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Perspectives of the Metabolic Engineering of Terpenoid Indole Alkaloids in *Catharanthus roseus* Hairy Roots

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Abstract This review looks back on how the terpenoid indole alkaloid pathway and the regulatory factors in *Catharanthus roseus* were identified and characterized, and how metabolic engineering, including genetic engineering and metabolic profiling, was conducted based on the gained knowledge. In addition, further examination of the terpenoid indole alkaloid pathway is proposed.

Keywords *Catharanthus roseus* · Genetic engineering · Metabolic engineering · Metabolic profiling · Terpenoid indole alkaloids

Abbreviation

16OMT	16-Hydroxytabersonine-*O*-methyltransferase
AACT	Acetyl-CoA *C*-acetyltransferase
ADH	Acyclic monoterpene primary alcohol dehydrogenase
AS	Anthranilate synthase
CaMV	Cauliflower mosaic virus
C. roseus	*Catharanthus roseus*
CMK	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol kinase
CMS	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol synthase
CPR	Cytochrome P450 reductase
CrBPF	*C. roseus* box P-binding factor
D4H	Desacetoxyvindoline 4-hydroxylase
DAT	Deacetylvindoline 4-*O*-acetyltransferase
DL7H	7-Deoxyloganin 7-hydroxylase
DXR	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
E4P	Erythrose 4-phosphate

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E. coli	*Escherichia coli*
ESI–MS-MS	Electrospray ionization tandem mass spectrometer
ET	Ethylene
G10H	Geraniol 10-hydroxylase
GA	Gibberellic acid
GBF	G-box binding factors
GES	Geraniol synthase
GPPS	Geranyl diphosphate synthase
HDR	1-Hydroxy-2-methyl-2-butenyl 4-diphosphate reductase
HDS	1-Hydroxy-2-methyl-2-butenyl 4-diphosphate synthase
HMGR	3-Hydroxy-3-methylglutaryl CoA reductase
HMGS	3-Hydroxy-3-methylglutaryl CoA synthase
HPLC	High-performance liquid chromatography
IDI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl diphosphate
JA	Jasmonic acid
LAMT	Loganic acid methyl transferase
MALDI-TOF	Matrix assisted laser desorption with time-of-flight detector
MAT	Minovincinine 19-hydroxy-*O*-acetyltransferase
MC	Monoterpene cyclase
MECS	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase
MeJA	Methyl jasmonic acid
MEP	2-*C*-Methyl-D-erythritol 4-phosphate
MFA	Metabolic flux analysis
MIAs	Monoterpenoid indole alkaloids
MVA	Mevalonic acid
MVD	Mevalonate 5-diphosphate decarboxylase
MVK	Mevalonate kinase
NMR	Nuclear magnetic resonance
NMT	*N*-methyltransferase
ORCAs	Octadecanoid-responsive *Catharanthus* AP2/ERF domain
PDA	Photo diode array
PEP	Phosphoenolpyruvate
PMK	5-Phosphomevalonate kinase
PRX1	Peroxidase 1
SA	Salicylic acid
SGD	Strictosidine β-D-glucosidase
SLS	Secologanin synthase
STR	Strictosidine synthase
T16H	Tabersonine 16-hydroxylase
T6,7E	Tabersonine 6,7-epoxidase
TDC	Tryptophan decarboxylase

TIAs	Terpenoid indole alkaloids
TSP-MS	Thermospray mass spectrometry
ZCT	Zinc finger *Catharanthus* transcription factor

Contents

1 Introduction

The terpenoid indole alkaloids (TIAs) are a class of over 3,000 natural products containing residues of trytophan or tryptamine (indole part) and terpenoid building blocks derived from secologanin. Some TIAs are proposed to protect plants from pests and pathogens [1]. In addition to the original benefit brought to the plants, these complex natural products and their derivatives are also used as anticancer, antimalarial, and antiarrhythmic agents. The better known and studied plants producing TIAs are *Catharanthus roseus*, *Tabernaemontana divaricata*, and *Rauvolfia serpentina* [2, 3].

The *Catharanthus* alkaloids comprise a large group of about 130 TIAs. Some major products found in *C. roseus* are anhydrovinblastine, vindoline, catharanthine, ajmalicine, and serpentine [4]. Some bisindole alkaloids can disrupt microtubule formation, causing dissolution of mitotic spindles and metaphase arrest in dividing cells, which make them ideal antineoplastic agents. The pharmaceutical *C. roseus* alkaloids are listed in Table 1.

Vinblastine, vincristine, and the many derivatives of vinblastine have been developed as leading pharmaceuticals [6]. The isolation of vinblastine and vincristine from *C. roseus* is costly because of the low levels *in planta* and the diverse

Table 1 Pharmaceutical *C. roseus* alkaloids

Name	Trade name	Treatment
Ajmalicine	Hydroserpan®, Lamuran®	Hypertension, circulatory disorders
Vinblastine	Vincaleukoblastine, Velbe®	Hodgkin's disease, non-Hodgkin lymphomas, testiscarcinomas, breast cancer, and chorio-carcinomas
Vincristine	Leurocristine, Oncovin®	Acute leukemia, Hodgkin's disease, non-Hodgkin lymphomas, rhabdomyosarcomas, Wilm's tumors in children, and breast cancer
Anhydrovinblastine		Antineoplastic agent for cervical and lung cancer [5]

alkaloids present. Approximately 500 kg of dried *C. roseus* leaves are required to isolate only 1 g of vinblastine [7]. This motivated a search for alternate approaches, such as chemical synthesis or production in micro-organisms. However, because of the complex compound structures and complicated biosynthesis pathway, these alternatives were not successful. Currently vinblastine, vincristine, and other valuable alkaloids are produced commercially by extraction of the whole plant. To produce these useful anticancer drugs more efficiently, scientists applied plant tissue culture technology [8]. However, the low yield of vinblastine and vincristine by de novo synthesis using callus or suspension cultured cells of *C. roseus* is not promising. A novel process to produce vinblastine developed by Allelix Inc. in Canada is the production of catharanthine by plant cell fermentation and a simple chemical or an enzymatic coupling with commercially available vindoline [9, 10]. This technology was transferred from the Canadian company to Mitsui Petrochemical Industries, Ltd. in Japan for further development, but has not been commercialized to date. In 1990, the yield of catharanthine through fermentation had increased to 230 mg/L per week with high-cell density [11]. Using the partially purified enzymes from the cultured cell of *C. roseus*, anhydrovinblastine was formed with a conversion yield of about 50 % [9]. In the absence of enzymes, the product of the chemical coupling catalyzed by ferric ions was not only anhydrovinblastine, but also vinblastine. The yields of both alkaloids were 52.8 and 12.3 %, respectively. Further development of the chemical catalysis increased the yield of vinblastine from anhydrovinblastine up to 50 % [12].

Several tissue culture strategies, such as cell suspension, embryo, immobilized cell, callus, and shoot cultures are available. One of the most promising tissue culture types is the hairy root culture, resulting from the transfer of T-DNA from the pathogenic soil bacterium, *Agrobacterium rhizogenes*, into the plant genome [13, 14]. The advantages of hairy root compared with whole plant cultures are rapid growth, ease of precursor feeding, inherent genetic stability, and amenability to genetic transformation. Hairy roots grow rapidly in hormone-free liquid media, with doubling times comparable with suspension cells and greater than other tissue cultures [13, 14]. In addition, hairy roots have been cultivated in large-scale

bioreactors [15] and compared with suspension cells, hairy roots have inherent genetic stability [14]. However, the major drawbacks of the *C. roseus* hairy root system are the low yields of valuable compounds and the absence of vindoline, a precursor of vinblastine and vincristine.

2 Terpenoid Indole Alkaloid Metabolism

2.1 Overview of TIAs Biosynthesis Pathway

Terpenoid indole alkaloid biosynthesis in *C. roseus* is a complex process with more than 50 biosynthetic events comprising the involved genes, enzymes, regulators, and intra-/intercellular transporters. The broad class of TIAs in *C. roseus* is derived from strictosidine, produced by the coupling of an indole, tryptamine, and a monoterpenoid, secologanin. The tryptamine derives from the shikimate pathway, whereas secologanin is formed from isopentenyl diphosphate (IPP) through the iridoid pathway with a number of steps, some of which are still unknown. IPP itself derives from either the cytosolic mevalonic acid (MVA) pathway or the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. Figure 1 shows an overview of the metabolic pathways of the precursors for TIA biosynthesis.

