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Review

Molecular regulation of terpenoid indole alkaloids pathway in the medicinal plant, *Catharanthus roseus*

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***Catharanthus roseus* (L.) is a plant species known for its production of many pharmaceutically terpenoid indole alkaloids (TIAs). The intricate networks of TIAs biosynthesis is regulated by all kinds of molecules such as plant signaling molecules (jasmonate acid, ethylene and nitric oxide), plant growth regulators, prenylated proteins and transcription factors. This fine-tuned regulation is also accompanied with compartmentalization and transportation of various alkaloids in different parts of *C. roseus*. In this paper, we present an analysis of the state of the art related to these molecules regulation mechanism in the TIAs pathway in *C. roseus*.**

Key words: Compartmentalization, indole alkaloid, jasmonate, nitric oxide, phytohormone, prenylated proteins.

INTRODUCTION

Catharanthus roseus (L.) (Madagascar periwinkle) is a dicotyledonous tropical perennial plant belonging to *Apocynaceae* family, and entirely self-pollinated species with a high heritability. It has been extensively investigated mostly because it produces many kinds of terpenoid indole alkaloids (TIAs), which have many physiological effects on humans and are used in pharmacy. It is especially true that the dimeric terpenoid indole alkaloids, such as 3', 4'-anhydrovinblastine, vincristine and vinblastine, have powerful effects as anticancer drugs, whereas the monomeric compounds (ajmalicine and serpentine) are used in the treatment of cardiac and circulatory diseases (Van der Heijden et al., 2004; Singh et al., 2001). Many efforts have been made to improve the production of therapeutically important alkaloids of *C. roseus* in the last few years; however, these alkaloids still cannot be industrialized successfully, mostly due to its sophisticated molecular mechanism. Unraveling the complexity of the genetic, the catalytic and transport processes of TIAs biosynthesis are one of the most stimulating intellectual challenges in *C. roseus*. TIAs biosynthesis pathway has been partly elucidated during the past decades, meanwhile, some of the enzymes involved have been characterized and several structural genes have been cloned. Schematic representation (Figure 1) of TIAs biosynthetic pathway in *C. roseus* is based on previous reviews and research results (Zhou et al., 2009).

The major routes of biosynthesis for TIAs pathway are now largely understood, but what is required is the complete elucidation of pathways by determining each intermediate and characterization of the enzyme involved in its formation; meanwhile, biosynthetic steps in TIAs pathway are often coordinately regulated at the level of

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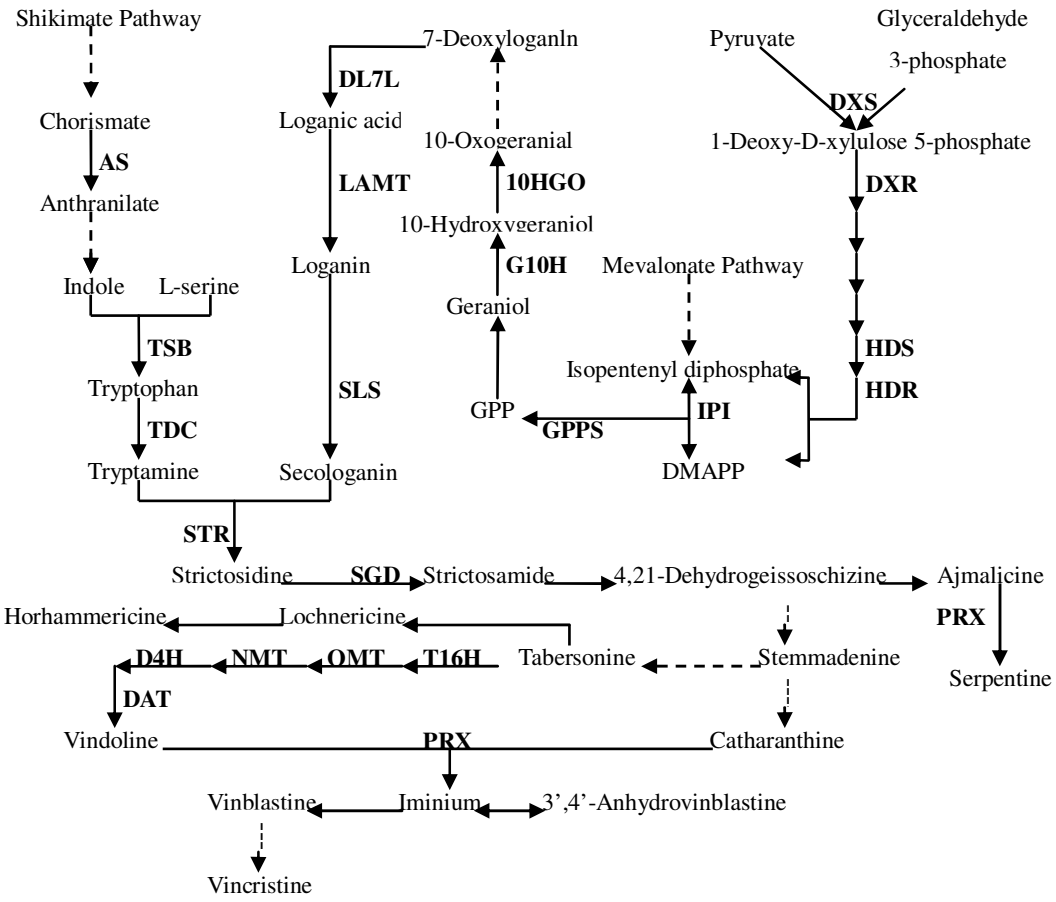

