH, Peacock R, Shanks JV, San KY (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. Met Eng 13:234–240
30. Mehrotra S, Srivastava V, Rahman LU, Kukreja AK (2013) Overexpression of a *Catharanthus* tryptophan decarboxylase (tdc) gene leads to enhanced terpenoid indole alkaloid (TIA) production in transgenic hairy root lines of *Rauwolfia serpentina*. Plant Cell Tiss Org Cult 115:377–384
31. Berlin J, Rugenhagen C, Dietze P, Fecker LF, Goddijn OJM, Hoge JHC (1993) Increased production of serotonin by suspension and root cultures of *Peganum harmala* transformed with a tryptophan decarboxylase cDNA clone from *Catharanthus roseus*. Transg Res 2:336–344
32. Hughes EH, Hong SB, Gibson SI, Shanks JV, San KY (2004) Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. Met Eng 6:268–276
33. Hong SB, Peebles CA, Shanks JV, San KY, Gibson SI (2006) Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. J Biotechnol 122:28–38
34. Geerlings A, Hallard D, Caballero AM, Cardoso IL, van der Heijden R, Verpoorte R (1999) Alkaloid production by a *Cinchona officinalis* 'Ledergriana' hairy root culture containing constitutive expression constructs of tryptophan decarboxylase and strictosidine synthase cDNAs from *Catharanthus roseus*. Plant Cell Rep 19:191–196
35. Peebles CAM, Sander GW, Li M, Shanks JV, San KY (2009) Five year maintenance of the inducible expression of anthranilate synthase in *Catharanthus roseus* hairy roots. Biotechnol Bioeng 102:1521–1525
36. Kim OT, Kim SH, Ohyama K, Muranaka T, Choi YE, Lee HY, Kim MY, Hwang B (2010) Upregulation of phytosterol and triterpene biosynthesis in *Centella asiatica* hairy roots overexpressed *ginseng* farnesyl diphosphate synthase. Plant Cell Rep 29:403–411
37. Zang YX, Kim DH, Park BS, Hong SB (2009) Metabolic engineering of indole glucosinolates in Chinese cabbage hairy roots expressing *Arabidopsis* CYP79B2, CYP79B3 and CYP83B1. Biotechnol Bioprocess Eng 14:467–473
38. Vranová E, Coman D, Gruissem W (2013) Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu Rev Plant Biol 64:665–700
39. Vaccaro M, Malafronte N, Alfieri M, Tommasi ND, Leone A (2014) Enhanced biosynthesis of bioactive abietane diterpenes by overexpressing *AtDXS* or *AtDXR* genes in *Salvia sclarea* hairy roots. Plant Cell Tiss Org Cult 119:65–77
40. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J (2011) Overexpression of the *Arabidopsis thaliana* squalene synthase gene in *Withania coagulans* hairy root cultures. Biol Plant 5:357–360
41. Argolo ACC, Charlwood BV, Pletsch M (2000) The regulation of solasodine production by *Agrobacterium rhizogenes*-transformed roots of *Solanum aviculare*. Planta Med 66:448–451
42. Kim YK, Kim JK, Kim YB, Lee S, Kim SU, Park SU (2013) Enhanced accumulation of phytosterol and triterpene in hairy root cultures of *Platycodon grandiflorum* by overexpression of *Panax ginseng* 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase. J Agric Food Chem 61:1928–1934

43. Tuan PA, Kwon DY, Lee S, Arasu MV, Al-Dhabi NA, Park Nam I, Park SU (2014) Enhancement of chlorogenic acid production in hairy roots of *Platycodon grandiflorum* by overexpression of an *Arabidopsis thaliana* transcription factor *AtPAP1*. Int J Mol Sci 15:14743–14752
44. Oller ALW, Agostini E, Milrad SR, Medina MI (2009) In situ and de novo biosynthesis of vitamin C in wild type and transgenic tomato hairy roots: a precursor feeding study. Plant Sci 177:28–34