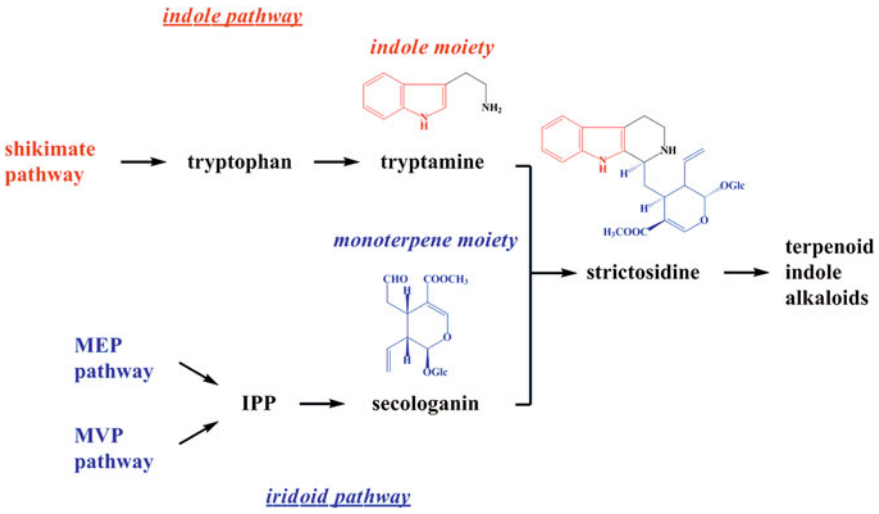

Fig. 1 An overview of the pathways leading to TIA biosynthesis

2.2 Two Metabolic Pathways for IPP Biosynthesis

The MVA pathway was originally considered the sole route leading to IPP; however, the discovery and study of the MEP pathway showed not only its involvement but greater contribution to IPP biosynthesis [16]. The MVA pathway only serves as a minor source of precursors for iridoid biosynthesis, but it contributes through protein prenylation to the regulation of MEP pathway gene expression [17].

The MEP pathway comprises seven steps, of which six have been characterized in *C. roseus*. Despite the impressive progress in the elucidation of the MEP pathway in bacteria and plants (Fig. 2), it is also important to analyze the contribution of the seven enzymes to controlling the flux of intermediates through the pathway. To date, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) is the only enzyme of the MEP pathway shown to have a regulatory role for IPP biosynthesis in all the systems analyzed, including *Arabidopsis*, tomato, and *E. coli* [18]. The role of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) cannot be generalized for all the systems studied. In *C. roseus* cell cultures, *DXS*, *DXR,* and 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*MECS*) transcript levels had a strong positive correlation with TIA biosynthesis [19, 20]. Other enzymes of the MEP pathway likely also contribute to the regulation of the supply of intermediates for IPP biosynthesis, but this remains to be established.

All the corresponding cDNA expressing enzymes in the MVA pathway in *C. roseus* have been isolated and characterized (Fig. 3), except for 3-hydroxy-3-methylglutaryl CoA synthase (HMGS), which was partially purified in 2004 [4]. 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) is one of the most highly regulated enzymes in animals and plants [21]. The activity of HMGR may be influenced, for example, by post-translational regulation by a protein kinase cascade that phosphorylates and thereby inactivates the enzyme. Allosteric modulation, proteolytic degradation, and the rate of turnover of the corresponding mRNA transcripts may all influence HMGR activity [22].

2.3 Iridoid Pathway

Secologanin is formed from IPP through a number of steps (Fig. 4), some of which remain unknown in *C. roseus*. The enzyme of interest in this pathway is Geraniol 10-hydroxylase (G10H). G10H is a cytochrome P450 monooxygenase thought to play a key regulatory role in TIA biosynthesis [23]. Cytochrome P450 reductase (CPR) transfers electrons from NADPH to G10H. CPR requires the co-factors FMN, FAD, and NADPH. G10H, together with CPR and crude *C. roseus* lipids, can hydroxylate geraniol at its C-10 position [24]. The first evidence that G10H could be a limiting step in the iridoid pathway was the reversible inhibition of G10H activity by catharanthine [25]. Later, G10H activity was found to be

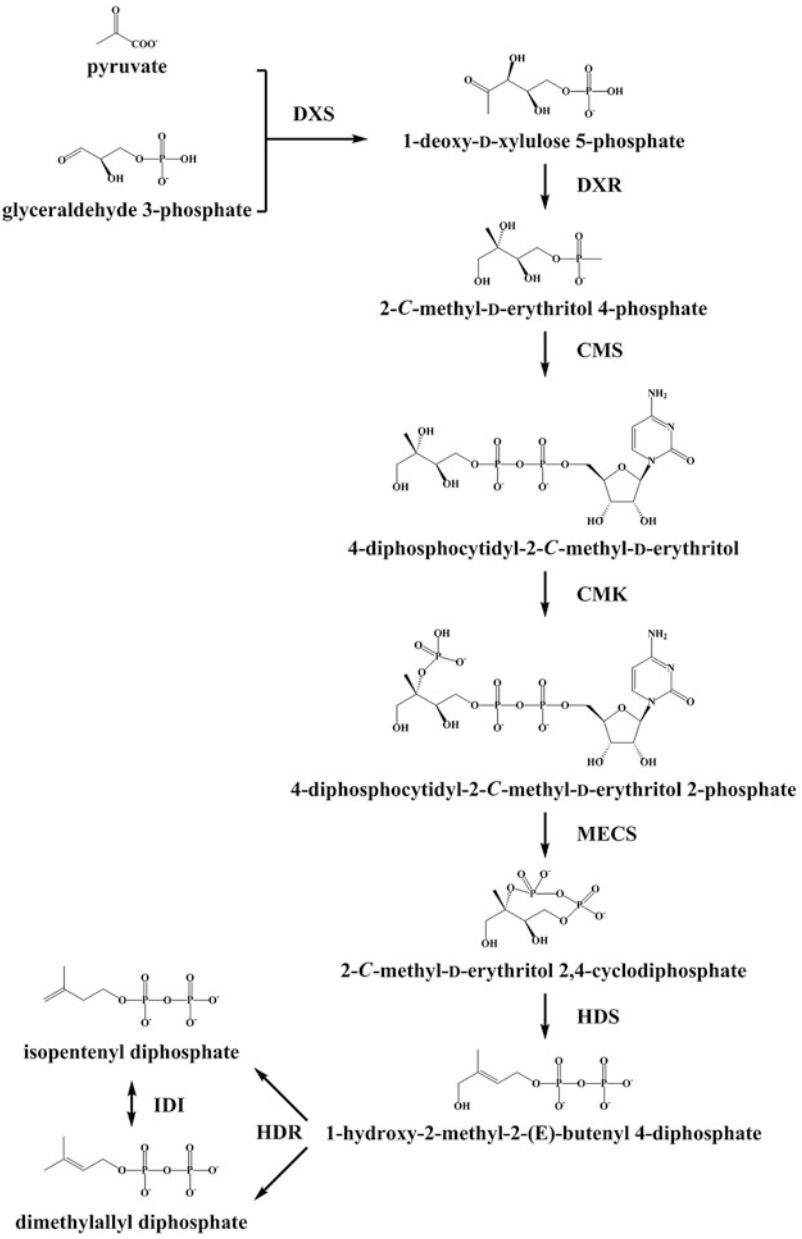

Fig. 2 The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. *DXS*: 1-deoxyD-xylulose 5-phosphate synthase; *DXR*: 1-deoxyD-xylulose 5-phosphate reductoisomerase; *CMS*: 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; *CMK*: 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase; *MECS*: 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *HDS*: 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase; *HDR*: 1-hydroxy-2-methyl-2-butenyl 4-diphosphate reductase; *IDI*: isopentenyl diphosphate isomerase

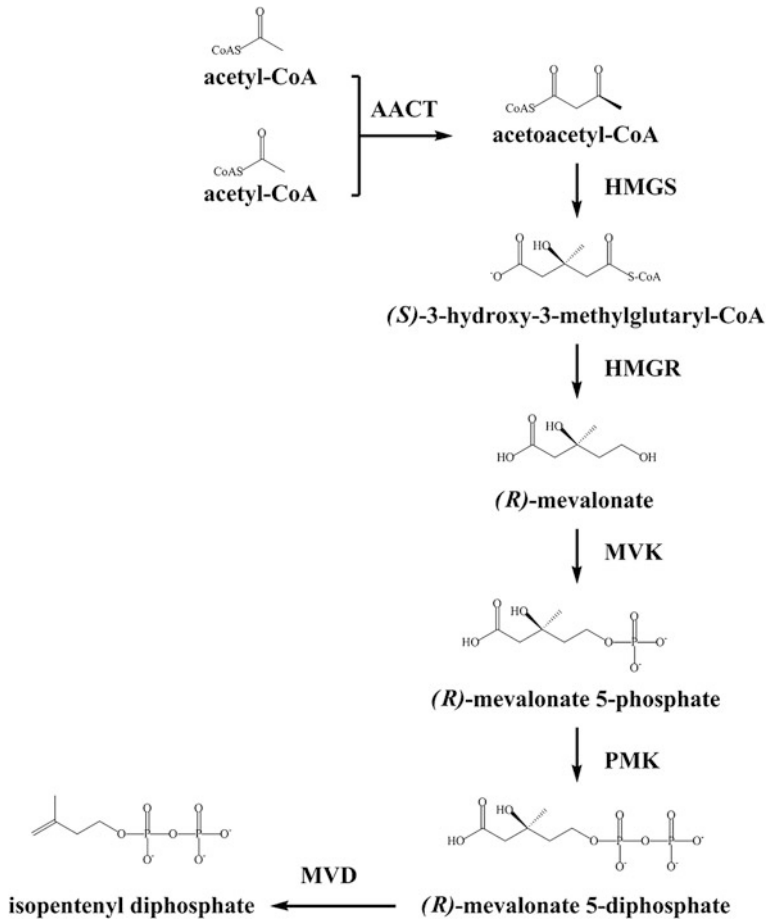

Fig. 3 The mevalonic acid (MVA) pathway. *AACT*: acetyl-CoA *C*-acetyltransferase; *HMGS*: 3-hydroxy-3-methylglutaryl CoA synthase; *HMGR*: 3-hydroxy-3-methylglutaryl CoA reductase; *MVK*: mevalonate kinase; *PMK*: 5-phosphomevalonate kinase; *MVD*: mevalonate 5-diphosphate decarboxylase

positively correlated to TIA accumulation [23]. Loganic acid methyl transferase (LAMT) and secologanin synthase (SLS), which are downstream of G10H, are also proposed as limiting for the synthesis of secologanin [26, 27].