Figure 1. Schematic representation of TIAs biosynthesis pathway in *C. roseus*, Solid lines represent a single enzymatic conversion, whereas dashed lines indicate multiple enzymatic conversions. The full names of the enzymes are presented in the text.

transcription of the structural genes so that some key transcription factors (for example, ORCA3) in this network regulation should be elucidated. Progress in the understanding of the TIAs biosynthesis pathway at the molecular level would offer novel opportunities to improve alkaloid yields by cell cultures. Here, we summarize the most recent advances in our understanding of this molecular regulation mechanism on TIAs pathway in *C. roseus*.

PLANT SIGNALING MOLECULES

Jasmonates and ethylene

Jasmonates (JAs) have been found to induce the biosynthesis of a variety of secondary metabolites in different plant species, including alkaloids, terpenoids, glucosinolates and phenylpropanoids (Memelink et al., 2001). Jasmonic acid (JA) and its methyl ester (MeJA), collectively called jasmonates (JAs), are synthesized via the lipid-based octadecanoid (ODA) pathway. The role of jasmonate in activating the expression of genes and the accumulation of phytoalexins and secondary metabolites has been confirmed in various plant systems including *C. roseus* (Lee-Parsons et al., 2004). Jasmonates, as secondary messengers, activate the expression of defense genes, including genes that code for enzymes catalyzing the formation of indole alkaloids, such as strictosidine beta-D-glucosidase (SGD), tryptophan decarboxylase (TDC), geraniol 10-hydroxylase (G10H), cytochrome P450 reductase (CPR), 1-deoxy-D-xylulo-sesynthase (DXS), strictosidine synthase (STR) and anthranilate synthase (ASα) (Van der Fits and Memelink, 2000). In addition, Ca^{2+} and jasmonate also interact in modulating the defense response. For example, Ca^{2+} plays a role in mediating jasmonate biosynthesis (Menke et al., 1999).

In TIAs metabolism and in the primary precursor pathways, jasmonate induce gene expression and metabolism via ORCAs (octadecanoid-responsive *Catharanthus* AP2/ERF-domain), which are members of the AP2/ERF-domain family of plant transcription factors. Treatment with MeJA could regulate strictosidine

synthase (STR) gene in *C. roseus* cell culture, and this process involved the change of Ca^{2+} flux. However, Ca^{2+} influx was required for jasmonate biosynthesis and the induction of two transcription factors, ORCAs and a MYB-like protein CrBPF-1 (*C. roseus* box P-binding factor-1) (Memelink et al., 2001; Menke et al., 1999; Van der Fits et al., 2000). Ajmalicine production in MeJA-induced *C. roseus* cultures depended on the intracellular Ca^{2+} concentration and a low extracellular Ca^{2+} concentration (3 mM) enhanced by MeJA-induced ajmalicine production (Lee-Parsons and Ertürk, 2005).

RNA gel blot analysis from MeJA treated cell cultures showed a transient suppression of *HMGR* mRNA (Maldonado-Mendoza et al., 1994). The activity of strictosidine β-D-glucohydrolase (SGD) is elevated in cultures treated by MeJA (Stevens et al., 1992). Vazquez-Flota and his co-worker observed that the cultures of transformed roots treated with MeJA can increase the production of catharanthine (Vázquez-Flota et al., 1994). *C. roseus* shoot exposure to jasmonate could shorten the time required for the maximal vindoline accumulation (Elizabeta et al., 2004). In the model plant of *A. thaliana*, the finding of E3-ubiquitin ligase SCF^{COI1} (a multi-protein E3-ubiquitin ligase, which is called SKP1-like, cullin and F-box proteins of the complex) led to the proposal that jasmonate signaling involves SCF^{COI1}-mediated ubiquitination of regulatory proteins that control the transcription of jasmonate-responsive genes (Devoto and Turner, 2005; Howe and Jander, 2008). High levels of JA-derived signals such as (+)-7-*iso*-JA-*L*-Ile could promote SCF^{COI1}-mediated ubiquitination and subsequent degradation of JAZ (jasmonate ZIM domain) repressor proteins via the 26S proteasome (26S), resulting in the derepression of transcription factors and the expression of early response genes (Thines et al., 2007). JAZ proteins, in addition to the highly conserved central ZIM motif, contain a highly conserved C-terminal Jas motif and a less conserved N-terminal region. The JAZ protein was shown to interact *in vitro* and in yeast with AtMYC2 (bHLH transcription factor). Based on these findings, it was postulated that JAZs is a repressor of AtMYC2 which is rapidly degraded in response to JA thereby activating AtMYC2 (Chini et al., 2007), AtMYC2 and JAZ proteins. Therefore, a jasmonate-responsive oscillator is formed, where JAZ proteins negatively regulate AtMYC2 activity at the protein level, JAs cause JAZ degradation and AtMYC2 activation, and AtMYC2 switches on the expression of JAZ repressors at the gene level (Thines et al., 2007; Chini et al., 2007). Afterwards, it then activates or represses the related gene expression, including secondary metabolites biosynthesis genes. These advances have been enabled by pioneering research on signal transduction of Jas, using the model plant known as *A. thaliana.* It provides a good perspective and contributes to a better understanding of how JA regulates TIAs biosynthesis pathway in *C. roseus*.