2.4 Indole Pathway

Tryptophan in the indole pathway (Fig. 1) is derived from the shikimate pathway. The shikimate pathway begins with two intermediates of carbohydrate metabolism, phosphoenolpyruvate (PEP) from glycolysis and erythrose 4-phosphate

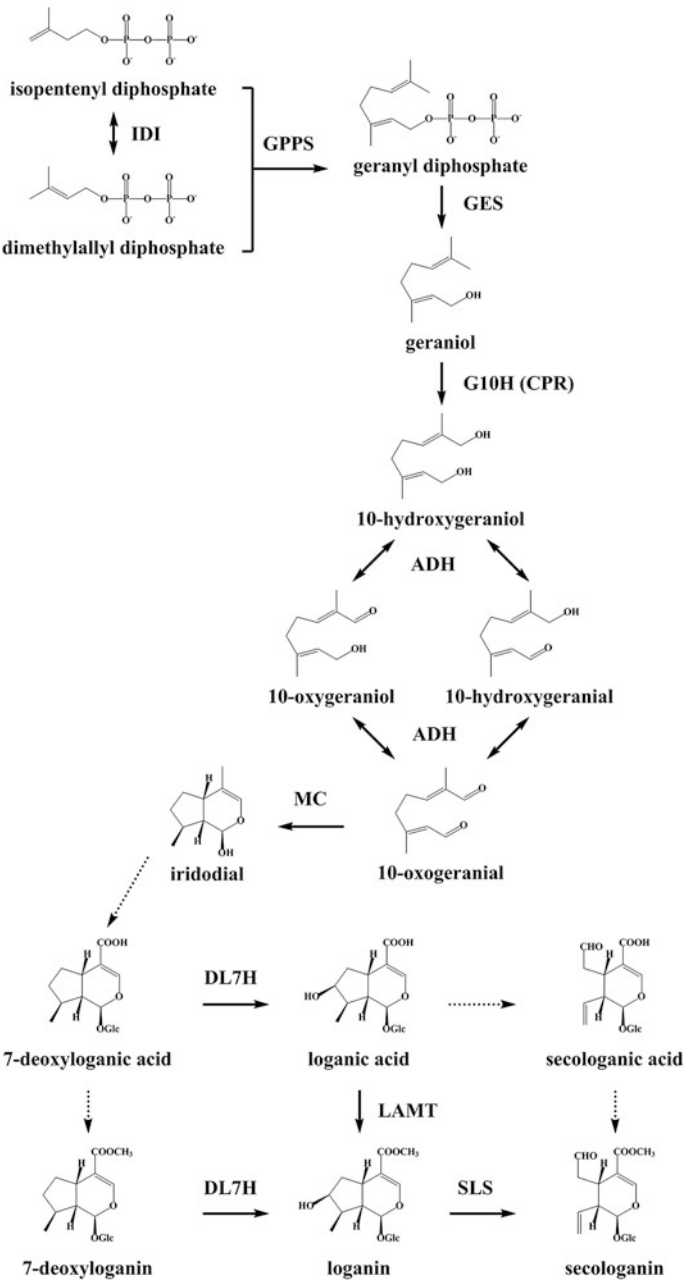

Fig. 4 The iridoid pathway. *IDI*: isopentenyl diphosphate isomerase; *GPPS*: geranyl diphosphate synthase; *GES*: geraniol synthase; *G10H*: geraniol 10-hydroxylase (Cytochrome P450-dependent monooxygenase); *CPR*: Cytochrome P450 reduactase; *ADH*: acyclic monoterpene primary alcohol dehydrogenase (10-hydroxygeraniol oxidoreductase); *MC*: monoterpene cyclase; *DL7H*: 7-deoxyloganin 7-hydroxylase; *LAMT*: loganic acid methyl transferase; *SLS*: secologanin synthase. *Dashed arrows* indicate uncharacterized steps

(E4P) from the pentose phosphate pathway. Tryptophan is converted into tryptamine by tryptophan decarboxylase (TDC). Two highly regulated enzymes in the production of tryptamine are anthranilate synthase (AS), which converts chorismate to anthranilate, and TDC, the last enzyme in this pathway. The AS enzyme is a tetramer composed of two α subunits (ASα) and two β subunits (ASβ), and is feedback-inhibited by tryptophan and tryptamine [28]. The α subunit catalyzes the aromatization of chorismate and binds tryptophan as a feedback inhibitor, and the β subunit donates an amino group from glutamine. TDC is located on the interface between primary and secondary metabolism, and is also feedback inhibited by tryptamine [29].

2.5 Terpenoid Indole Alkaloid Biosynthesis

An overview of the downstream TIA pathway is shown in Fig. 5. Strictosidine is the central intermediate in TIA biosynthesis and is formed by the condensation of secologanin and tryptamine by strictosidine synthase (STR). It is the first committed step in the biosynthesis of TIAs. The expression of the *STR* gene is down-regulated by auxin [30] and up-regulated by fungal elicitation and methyl jasmonic acid (MeJA) [31]. Further studies revealed that hormones and elicitors regulate several transcription factors, which repress or activate the *STR* gene and other genes in the TIA pathway. The transcriptional regulation of the TIA pathway is described in the next section.

Strictosidine β-D-glucosidase (SGD) catalyzes strictosidine and after several spontaneous steps forms 4,21-dehydrogeissoschizine, the precursor for all monoterpenoid indole alkaloids (MIAs; Fig. 6). In one branch, 4,21-dehydrogeissoschizine is converted to form the terminal metabolites serpentine and tetrahydroalstonine. Other branches lead to the important metabolites catharanthine and tabersonine. Eight tabersonine-like MIAs, including lochnericine, all derive from tabersonine and compose a complex pathway where the terminal metabolites are 19-acetylhörhammericine and echitovenine (Fig. 7). The most important pathway starting from tabersonine ends at vindoline after six successive reactions. The MIAs vindoline and catharanthine are condensed to produce the bisindole alkaloids, including vincristine and vinblastine (Fig. 8).

2.6 Regulation of the TIA Pathway

The TIA pathways involve at least 35 intermediates, 30 biosynthetic enzymes, eight regulatory genes, and several cellular compartments [4, 32, 33]. Perhaps not surprisingly, the genetic manipulation of the TIA pathway in *C. roseus* is rarely successful in increasing the final levels of TIA metabolites. Because the TIA pathway is highly regulated, a complete understanding of the regulatory

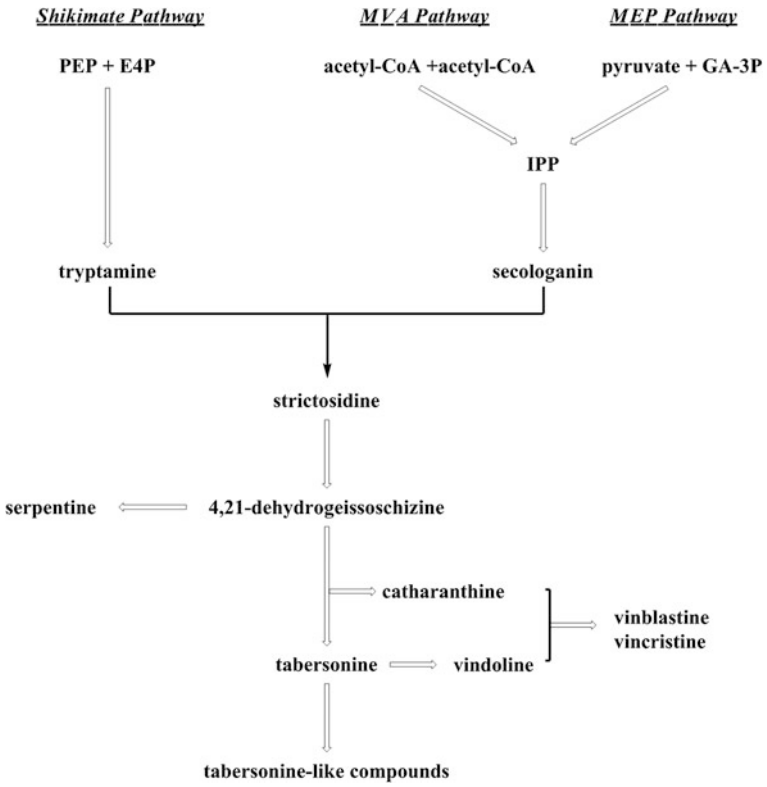

Fig. 5 An overview of the TIA biosynthesis pathway. *PEP*: phosphoenolpyruvate; *E4P*: erythrose 4-phosphate; *IPP*: isopentenyl diphosphate. *Solid arrows* indicate single reactions; hollow arrows indicate multiple steps

mechanisms of the TIA pathway may be a requirement for successful metabolic engineering. Despite the complicated regulation, recent research has shed light on this area.

Extensive research has studied the influence of signaling molecules on TIA biosynthesis. Although the elucidation of the entire regulatory mechanism is not complete, those signaling molecules, such as jasmonate (JA), ethylene, nitrous oxide (NO), and salicylic acid, not only demonstrate involvement in TIA biosynthesis, but also mediate TIA production by either synergistic or antagonistic effects [34].

The most direct regulation of the TIA pathway happens at the transcriptional level. Transcription factors, binding to a specific element and regulating the expression of genes, are a major mechanism controlling the biosynthetic genes of the TIA pathway. Transcription factors are usually regulated by signaling molecules or other elements. During the past few years, much effort was made to identify the transcription factors regulating the TIA pathway and to elucidate which genes are regulated by specific transcription factors.

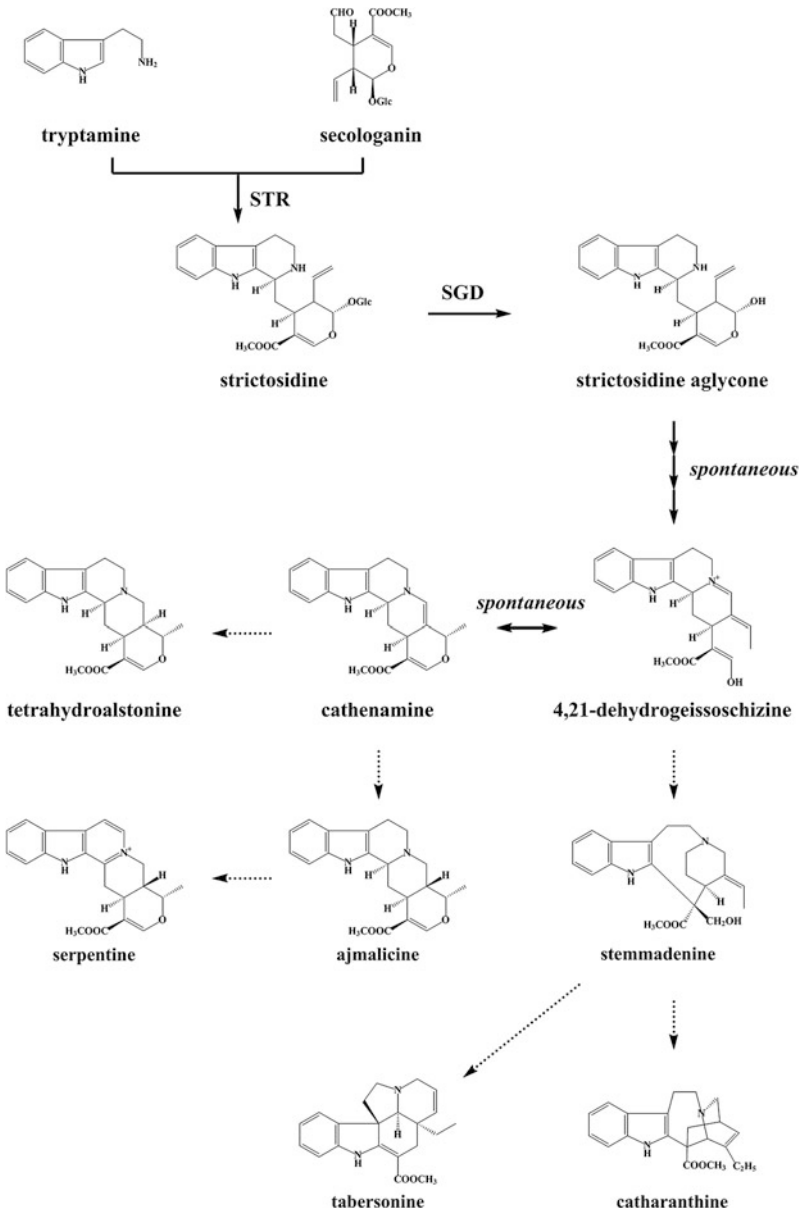

Fig. 6 The main branches of the downstream TIA biosynthesis pathway. *STR*: strictosidine synthase; *SGD*: strictosidine β-D-glucosidase. *Dashed arrows* indicate uncharacterized steps

The most well-known transcription factors are the octadecanoid-responsive *Catharanthus* AP2/ERF domain (ORCAs), including ORCA1, ORCA2, and ORCA3, which are members of the AP2/ERF transcription factor family. ORCA3,

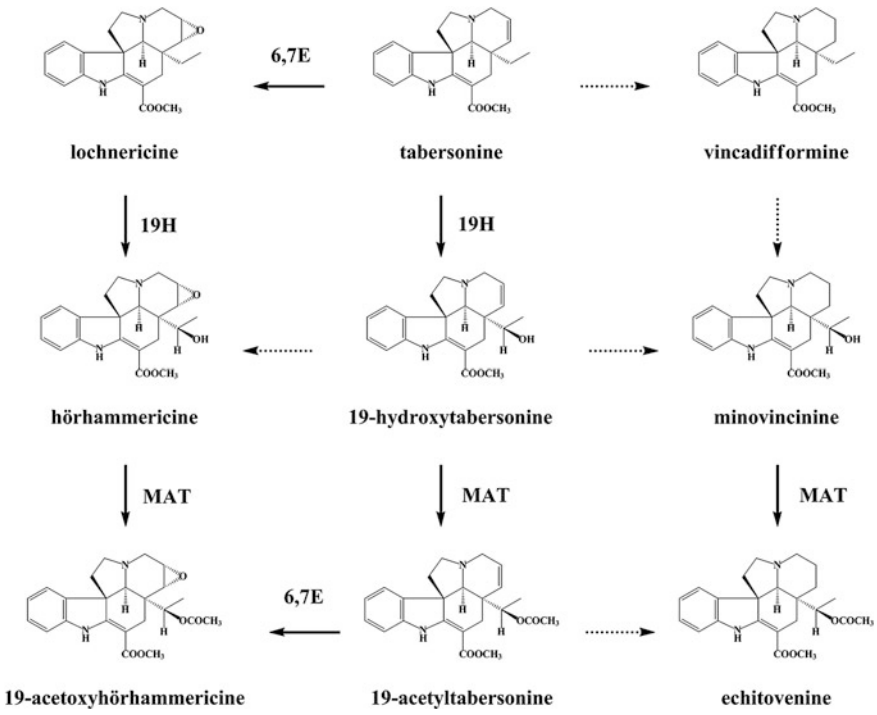

Fig. 7 The biosynthesis of tabersonine-like compounds. 6,7*E*: 6,7-epoxidase; *19H*: 19-hydroxylase; *MAT*: minovincinine 19-hydroxy-*O*-acetyltransferase. *Dashed arrows* indicate uncharacterized steps

for which expression is inducible with JA and fungal elicitation, is the most widely studied. The overexpression of ORCA3 in *C. roseus* hairy roots enhanced the transcript levels of several biosynthetic genes (*AS*, *TDC*, *DXS*, *CPR*, *G10H*, *SLS*, *STR*, *SGD*, and *D4H*) involved in the TIA pathway and consequently increased the accumulation of some TIAs [35]. Previously, overexpression of ORCA3 in *C. roseus* suspension cells also enhanced the expression of *AS*, *TDC*, *DXS*, *CPR*, *STR*, and *D4H* genes[36].

Yeast one-hybrid screening with a *Str* promoter indicated that ORCA2 activates the *Str* promoter and its expression is rapidly inducible with JA and fungal elicitation; in contrast, ORCA1 is expressed constitutively and is not involved in JA- and fungal elicitor-induced *Str* gene expression [31]. Recently, the overexpression of ORCA2 in *C. roseus* hairy roots enhanced the transcript levels of *AS*, *TDC*, *G10H*, *STR*, *D4H*, *T16H*, and *PRX1* genes [37]. In addition to the three ORCAs, another transcription factor, the *C. roseus* box P-binding factor (CrBPF-1) can also bind the *Str* promoter but at a different position. Research suggests that CrBPF-1 may enhance the expression of the *Str* gene when the ORCAs are already bound to the promoter [38]. CrMYC1 [39] and CrMYC2 [40] are basic helix–loop–helix transcription factors. Both of these two transcription factors are induced by JA and