The plant hormone ethylene regulates a wide range of developmental processes and the response of plants to stress and pathogens. Ethylene (ET) is synthesized from the amino acid methionine, and this biosynthetic pathway was unraveled to a large extent by the pioneering work of Yang et al. in the 1970 to 1980s (Kende et al., 1993). Ethylene induced signaling transduction, through a single conserved pathway, has been well characterized in the model plant, *Arabidopsis thaliana* (Willem et al., 2006), and as such, the ethylene signal pathway is negatively regulated (Hua and Meyerowitz, 1998). When ethylene occupies a receptor, the receptor is thought to undergo a conformational change and interacts with the Raf-like kinase CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) which is a negative regulator of the signal transduction pathway and is controlled at the posttranscriptional level by ethylene. Inactivation of CTR1 releases suppression of the MAPK cascade and results in the activation of EIN2 (ETHYLENE INSENSITIVE 2) protein, a key positive regulator in the ethylene signal transduction pathway. Activation of EIN2 leads to a cascade of transcription factors consisting of primary EIN3-like regulators and downstream ERF-like transcription factors, which then activates transcription of ethylene responsive genes such as PDF1.2 (Guo and Ecker, 2003; Kendrick and Chang, 2008). EIN3-BINDING F-BOX 1 and 2 (EBF1/2) coordinately control 26S proteasome degradation of the critical transcription factors EIN3 and EIL1. EBF1/2 expression is repressed by ETHYLENE-INSENSITIVE 5 (EIN5), which encodes the exoribonuclease XRN 4 (Guo and Ecker, 2003; Potuschak et al., 2003; Gagn et al., 2004). In the medicinal plant, *C. roseus*, few literatures could be found on this knowledge. *CrETR1 cDNA* was isolated by PCR amplification of a *cDNA* library from periwinkle cell cultures. It was found that *CrETR1* transcripts are strongly accumulated in petals and ovaries of *C. roseus* young plants, whereas no significant changes are detected in cell cultures treatment with various stress or hormonal stress (including ethylene). They found that addition of inhibitors of histidine kinases could decrease the ethylene enhanced accumulation of monoterpenoid indole alkaloids in periwinkle cell cultures, which show a possible involvement of *CrETR1* protein in the ethylene-related signaling pathway (Papon et al., 2004). This experiment demonstrates that ET could increase the production of indole alkaloids in *C. roseus*. In many cases, the interaction between JA and ET signaling is a synergistic one. A classic example is the regulation of Arabidopsis plant defensin gene *PDF 1.2*, which requires concomitant activation of the JA and ET response pathway. Two members of the large plant-specific APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily (ERF1 and ORA59) emerged as the principal integrators of the JA and ET signaling pathway (Pré et al., 2008). The expression of both *ERF1* and *ORA59* is induced by JA and ET and can be activated synergistically by both hormones. Another basic

helix-loop-helix leucine zipper transcription factor MYC2 has been demonstrated to play an important role in the regulation of JA-responsive genes. MYC2 functions as a positive regulator of JA-responsive genes (*VSP2* and *LOX2*), whereas it acts as a negative regulator of JA/ET-responsive genes such as PDF1.2 which are activated by ERFs (ERF1 and ORA59) (Pieterse et al., 2009). However, we still have a long way to go to get the detail information on ethylene molecular regulation mechanism in TIAs pathway.