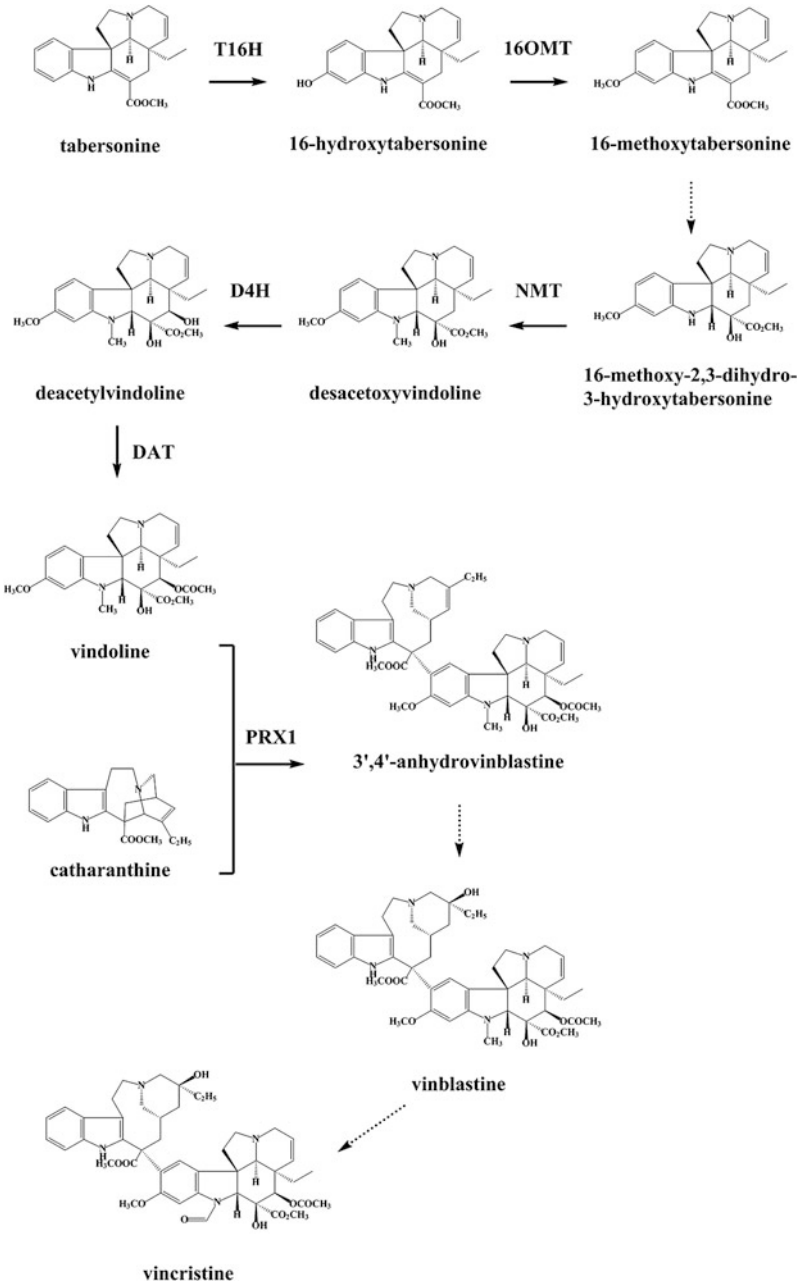

Fig. 8 The biosynthesis of vinblastine and vincristine. *T16H*: tabersonine 16-hydroxylase; *16OMT*: 16-hydroxytabersonine-*O*-methyltransferase; *NMT*: *N*-methyltransferase; *D4H*: desacetoxyvindoline 4-hydroxylase; *DAT*: deacetylvindoline 4-*O*-acetyltransferase; *PRX1*: peroxidase 1. *Dashed arrows* indicate uncharacterized steps

fungal elicitation. The mRNA levels of *CrMYC1* and *Str* genes were enhanced after induction by JA and fungal elicitation, which suggests that CrMYC1 activates the expression of the *Str* gene [39]. CrMYC2 is postulated to act upstream of ORCA2 and ORCA3, activating their transcription [40].

ORCA2, ORCA3, CrBPF-1, CrMYC1, and CrMYC2 act as transcriptional activators for some TIA biosynthetic genes as well as for several transcription repressors for TIA biosynthesis. Three members of the Cys2/His2-type (transcription factor IIIA-type) zinc finger protein family from *C. roseus*, ZCT1, ZCT2, and ZCT3, are activated by ORCA2 and ORCA3, and repress the activity of the promoters of *TDC* and *STR*. In addition, the ZCT proteins can repress the activating activity of the AP2/ERF domain of the ORCAs [41]. In addition to the ZCT proteins, G-box binding factors 1 and 2 (GBF-1 and GBF-2) also act as repressors of *STR* gene expression [42].

CrWRKY1 and CrWRKY2 are jasmonate responsive WRKY transcription factors that activate several genes involved in TIA biosynthesis [43, 44]. Overexpression of *CrWRKY1* in *C. roseus* hairy roots up-regulated *TDC*, as well as the transcriptional repressors *ZCT1*, ZCT2, and *ZCT3*, and down-regulated the transcriptional activators *ORCA2*, *ORCA3*, and *CrMYC2* [43]. In contrast, overexpression of *CrWRKY2* in *C. roseus* hairy roots led to an increase in mRNA transcripts of *TDC*, *NMT*, *DAT*, *MAT*, as well as both specific TIA transcriptional activators (*ORCA2*, *ORCA3*, and *CrWRKY1*) and repressors (*ZCT1* and *ZCT3*) .

The complex interactions of the regulation of TIA biosynthesis is summarized as Fig. 9. Although the isolation of transcription factors regulating the TIA biosynthesis pathway is progressing, further characterization of the regulatory pathways is needed.

3 Metabolic Engineering of TIA Biosynthesis in *C. roseus* Hairy Roots

The metabolic engineering of medicinal plants focuses primarily on increasing the biosynthesis of pharmaceutical metabolites. As more enzymes in the TIA pathway and their corresponding genes have been identified and characterized, as well as the deepening understanding of the whole pathway, it becomes feasible for the metabolic engineering of the TIA pathway to increase the production of valuable pharmaceutical metabolites [45]. Inasmuch as creating transgenic plants of Catharanthus is currently not feasible, tissue and cell culture is the only viable option for an engineered system. The previous metabolic engineering studies of the TIA pathway in *C. roseus* hairy roots are listed in Table 2.

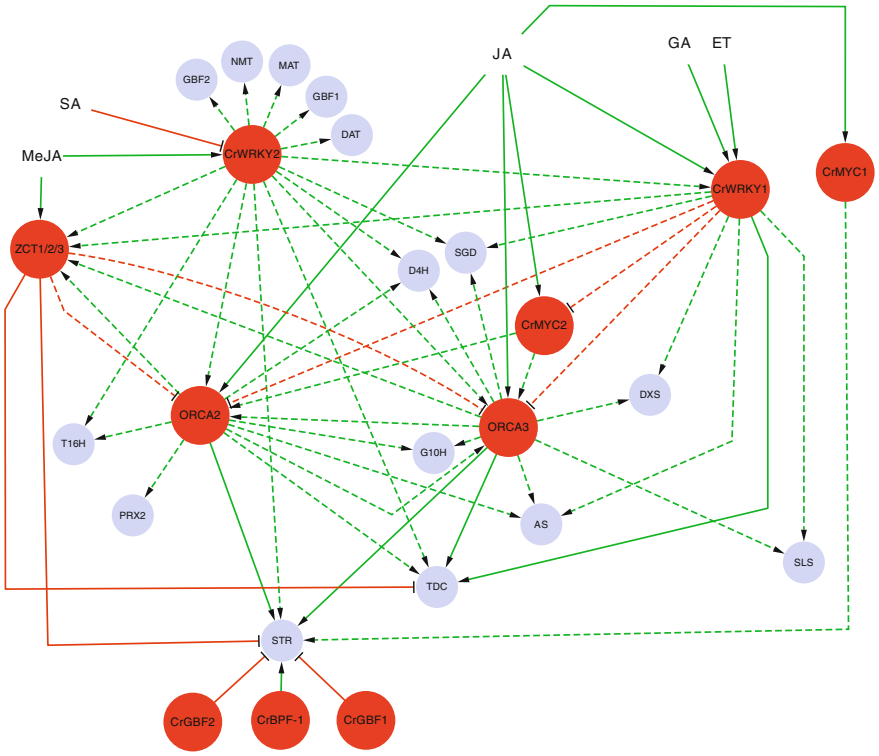

Fig. 9 Regulation map of the TIA biosynthesis pathway. *MeJA*: methyl jasmonic acid; *SA*: salicylic acid; *JA*: jasmonic acid; *ET*: ethylene; *GA*: gibberellic acid. The nodes with *empty* background are signaling molecules; the nodes with *red* background are transcriptional factors; the nodes with *grey* background are genes in the TIA biosynthesis pathway; *dashed lines* represent interactions that may be direct or indirect; *solid lines* indicate potentially direct interactions. *Green lines* with arrows represent transactivation and red lines with bars represent transrepression. *Note* CrWRKY2 overexpressed line has great positive effect on ZCT1, ZCT3 but only slight positive effect on ZCT2; however, in order to make this figure clear, ZCT1, ZCT2, and ZCT3 are lumped

3.1 Metabolic Engineering

AS, the regulatory step of the tryptophan biosynthesis pathway, is under the feedback control of both tryptophan and tryptamine [28]. To test how enhanced tryptophan availability affects the accumulation of indole alkaloids, an *Arabidopsis* feedback-resistant AS α subunit with a glucocorticoid-inducible promoter was overexpressed in *C. roseus* hairy roots. Enzyme assays demonstrated that the heterogeneous α subunit is compatible with the native β subunit and that AS activity is more resistant to tryptophan inhibition in induced than in uninduced cultures. After a 6-day induction, tryptophan and tryptamine yields increased from undetectable levels to 2,500 μg/g dry weight and from 25 to 267 μg/g dry weight,

Table 2 Summary of the generation and metabolic characterization of transgenic *C. roseus* hairy roots lines