Nitric oxide

Nitric oxide (NO) is a small highly diffusible gas and a ubiquitous bioactive molecule. NO is generated mainly by nitric oxide synthase (NOS), which catalyzes the NADPH dependent oxidation of L-Arg to L-citrulline and NO in animal (Stuehr et al., 2004). Although the precise NO biosynthetic pathway has not been fully understood in plant cells, it has been established that there are three pathways, namely, NOS, nirtrate reductase (NR) and non-enzymatic chemical reduction (Qiao and Fan, 2008). In plant, NO governs an impressive number of physiological and pathophysiological reactions involved in later growth and developmental processes, which opens a fantastic window for an as-yet unexplored field of NO's function in the plant kingdom. Until recently, we still know little about the mechanisms on how NO exert its effects. In the past few years, part of the gap has been bridged. NO modulates the activity of proteins through nitrosylation and probably tyrosine nitration. Furthermore, NO can act as a Ca^{2+}-mobilizing messenger (Besson-Bard et al., 2008). It was demonstrated that NO played key roles in the signaling network leading to plant secondary metabolite biosynthesis (Xu and Dong, 2007a). Treatment with fungi elicitor in *C. roseus* cell suspensions could induce NO generation, meanwhile, the content of catharanthine was enhanced. It was demonstrated that NO was essential for triggering catharanthine synthesis (Xu and Dong, 2005). The over-expression of mouse Bax (a mammalian pro-apoptotic member of the Bcl-2 family) could induce nitric oxide synthase (NOS) and stimulates NO generation in *C. roseus* cell culture. Then treatment with NO scavenger (cPITO: 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) found that it inhibits not only NO accumulation, but also the Bax-triggered TIA production. This could be explained that mouse Bax may induce TIA production through the NO-dependent signaling pathway (Xu and Dong, 2007b). The latest report showed that nitric oxide could down-regulate catharanthine biosynthesis through a MeJA-dependent or an independent pathway in *C. roseus* hairy root culture. The results demonstrated that the nitric oxide induced high levels of ZCTs (zinc finger-binding proteins) could bind to the promoters of *STR* and *TDC* and inhibit their transcription. At the same time, nitric oxide could reduce the transcription of the MIA pathway genes and the *ORCA3* transcription through the inhibition of *PGGT-I* (type-I geranylgeranyltransferase) (Zhou et al., 2010). These contradictions can be explained by the existence of different regulatory mechanisms in cell suspension cultures and in differentiated hairy root cultures. It is possible that the fungal elicitor can increase the content of catharanthine in *C. roseus* cell suspensions through another signaling pathway such as the JA pathway which plays a vital role in plant immunity (Pieterse et al., 2009). In future studies, it will be most important to know about the mechanisms of NO involved signaling transduction, and to identify and characterize its direct targets and functions.

Exogenous H_2O_2 was observed to induce NO generation in plant guard cells, accompanied by an increase of NOS like activity by H_2O_2 treatment (Bright et al., 2006). Experimental exposure of plants to different concentrations of H_2O_2 showed that endogenous H_2O_2 and vinblastine alkaloid concentrations in leaves were positively elevated (Tang et al., 2009). Biosynthesis of TIAs is regulated by multiple endogenous signaling pathways. The signaling molecules such as jasmonic acid, ethylene, NO and salicylic acid have been well demonstrated not only to be involved in TIAs biosynthesis, but also to interact in mediating TIAs production by either synergistic or antagonistic (Figure 2) (El-Sayed and Verpoorte, 2004). Our latest research results found that the antagonistic effects of NO and MeJA exist on catharanthine biosynthesis in *C. roseus* hairy roots (Zhou et al., 2010).

PLANT GROWTH REGULATORS

Phytohormones influence many diverse developmental processes ranging from seed germination to root, shoot and flower formation, involving plant growth, morphogenesis and metabolism. Under most circumstances, a single hormone can regulate many different processes, and at the same time, different hormones can influence a single process in plant. The role of phytohormones in the regulation of *C. roseus* indole alkaloids have been extensively studied (Zhou et al., 2009). They affect both culture growth and secondary metabolite production. Auxin negatively influences alkaloid biosynthesis at all levels. In cell suspension cultures, the mRNA levels of *STR* and *TDC* are rapidly down-regulated by auxin (Pasquali et al., 1992). Auxin also down-regulates the expression of *CrG10H* (Papon et al., 2005). The *CrDXS* and *CrDXR* (1-deoxy-D-xylulose 5-phosphate reductase) expressions were repressed and *CrMECS* (2-C-methyl-D-erytritol 2,4-cyclodiphosphate synthase) mRNA was at a low level in suspension cells cultured with 2,4-D (2,4-dichlorophenoxyacetic acid) (Veau et al., 2000). 2,4-D strongly inhibits the biosynthesis of all indole alkaloids

and peroxidase activity (Zhao et al., 2001). Transcriptional repression, triggered by addition of 2,4-D, may also be responsible for the reduced abundance of a group of polypeptides that appeared to be related to TIA biosynthesis (Ouelhazi et al., 1993). The latest research found that an opposite and coordinated action of multiple Ca^{2+}-release pathways exist in 2,4-D signal transduction, meanwhile, RyR (ryanodine receptor) and IP3 (inositol-triphosphate) channels in animals may also exist in plants. Consequently, there would be an addition of a new level of complexity to calcium signaling in plants (Poutrain et al., 2009). However, an addition of cytokinins to an auxin-free *C. roseus* cell cultures resulted in an increase in alkaloid accumulation and enhancement of *DXS*, *DXR* and *G10H* expression (Decendit et al., 1992; Oudin et al., 2007).Furthermore, synergistic interaction between cytokinins and ethylene transduction pathways has been reported since the addition of these two hormones further enhance *G10H* expression (Papon et al., 2005). It was found that the antagonistic effects of gibberellins and cytokinins exist on monoterpenoid indole alkaloids biosynthesis and their possible impact on elements of the signal transduction in *C. roseus* cell suspensions (Amini et al., 2009).

An addition of zeatin to a 2,4-D containing medium decreased tryptamine levels and increased the bioconversion of secologanin to ajmalicine, and also enhance the G10H activities (Papon et al., 2005; Decendit et al., 1993). Abscisic acid (ABA) could stimulate the accumulation of catharanthine and vindoline in *C. roseus* (Smith et al., 1987). However, treatment of precursors (tryptamine and loganin) fed *C. roseus* cells with ABA did not induce the accumulation of alkaloids, but it delayed the catabolism of strictosidine (El-Sayed and Verpoorte, 2002). The latest report displayed that salicylic acid and ethylene (ethephon) treatments resulted in a significant increase of vinblastine, vindoline and catharanthine, while ABA and gibberellic acid had a strongly negative influence on the accumulation of the three important alkaloids in *C. roseus* plant (Pan et al., 2010). Still, the molecular mechanisms of TIAs biosynthesis regulation by these phytohormones remain unknown.From related research at home and abroad, we may state that these phytohormones would involve transcriptional and post-transcriptional mechanisms of related key enzymes, and also define a regulatory interplay between the MVA and MEP pathways.

PRENYLATED PROTEINS

Isoprenylation is a posttranslational lipid modification of proteins that consists of the formation of a covalent attachment of isoprenyl groups which are derived from pyrophosphate intermediates of the cholesterol biosynthetic pathway. Prenylation involves the attachment of either C15 (farnesyl) or C20 (geranylgeranyl) groups to specific cysteine residues located near the carboxy termini of target proteins. This process was catalyzed by CaaX-PTases (CaaX-prenyltransferases, where C is a cysteine that becomes alkylated, A residues are aliphatic amino acids and X is a residue that controls whether the protein is farnesylated or geranylgeranylated) which contain protein farnesyltransferase (PFT) and type-I geranylgeranyltransferase (PGGT-I) that are heterodimeric enzymes composed of a common α subunit and a specific β subunit. Isoprenylation promotes the anchoring of proteins to specific cell membranes, a process that is essential for the normal function of these proteins such as sub-cellular localization, signal transduction and membrane trafficking machinery (Schafer and Rine, 1992; Casey, 1995).

Several pieces of evidences demonstrate that isopenthenyl diphosphate (IPP) for monoterpenoid indole alkaloids production originates mainly from the plastidial MEP pathway and not from the MVA pathway in *C. roseus* (Imbault et al., 1996). Crosstalk between the two terpenoid pathways cannot be explained in detail by a simple two compartment model. Consequently, this result shows that the IPP produced via the MVA pathway is not incorporated into secologanin biosynthesis and it suggested that compounds originating from the MVA pathway may be involved in the regulation of secologanin biosynthesis (Hedhili et al., 2007). Protein prenylation with prenyl moieties derived from MVA pathway seems to be required for the biosynthesis of iridoids. It was reported that protein targets for both PFT and PGGT-I are required for the expression of the early stage of monoterpenoid biosynthetic pathway (ESMB) genes and monoterpenoid biosynthesis in *C. roseus* (Courdavault et al., 2005). It appears that proteins, prenylated by both PFT and PGGT-I, are involved in the induction of the expression of *DXS*, *DXR* and *G10H*.

Trusov Y and his co-worker have recently reported that prenylated proteins function as part of the JA signaling pathway (Trusov et al., 2007). Previous works have shown that the MeJA-induced expression of TIAs biosynthesis genes were dependent on activation of pre-existing ORCA3 from the result of cycloheximide-mediated inhibition of *de novo* ORCA3 expression (Van der Fits and Memelink, 2001). However, inhibition of PGGT-I abolishes the MeJA-induced up-regulation of *DXR*, *HDS* and *G10H* that causes the decrease of TIAs biosynthesis.

Jointly, it also inhibits the MeJA-induced expression of the AP2/ERF transcription factor ORCA3 that acts as a central regulator of TIAs biosynthesis. Consequently, PGGT-I prenylated proteins are part of the early steps of jasmonate signaling leading to TIAs biosyn-thesis (Figure 2) (Courdavault et al., 2009). Meanwhile, some results suggested that PFT would be involved in the abscissic acid (ABA) signaling pathway (Allen et al., 2002). There are still ambiguities about the detailed molecular mechanism that controls the flux of metabolites and the precise relationship between the mevalonate and MEP