Description of Transgenes	Major Observations
Feedback-insensitive *Arabidopsis* ASα [46]	Tryptophan ↑↑↑↑↑, tryptamine↑↑, lochnericine↑
Feedback-insensitive *Arabidopsis* ASα + *C. roseus* Asβ [47]	Tryptophan ↑↑↑↑↑, tryptamine↑↑↑
C. roseus TDC [48]	Serpentine↑
Feedback-insensitive *Arabidopsis* ASα + *C. roseus* TDC [48]	Tryptamine↑↑
Feedback-insensitive *Arabidopsis* ASα + *C. roseus* TDC, *C. roseus* ASβ [47]	Tryptophan ↑↑↑↑↑, tryptamine↑↑↑
Arabidopsis DXS [49]	Serpentine↑, ajmalicine↑, lochnericine↓, hörhammericine↓, tabersonine↓
Arabidopsis DXS + *C. roseus* G10H [50]	Ajmalicine↑, lochnericine↑, tabersonine↑
Arabidopsis DXS + feedback-insensitive *Arabidopsis* ASα [50]	Lochnericine↑, hörhammericine↑, tabersonine↑
C. roseus T16H [49]	Strictosidine↓, catharanthine↓, lochnericine↑, hörhammericine↓, tabersonine↓
C. roseus DAT [51]	Hörhammericine↑
C. roseus ORCA3 [35]	Lochnericine↓, hörhammericine↓, tabersonine↓
C. roseus ORCA2 [37]	Strictosidine↓, secologanin↓, serpentine↑, tabersonine↓↓
C. roseus WRKY1 [43]	Serpentine↑↑, catharanthine↓, tabersonine↓
C. roseus WRKY2 [44]	Serpentine↑, tabersonine↑↑, vindoline↑↑↑

respectively. Although the yield levels of tryptophan and tryptamine increased dramatically, the accumulation levels of most TIAs were not changed, except for lochnericine, which increased 81 % after a 3-day induction [46]. The results indicated that TIA levels are tightly controlled and there are other possible rate-limiting steps in the TIA biosynthesis pathway. Overexpression of TDC with a glucocorticoid-inducible promoter in *C. roseus* hairy roots showed a 48 % increase of TDC activity, a 129 % increase of serpentine yield, and no significant increase in tryptamine levels [48]. In the same study, co-expression of TDC and *Arabidopsis* feedback-resistant ASα resulted in an 87 % increase of TDC activity, sixfold increase of tryptamine for a 3-day late exponential induction, and no effects on TIAs measured. The results suggested that the flux to tryptamine in *C. roseus* hairy roots can be increased, but further manipulation of other biosynthetic steps will be needed to increase the yield of TIAs. To further engineer the indole pathway, an *Arabidopsis* ASβ subunit (ASB1) cDNA was constitutively overexpressed together with the glucocorticoid-inducible overexpression of feedback-resistant ASα and TDC in *C. roseus* hairy roots. Transgenic hairy roots overexpressing the *Arabidopsis* feedback-resistant AS holoenzyme showed significantly greater resistance to

feedback inhibition of AS activity than expressing ASα alone, and the AS holoenzyme transgenetic line produced 1.8 times more tryptophan after a 3-day induction. In addition, compared to a fourfold increase of tryptamine levels in the induced ASα line, tryptamine levels in the induced ASα:ASβ line increased up to ninefold. When TDC was expressed together with ASα and ASβ, the induced ASα:ASβ:TDC line produced up to 14-fold higher tryptamine levels than the uninduced lines. Compared with the dramatic increase of tryptophan and tryptamine, the change in alkaloid levels in the induced lines depended on the duration and dosage of induction [47].

Engineering increased flux towards the iridoid pathway, the overexpression of *Arabidopsis* DXS under the control of a glucocorticoid-inducible promoter in hairy root cultures of *C. roseus* led to an increase of loganin, serpentine, and ajmalicine levels and a decrease in tabersonine, lochnericine, and hörhammericine levels [52]. To increase the flux towards downstream metabolites of the TIA pathway, DXS, G10H, and ASα were overexpressed in *C. roseus* hairy roots alone or in combination. The study showed that overexpression of DXS led to an increase in ajmalicine by 67 %, serpentine by 26 %, and lochnericine by 49 % and a decrease in tabersonine by 66 % and hörhammericine by 54 %. Co-overexpression of DXS and G10H resulted in an increase of ajmalicine, lochnericine, and tabersonine by 16, 31, and 13 %, respectively. Similarly, co-overexpression of DXS and ASα showed a significant increase in hörhammericine, lochnericine, and tabersonine by 30, 27, and 34 %, respectively. These results suggested that multiple enzymes may need to be co-overexpressed to increase the accumulation of the downstream metabolites of the TIA pathway [50].

In the *C. roseus* hairy roots system, the pathway from tabersonine to vindoline is blocked. Overexpression of enzymes in the vindoline biosynthesis pathway could be a method to increase the accumulation of valuable metabolites, such as vindoline or even vincristine and vinblastine. Overexpression of tabersonine 16-hydroxylase (T16H), the first enzyme in the vindoline biosynthesis pathway starting from tabersonine, in *C. roseus* hairy roots under the control of a glucocorticoid-inducible promoter was studied. After induction, the level of lochnericine increased, and the levels of strictosidine, catharanthine, hörhammericine, and tabersonine decreased [52]. The last enzyme involved in the vindoline biosynthesis pathway is deacetylvindoline-4-*O*-acetyltransferase (DAT), which converts deacetylvindoline into vindoline. Overexpression of DAT alone with the strong cauliflower mosaic virus (*CaMV*) 35S promoter in *C. roseus* hairy roots did not lead to the accumulation of vindoline. Interestingly, around fourfold higher levels of hörhammericine were observed in the DAT overexpressing line compared with the control line. Enzyme assays showed that the DAT protein could inhibit the enzyme activity of minovincinine-19-*O*-acetyltransferase (MAT), which caused the accumulation of hörhammericine [51].

Transcriptional regulators usually control the expression of multiple genes in a metabolic pathway. It is a reasonable idea metabolically to engineer these transcriptional regulators to enhance the yield of targeted metabolites. In the TIA pathway, several transcriptional regulators have been identified. Among these

transcription factors are some that are activators of several TIA biosynthetic genes, such as ORCA2 [53], ORCA3 [36], CrBPF1 [38], CrMYC1 [39], CrMYC2 [40], CrWRKY1 [43], and CrWRKY2 [44], and some that act as repressors, such as the three zinc finger proteins [41], GBF-1, and GBF-2 [42]. The interaction among all the transcription factors is complex, and all of them can be induced by either JA or fungal elicitation. Although many transcriptional regulators have been identified, metabolic engineering with these regulators is still limited. So far, only ORCA2, ORCA3, CrWRKY1, and CrWRKY2 have been overexpressed separately in *C. roseus* hairy roots to study their effect on the regulators, genes, and metabolites in the TIA metabolic pathway [35, 37, 43, 44].

JA treatment and the overexpression of ORCA3 in *C. roseus* hairy roots under a glucocorticoid-inducible promoter were investigated for their effect on TIA metabolite levels and gene expression [35]. The ORCA3 transcripts increased 170-fold after glucocorticoid-induced overexpression and JA elicitation, whereas ORCA3 overexpression without JA elicitation increased ORCA3 transcripts 89-fold, and JA elicitation alone caused a 5-fold increase. Elicitation with JA caused the greatest increase of TIA pathway gene transcripts, including *ASα*, *TDC*, *DXS*, *G10H*, *SLS*, *STR*, and *SGD*, and also caused the greatest increase in tabersonine, lochnericine, hörhammericine, catharanthine, and serpentine concentration. In contrast, induction of ORCA3 overexpression or overexpression plus JA elicitation caused a decrease in tabersonine, lochnericine, and hörhammericine, and did not show a significant effect on transcript levels of TIA pathway genes. The large increase of ORCA3 transcripts did not lead to a significant increase of TIA pathway gene transcripts or TIA metabolites. The observation that the expression of transcriptional repressors ZCT1 and ZCT2 increased along with the increase of the ORCA3 transcripts could possibly explain this.

In addition to ORCA3, the effect of another transcriptional activator, ORCA2, on the TIA pathway has also been studied. ORCA2 was overexpressed under an ethanol-inducible promoter in *C. roseus* hairy roots, and the expression levels of key genes involved in the TIA pathway, as well as the important metabolites, were examined [37]. Compared with the uninduced ORCA2 line, the induced ORCA2 line produced more serpentine, 16-hydroxytabersonine, and 19-hydroxytabersonine and less secologanin, strictosidine, tabersonine, and hörhammericine. Based on the time course analysis of TIA regulatory gene expression, the expression levels of the activator ORCA3 and the repressors ZCT1, ZCT2, and ZCT3 all increased. Although overexpression of the ORCA2 gene could increase the expression of key TIA biosynthetic genes (the side-effect of increasing expression levels of transcriptional repressors produced TIA metabolites), level changes there were transient and limited in magnitude.

A number of independent hairy root lines expressing *CrWRKY1* under the control of the constitutive *CaMV* 35S promoter were generated by Suttipanta et al. [43]. The expression levels of the *CrWRKY1* and the *TDC* genes increased dramatically, 22-fold higher and 7- to 9-fold, respectively, in the CrWRKY1-overexpressing lines compared with the empty vector control lines. Although to a lesser extent than *TDC*, the expression of *AS*, *DXS*, *SLS*, and *SGD* also increased

by an average of 1.2-, 0.7-, 2.3-, and 2.4-fold, respectively, compared with the control. For the transcription factors, the expression of ORCA2, ORCA3, and CrMYC2 were suppressed by an average of 90, 80, and 60 %, respectively, and the expression of ZCT1, ZCT2, and ZCT3 increased by an average of 1.4-, 0.3-, and 3.2-fold. To further clarify the role of CrWRKY1 in the regulation of *TDC*, gene-silencing technology was applied and opposite expression patterns were observed for each gene compared with the CrMRKY1 overexpression lines. In the metabolic profiling experiments, the significant increase of serpentine and the reduced production of catharanthine and tabersonine in CrWRKY1 hairy roots indicated that overexpression of CrWRKY1 appears preferentially to activate the serpentine pathway at the expense of the catharanthine and tabersonine pathways.