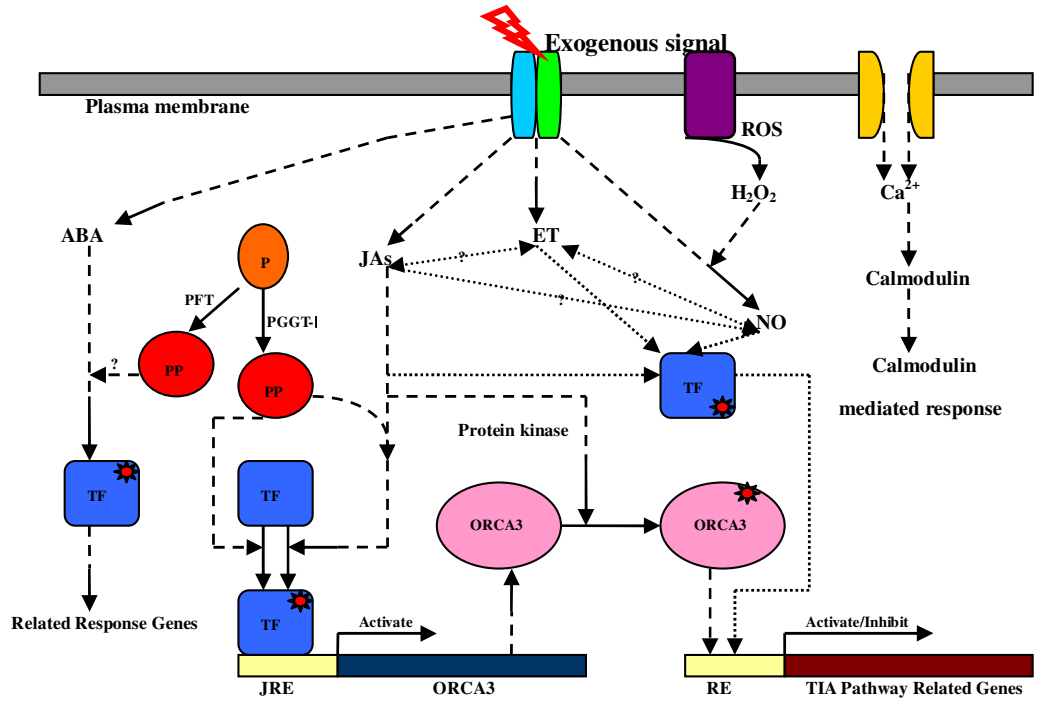

Figure 2. Overview of plant signal molecules (JAs, ET, NO and ABA) and prenylated proteins (PP) that can regulate TIAs biosynthesis pathway in *C. roseus*. JRE: JA-responsive element; TF: transcription factor; Red star represents phosphorylation.

pathways. Other key transcription factors and post-modification models should be involved in the regulation of ESMB genes in TIAs biosynthesis in *C. roseus*.

TRANSCRIPTION REGULATION

Plants produce secondary metabolites in response to various external signals. Coordinated transcriptional control of biosynthetic genes emerges as a major mechanism dictating the accumulation of secondary metabolites in plant cells. The establishment of a causal relationship between the binding of a transcription factor to a specific element and regulation of target gene expression *in vivo* is a prerequisite to generate the conclusive data about regulation of gene expression. Over the last few years, much effort has been made in relation to some transcription factors on how to regulate TIAs biosynthesis pathway in *C. roseus*.

Ocatdecanoid-responsive *Catharanthus* AP2/ERF domain (ORCAs), a member of the AP2/ERF transcription factor family have been shown to regulate the MeJA-responsive activation of several TIA biosynthetic genes in *C. roseus*. The gene for ORCA3 was isolated by transferred DNA activation tagging. ORCA3 overexpression resulted in an enhanced expression of several metabolite biosynthetic genes (*TDC, STR, SGD, D4H* and *CPR*) and, consequently, in an increased accumulation of TIAs (Van der Fits and Memelink, 2000). ORCA2 and ORCA1 were identified through yeast one-hybrid screening using JA-responsive regions containing a GCC-box-like element of *CrSTR* as a bait. ORCA2 trans-activates the *STR* promoter and its expression is rapidly inducible with JA and elicitor, whereas ORCA1 is expressed constitutively. The results indicate that a GCC-box-like element and ORCA2 play key roles in JA- and elicitor-responsive expression of the TIA biosynthetic gene *STR* (Menke et al., 1999). A C-repeat binding factor from *C. roseus* (*CrCBF*), which is another member of AP2 family transcription factor, would involve a low temperature response and TIA pathway (Dutta et al., 2007).

ORCAs and a MYB-like protein CrBPF-1, bind to two different positions on the *Str* promoter to modulate the expression of the *STR* gene. The binding of ORCAs, whose production is induced by jasmonate, is critical for the expression of *STR* gene, whereas the binding of MYB-like protein CrBPF-1, whose production is induced by Ca^{2+} influx, is not critical. CrBPF-1 is postulated to enhance the expression of *STR* gene when ORCA is already bound to the promoter (Van der Fits et al., 2000; Memelink et al., 2001**)**. The induction of *STR* expression by JA is sensitive to protein kinase inhibitor (Menke et al., 1999), which is compatible with the possibility that ORCA3 is phosphorylated. However, protein-protein interaction may be one of the mechanisms in the process

of ORCA3 modification. A *cDNA* encoding bHLH transcription factor (CrMYC1) was isolated by the yeast one-hybrid system from a *C. roseus cDNA* library using the G-box (CACGTG) element of the *STR* gene promoter as bait. In *C. roseus* suspension cells, *CrMYC1* mRNA concentrations are induced by fungal elicitors and jasmonate suggesting that CrMYC1 may be involved in the regulation of gene expression in response to these signals (Chatel et al., 2003). Interestingly, the *TDC* gene promoter sequence also possesses a G-box-like sequence (AACGTG) at -98 to -93 (Ouwerkerk and Memelink, 1999).

Therefore, G-box (G-box-like) promoter sequences might be involved in the co-ordinate control of *STR* and *TDC* gene expression patterns. It was astonishing that three members of the Cys2/His2-type (transcription factor IIIA-type) zinc finger protein family from *C. roseus*, ZCT1, ZCT2 and ZCT3 were identified using a yeast one-hybrid technique after the induction by yeast extract and methyl jasmonate. These proteins repress the activity of the promoters of *TDC* and *STR*. In addition, the ZCT proteins can repress the activating activity of AP2/ERF domain. These results suggest that the ZCT proteins act as repressors in the regulation of elicitor-induced secondary metabolism in *C. roseus* (Pauw et al., 2004). 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 by direct interaction with the promoter of *STR* in *C. roseus* cell cultures (Sibéril et al., 2001).