Suttipanta also did a similar analysis for CrWRKY2-overexpressed hairy root lines as the CrWRKY1 lines [44]. A comparision of the qRT-PCR results of the CrWRKY2 lines under the control of the constitutive *CaMV* 35S promoter with the empty vector control lines revealed that the mRNA transcripts of *TDC*, *STR*, *SGD*, *D4H*, *T16H*, *NMT*, *DAT*, and *MAT* increased. For the transcription factors, ORCA2 expression and ORCA3 expression were significantly enhanced by 4.6- to 16.2-fold and 14.0- to 17.30-fold, respectively. CrWRKY1 (2.5- to 7.4-fold) was moderately up-regulated, and the transcriptional repressors, ZCT1 (4.75- to 7.92-fold), ZCT2 (0.87- to 0.92-fold), ZCT3 (2.47- to 5.15-fold), GBF1 (0.67- to 0.96-fold), and GBF2 (0.74- to 0.82-fold), were also increased to different extents. In the metabolic profiling analysis, the increased accumulation of serpentine (1.6-fold) and tabersonine (5.1- to 6.0-fold) was observed in the transgenic hairy root lines compared with the empty vector control hairy root lines.

All previous research showed that overexpressing the transcriptional activators increased the accumulation of some TIA metabolite levels, while also increasing the transcriptional activators and repressors in the TIA biosynthesis pathway. This indicates that the interaction network of transcription factors is complex. In order to further increase TIA accumulation, the down-regulation of one or more transcriptional repressors in combination with the up-regulation of transcriptional activators may be a promising direction for achieving a sustained increase of TIA gene expression and metabolite levels.

3.2 Metabolic Profiling

Metabolic profiling, the quantification of small biomolecules or metabolic fluxes, provides a direct view of the physiological status of the cell at a certain time point and under specific circumstances [54]. Metabolic profiling is important for understanding cellular regulation, identification of bottlenecks in biosynthetic pathways, and even gaining more knowledge of biological processes.

C. roseus is a well-studied medicinal plant and the two well-known anticancer pharmaceuticals, vincristine and vinblastine, are just 2 of 130 alkaloids produced. However, only a small number ($\sim$11) of the alkaloids are frequently analyzed and

even fewer ($\sim$8) are available commercially. In the last decade, the improvement in analytical equipment and protocols provides the ability to detect, separate, identify, and quantify intermediates and end metabolites. The development of large spectral analytical techniques allows the identification of more *C. roseus* secondary metabolites. On the other hand, with the recent perspectives of metabolic engineering to enhance the yield of secondary metabolites [48, 55, 56], it is crucial to be able to identify and quantify the fluxes controlling the secondary metabolic pathways. Therefore, the quantification of the TIAs and of the indole and iridoid precursors enables the process of TIA metabolic engineering.

High-performance liquid chromatography (HPLC) coupled with different detectors, such as the photo diode array (PDA), fluorescence detector, electrospray ionization tandem mass spectrometer (ESI–MS-MS), nuclear magnetic resonance (NMR), and circular dichroism (CD) [57], is efficient for precise identification and/or quantification of alkaloids. Because of the aromatic rings in TIA structures, UV absorbance is effective in TIA detection. The presence of conjugated π-electrons, especially in the aromatic rings, provides intense fluorescent activity, allowing for the possibility of fluorescence detection. Alkaloids are also easily ionized by electrospray in positive mode, therefore ESI (+) MS or MS/MS detection is recommended. Among all the detectors, UV and fluorescence are efficient and common for the quantification of TIAs, whereas the other detectors, such as MS or NMR, are usually used for identifying or more precisely quantifying TIA metabolites.

For UV absorbance detection, 218 nm is the optimal wavelength for maximizing the absorbance level for most of the alkaloids if only a single wavelength is available. However, a higher precision of detection is possible by using 254 nm to quantify strictosidine, ajmalicine, serpentine, catharanthine, and vindoline and 329 nm to quantify tabersonine-like compounds, with signal-to-noise ratios of 4 and 60 times higher than at 218 nm, respectively. In addition to UV detection, fluorescence detection is applied because it is more sensitive, having a lower limit of detection at the micromolar level. The optimal excitation and emission wavelengths in fluorescence detection [58] and the absorption UV wavelengths in UV detection [52] for several *C. roseus* TIAs and precursors are listed in Table 3. Recent developments in the separation column, PDA detectors, as well as fluorescence detectors have contributed to decreasing the limits of detection significantly. However, the common bottleneck of these two detectors for metabolite quantification is the presence of other compounds at the peaks of interest (co-elution) [59].

The major limitation in developing effective separation and identification methods relies on the lack of standards for most of the *C. roseus* secondary metabolites. In order to solve this problem, a semi-preparative (semi-prep) scale HPLC method was developed to purify standards of interest. An example of an analytical method scaled up to obtain tabersonine, lochnericine, and hörhammericine standards from *C. roseus* hairy root tissue is given by Sander [52]. To collect the fractions, the same HPLC system as the analytical method was used with the addition of a fraction collector. Data extracted at 329 nm were used to identify

Table 3 Fluorescence and UV properties of *C. roseus* TIAs and precursors

Alkaloid	Fluorescence [58] (Max Ex/Em in nm)	Absorption UV [52] (nm)
Tryptophan	270/370	218, 278
Tryptamine	270/370	218, 278
Geraniol		199
10-Hydroxygeraniol		199
Loganin		223, 241
Secologanin		222, 240
Strictosidine	280/354	222
Ajmalicine	270/370	225, 279
Serpentine	350/450	248, 305, 363
Catharanthine	290/363	225, 281
Tabersonine		224, 298, 323
Lochnericine		225, 298, 327
Hörhammericine		225, 298, 325
Vindoline	307/357	216, 251, 304
Vinblastine	297/364	216, 266
Vincristine		220, 254, 295

tabersonine, hörhammericine, and lochnericine using retention time standards [60, 61]. The UV chromatograms of the analytical and semi-preparative scale HPLC methods at 329 nm are shown in Fig. 10. The time scales of the analytical chromatogram and the semi-prep chromatogram are different due to the difference in column diameter and flow rate.

Another similar semi-prep method, developed from the Tikhomiroff and Jolicoeur [62] analytical method, separated serpentine, catharanthine, lochnericine, tabersonine, and several unknown compounds. The fractions achieved from a semi-prep method may not be pure because it is possible that several compounds co-elute [63]. The fractions can be separated again under further modified HPLC conditions. The collected fractions could then be further analyzed through MS–MS or ^{1}H-NMR detection.

Widely utilized UV and fluorescence detection applications focus on identifying and quantifying compounds with standards. MS and NMR techniques identify uncharacterized compounds and quantify more precisely. Utilization of MS detection is well established for the *C. roseus* hairy root extracts where all kinds of MS instruments, such as TSP-MS (Thermospray Mass Spectrometry), ESI–MS (Electrospray Ionization Mass Spectrometry), or MALDI-TOF (Matrix-assisted Laser Desorption with Time-of-Flight detector), enhance the identification and quantification of low concentration compounds and of nonpure peaks with a higher precision than UV and fluorescence spectra. In addition, tandem mass or ^{1}H-NMR spectrometry identifies fragment patterns for metabolites at the same molecular weight. For instance, 13 alkaloids from *C. roseus* have the same molecular weight of 352.432 g/mol. The MS/MS fragmentation patterns of some TIAs are listed in Table 4 [64]. HPLC-NMR has been used for the screening of

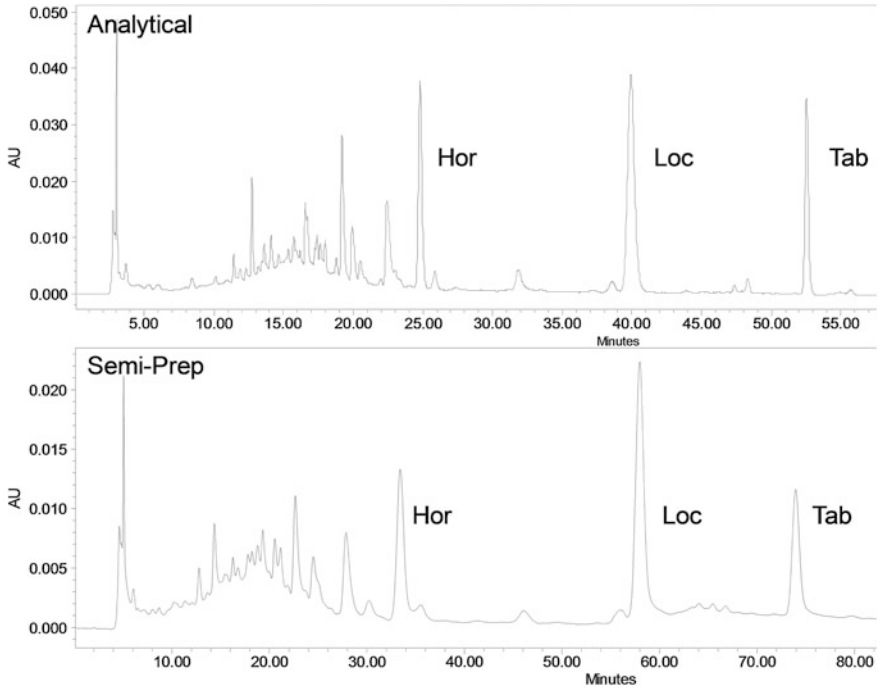

Fig. 10 Chromatograms of analytical and semi-preparative scale HPLC methods at 329 nm

plant extracts [57, 65]. However, this technique has never been used for *C. roseus* hairy root extracts.

Metabolic flux analysis (MFA), the quantification of the intracellular fluxes in a metabolic network, is an important cornerstone for metabolic engineering and systems biology. MFA in one organism determines the phenotype [66] or cell physiology [67]. Comparison of fluxes between phenotypes is of the utmost

Table 4 MS/MS fragmentation patterns of *C. roseus* TIAs

Alkaloid	MS/MS fragmentation pattern [64]	
	Precursor ion $[M + H]^+$ (m/z)	Main fragment ion (m/z)
Ajmalicine	353	222; 210; 178; 144; 117
Serpentine	349	317; 289; 263
Catharanthine	337	174; 144
19*S*-Vindolinine	337	320; 308
Vindolinine	337	320; 308
Vindolidine	427	409; 367; 158; 143
Tabersonine	337	323; 305; 274
Vindoline	457	439; 397; 188
Vinblastine	811	793; 751; 733; 680; 649; 542; 522; 355; 337
Vincristine	825	807; 765; 747; 723; 705; 687

importance to elucidate metabolic regulation [68] and the impact of genetic manipulation to the phenotypes [69], as well as the identification of bottlenecks in product formation, which provides the basis for further metabolic engineering [70].

In MFA of central carbon metabolism, the intracellular fluxes are calculated by using a stoichiometric model for the major intracellular reactions and applying mole balances around intracellular metabolites. A set of measured extracellular fluxes and the growth of the organism are used as inputs to the calculations. In addition, this method requires the assumption of biological steady-state, or homeostasis. One of the advantages of this method is that it does not require the intracellular metabolite concentrations and the enzyme kinetics of the system. However, as the metabolic pathways in plant cell and tissue cultures are far less characterized than microbial or mammalian cultures, this method is currently not feasible for many plant systems. Extension of these MFA models to include secondary metabolism is problematic, inasmuch as the flux to secondary metabolites is frequently less than a few percent of the total carbon flux. Thereby, error in measurement of secondary metabolites would increase dramatically compared to the primary metabolites. Consequently, flux is difficult to calculate using the traditional stoichiometric model. Therefore, the basis of the flux estimations is made directly from intracellular alkaloid or intermediate concentration measurements and focuses specifically on a single class of secondary metabolites and pathways.

Morgan et al. [71] and Sander [49] performed this type of MFA on the TIA pathway in the *C. roseus* hairy roots. The MFA model was based on the following equation,

$$r_i = \frac{d(C_i X)}{dt}$$

where r_i is the flux of a specific alkaloid (mmol alkaloid/h), C_i is the alkaloid specific yield (mmol/g DW), X is the biomass (g DW), and t is the time (h). The values for the variables C_i and X are taken directly from alkaloid and biomass measurements. Metabolic flux maps were derived through a software platform after the input of the model and experimental parameters [49, 71].

4 Conclusion and Outlook

In this review, we have summarized the TIA pathway, transcriptional regulatory networks, and the metabolic engineering of the TIA pathway based on the current progress made in *C. roseus*. Although extensive efforts have been conducted in the last few years, more needs to be done in order to further increase TIA production to a commercialized application.

4.1 Further Elucidation of the TIA Biosynthesis Pathway

Complete elucidation of the TIA biosynthesis pathway is of significant importance for the metabolic engineering of this pathway in *C. roseus* plants or hairy roots. Alternatively, in the future it may be possible to construct large portions of the TIA pathway in microbes for the production of valuable TIAs. Although more and more enzymes and their encoding genes involved in the TIA pathway have been characterized, many steps are still obscure. The vindoline biosynthesis pathway starting from tabersonine involves six enzymatic steps, among which five steps have been characterized; another step converting 16-methoxytabersonine to 16-methoxyl-2,3-dihydro-3-hydroxyltabersonine remains to be elucidated. If the missing genes involved in this pathway can be identified, it would be possible to produce vindoline or even vincristine and vinblastine in *C. roseus* hairy roots. Besides the vindoline biosynthesis pathway, the enzymes involved in many other TIA pathways have not been identified. With the elucidation of these TIA pathways, overexpressing the pathway genes and blocking the competitive pathway by RNAi can be combined to increase the yields of many valuable TIAs.

Identification of the unknown genes involved in the TIA pathway in *C. roseus* is not easy compared to other model plants such as *Arabidopsis*, because the genome sequence is not available yet and there is no mutant library of *C. roseus*. However, recent efforts have been exerted to discover new genes involved in this pathway. By sequencing the cDNA library generated from the RNA of *C. roseus* leaf epidermis cells, the LAMT gene was identified and characterized [72]. Indeed, the identification of an unknown gene requires the integration of several types of information, such as the metabolite profiles of *C. roseus* plants under different growth conditions or different elicitor treatment, the tissue-specific accumulation of certain metabolites, and the related gene expression and regulation patterns. The basic assumption is that the differential accumulation of the TIA metabolites is caused by the differential expression of pathway genes. So, identifying the differentially expressed genes provides a candidate list for the target unknown pathway genes. Many approaches can be used to identify differentially expressed genes, such as cDNA microarray and the newest RNA sequencing technology. The candidate list can be narrowed down by comparing the gene expression in many different samples (RNA extracted from different tissues, plants under different treatment). When a handful of candidate genes are identified, their accurate expression patterns can be analyzed and more detailed bioinformatic analysis can be performed to choose the most promising candidate for further biochemical characterization. By combining all these techniques, the unknown step may eventually be elucidated.

As the cost of genome sequencing decreases, the genome sequence of *C. roseus* may be reported in the near future. Then, the identification of unknown genes may become easier. Eventually, all steps of the TIA pathway may be elucidated.

4.2 Further Elucidation of Regulatory Mechanisms

Limiting improvements to the TIA pathway in *C. roseus* is the unclear correspondence of the DNA, RNA, protein, and metabolite levels to the regulatory mechanisms. Phytohormones are involved in many aspects of plant growth and development, including TIA's biosynthesis pathway, however, how to regulate the molecular mechanism of phytohormones in the TIA pathway is unclear. Forward and reverse genetic strategies should be used to identify important related molecular components in plant hormone perception, signaling, and transport involved in the TIA pathway. For example, it was demonstrated that auxin down-regulated the transcription level of the STR gene in *C. roseus* cell culture [73]. Research on the auxin signaling pathway [74, 75] would help to explore the relationship between plant hormones and TIAs. Calcium signaling is also involved in the regulation of TIA accumulation [76]. The identification of the calcium signaling components involved in the TIA pathway would also be important in the regulation. The use of some new related techniques, such as reverse-genetics approaches, gene-to-metabolite networks, metabolomics analysis, expressed sequence tags, and transcriptome, proteome, and metabolomic analysis, would deepen our understanding of the interrelationships between the TIA pathway and regulatory factors [77].

4.3 Further Development of Metabolic Engineering

In this review, the metabolic engineering of the overexpression some of the key enzymes in the TIA pathway and the two transcriptional activators, ORCA2 and ORCA3, did not increase the accumulation of TIA metabolites significantly. The transcript and metabolic profiling experiments drew a conclusion that down-regulation of transcriptional repressors in combination with up-regulation of transcriptional activators may be a promising direction for achieving higher and more sustained increases in TIA gene expression and metabolite levels.

In the previous metabolic engineering attempts of the TIA pathway in *C. roseus* hairy roots, the maximal number of manipulated genes is three [47]. Multigene transformation in plants is very difficult due to the technical limitations of existing methods. In the past several decades, various plasmids suitable for the cloning, transfer, and expression of foreign genes in plant cells have been developed [78]. The multigene overexpression could be achieved either by constructing a vector containing multiple genes or transferring multiple single-gene vectors in one host cell. For example, a constructed vector with 10 foreign DNA fragments was transferred into the rice genome in 2002 [79], and a mixture of 12 different single-gene plasmids was transformed into soybean embryogenic suspension cultures [80]. If we could manipulate multiple important genes in the TIA pathway as well as the regulatory factors using those techniques in the *C. roseus* hairy root system, it is possible to compel the hairy roots to produce vinblastine and vincristine.

4.4 Further Development of Metabolic Profiling Technology

The full potential of metabolic profiling in plant sciences has not been achieved yet. Several methodological limitations hamper the development of metabolic profiling and need to be addressed in the near future. The major obstacle of MS-based metabolic profiling is that the rate of the analyte identification is currently low because of the lack of structural information obtainable by any kind of mass spectrometer. Although NMR is commonly used to identify small organic molecules, due to the low sensitivity the technique requires time and expense to enrich the natural products for NMR structure analysis. Recently, a new technology, high-performance liquid chromatography combined with solid-phase extraction and NMR spectroscopy (HPLC-SPE-NMR), has been applied to rapidly identify secondary metabolites in some plants. For example, the structures of a total of 15 compounds, some major as well as some minor constituents, of *Harpagophytum procumbens* (Devil's claw) extracts, have been determined by using this technique [81]. Although there are limits to the sensitivity of the HPLC-SPE-NMR technique, it still provides a rapid strategy to identify the metabolites in a complex plant extract. In conclusion, NMR, MS-based technologies coupled with chromatographic separation assays present a chance to develop metabolic profiling to the comprehensive analysis of complex biological systems, such as the TIA metabolites in *C. roseus* hairy roots.

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