COMPARTMENTALIZATION AND TRANSPORTATION

Up to date, none significant amounts of final dimeric monoterpenoid indole alkaloids products such as vinblastine or vincristine were produced in undifferentiated or partially differentiated *in vitro* cultures. The main products that accumulate are usually monomeric monoterpenoid indole alkaloids such as ajmalicine and serpentine. Part of the explanation for the discrepancies of dimeric monoterpenoid indole alkaloids between the whole plant and the *in vitro* plant models relies on the fact that very accurate spatial and temporal regulation processes occur during the TIAs biosynthetic pathway (Table 1). Understanding the details of the regulation of TIA accumulation is a key aspect for achieving improved yields in *C. roseus*.

Much progress about compart-mentalization and transportation of TIAs in *C. roseus* have been made through *in situ* hybridisation and immunocytochemical localisation studies in the last few years (Mahroug et al., 2007). The uses of *in situ* RNA hybridization and immunolocalization have revealed that TIA biosynthesis is a highly dynamic, complex and compartmentalized process in *C. roseus* (Kutchan, 2005). The enzymes are localized in different cell types in the leaves and in root tips, as well as in different cellular compartments into the cells (Murata and De Luca, 2005). The compart-mentalizetion and localization involved in this metabolic pathway can be considered as a regulatory mechanism, since this location requires the transport of different metabolites from one point to another for its trans-formation. Recently, it has been suggested that different types of pumps are involved in the trafficking of natural products biosynthetic intermediates (Yazaki, 2006).

In situ RNA hybridization and immunocytochemistry were used to establish the cellular distribution of monoterpenoid indole alkaloid biosynthesis in *C. roseus*. *TDC* and *STR1* mRNAs were present in the epidermis of stems, leaves and flower buds, and in the protoderm and cortical cells around the apical meristem of root tips (Pasquali et al., 1992; Collu et al., 2001). In contrast, *D4H* and *DAT* mRNAs were present in the laticifer and idioblast cells of leaves, stems and flower buds. The immunocytochemical localization of TDC, D4H (desacetoxyvindoline-4-hydroxylase) and DAT (deacetylvin-doline 4-O-acetyltransferase) proteins confirmed the differential localization of the early and late stages of vindoline biosynthesis, indicating that at least two cell types are involved and that there is a need for the translocation of a pathway intermediate (St-Pierre et al., 1999).

In the roots, the enzymatic activity of TDC is located in the cytosol and in the apoplastic region of the roots' meristematic cells with a slight enrichment in the epidermal cells of the root cap and in the meristematic region. In the enlargement zone, TDC was localized only in the first three layers of the cortex. In the maturation zone, the enzyme was not present (Moreno-Valenzuela et al., 2003). Three MEP pathway genes, *DXS*, *DXR* and *MECS*, as well as the *G10H* gene display identical cell-specific expression patterns using northern blot and *in situ* hybridization.

These four transcripts were restricted to the internal phloem parenchyma of young aerial organs of *C. roseus* (Burlat et al., 2004). The expression of SLS (CYP72A1) (secologanin synthase) has been localized in the epidermis of leaves in *C. roseus* (Irmler et al., 2000). Tabersonine gives rise to end products that are in different tissues in the plant. In this case, the mRNA for the minovincinine 19-O-acetyltransferase is only expressed in the cortical cells of the radical meristems (Rodriguez et al., 2003). Analysis of *G10H* promoter demonstrated that fusion GUS expression was tissue-specific, restricted to the leaf and actively growing cells around the root tip, and are not detected in the hypocotyls, root cap and older developing areas of the root in transgenic tobacco seedlings (Suttipanta et al., 2007).

We may conclude that the translocation of pathway intermediates from the internal phloem parenchyma to the epidermis and, ultimately, to laticifers and idioblasts during TIAs biosynthesis is required. Similarly, the trans-location of intermediates from the phloem parenchyma is

Table 1. Organ-specific, cell-specific and sub-cell-specific distribution of TIA-related mRNAs, proteins and enzymatic activities in *C. roseus*.

Pathway branch enzymatic abbreviation	GeneBank access number	Organ-specific distribution			Cell-specific localisation		Sub-cell-specific localisation	Reference
Indoles		**mRNA**	**Protein**	**Enzyme**	**mRNA**	**Protein**		
TDC	X67662	ESd, R,L, YL ,CC			E, RT	E	Cy	(Pasquali et al., 1992; St-Pierre et al., 1999)
Methyl erythritol phosphate								
DXS	AJ011840	R,YL, FB, S,CC			PP		PI	(Courdavault et al., 2005)
DXR	AF250235	YL,CC FB, S, R			PP		PI	(Veau et al., 2000; Burlat et al., 2004)
MECS	AF250236	YL,CC FB, S, R			PP		PI	(Veau et al., 2000; Burlat et al., 2004)
HDS	AY184810				PP		PI	(Oudin et al., 2008)
Monoterpene-secoiridoids								
G10H	AJ251269	YL,CC FB, S, R		CC	PP		ER	(Burlat et al., 2004; Guirimand et al., 2009)
CPR	X69791	CC,F, R,S,L					V	(Collu et al., 2001)
SLS	L10081				E,P	E	V	(Irmler et al., 2000)
Monoterpene indole alkaloids								
STR	X53602	R,L,F, S,CC	R,L, CC		E,RT	E	Cy	(Pasquali et al., 1992; Collu et al., 2001)
SGD	AF112888	R,L,F, S,CC		L,R, S,F			ER	(Collu et al., 2001; Geerlings et al., 2000)
MAT	AF253415	HRT, H Esd, R		R	HRT			(Laflamme et al., 2001)
T16H	AJ238612	CC		L,C C			ER	(St-Pierre et al., 1999; St-Pierre and De Luca, 1995)

Table 1. Contd.

D4H	AF008597	L,Sd,C EC,H,Fr,S EH,Ra,Era	C,EC	Sd,Fr S,EC L	L+I	L+I		(St-Pierre et al., 1999)
DAT	AF053307	YL,Sd F,L,P,S	YL,Sd F,L,S	YL,Sd F,L,S	L+I	L+I	Cy	(St-Pierre et al., 1999)
PRX1	AM236087	L	L	L			V	(Costa et al., 2008)

E, epidermis of aerial organs; PP, internal phloem parenchyma of aerial organs; L + I, laticifers and idioblasts of aerial organs; P, palisadic parenchyma; HRT, hairy root tips; HR, root tips; C, cotyledons; CC, cell culture; EC, etiolated cotyledons; EH, etiolated hypocotyls; Era, etiolated radicles; ESd, etiolated seedlings; F, flowers; FB, flower buds; Fr, fruits; H, hypocotyls; HRT, hairy root tips; L, leaves; P, petals; R, roots; Ra, radicles; S, stems; Sd, seedlings; YL, young leaves; Cy, cytoplasm; PI, plastid; ER, endoplasmic reticulum; V, vacuole.

probably also required during the biosynthesis of hormones and photosynthetic primary metabolites derived from the MEP pathway.

The spatial control of TIA biosynthesis is also observed at the subcellular level in recent years. The compartmentation of different portions of TIAs pathways of *C. roseus* involves chloroplast, cytosol, vacuoles and endosplasmic reticulum (ER). Relatively, poor performance of bisindoles contents is partly due to the lack of appropriately differentiated subcellular compartments in hairy root culture or cell culture. An understanding of the subcellular compartmentation of TIA pathways will reveal whether various enzyme characteristics observed *in vitro*, such as their inhibition by pathway intermediates, represent a true regulatory function *in vivo*. Enzymes of the MEP pathway involved in IPP biosynthesis are located in the chloroplast or plastid (Lange et al., 2000), while enzymes involved in vindoline biosynthesis of *C. roseus* provide rich subcellular compartmentation of monoterpenoid indole alkaloid pathway. The conversion of tryptophan to tryptamine by TDC occurs in the cytosol (Stevens et al., 1993), whereas G10H is associated with vacuolar membranes (Madyastha, 1977).

However, the latest result showed that the subcellular localization of G10H is at ER level (Guirimand et al., 2009). Since STR is localized in the vacuole, tryptamine must be transported across the tonoplast before coupling to secologanin can occur (McKnight et al., 1991). *In vivo* localization studies showed that SGD is associated with the endoplasmic reticulum (Geerlings et al., 2000). T16H (tabersonine 16-hydroxylase) was also shown to be associated with ER (St-Pierre and De Luca, 1995), while NMT (2,3-dihydro-3-hydroxy-tabersonine- N-methyltransferase) was associated with thylakoid membranes. Conversely, D4H, DL7H (7-Deoxyloganln 7-hydroxylase) and DAT (deacetylvindoline 4-O-acetyltransferase) were localized in the cytosol (De Carolis et al., 1990). Peroxidase enzyme, which is involved in the coupling of vindoline with catharanthine to form the bisindole alkaloid anhydrovinblastine, was in the vacuole (Costa et al., 2008).

Northern-blot analyses showed that MAT (minovincinine-19-O-acetyltransferase) is expressed in the cortical cells of the root tip, whereas DAT is only expressed in specialized idioblast and laticifer cells within light exposed tissues like leaves and stems (Laflamme et al., 2001). Tabersonine 6,7-epoxidase activity converts tabersonine to lochnericine by selective epoxidation at positions 6 and 7 via a reaction dependent of NADPH and molecular oxygen. This activity was found in microsomes of the transformed hairy root cultures of *C. roseus* (Rodriguez et al., 2003). The reaction catalyzed by the enzyme AVLBS (anhydrovinblastine synthase) is carried out in the vacuole (Sottomayor et al., 1996). Consequently, there is a need for significant transport of pathway intermediates among different compartmentsin *C. roseus*.

As different cellular compartments and even different cells may be involved in certain steps of a biosynthetic pathway, the regulation of the flux is not only dependent on structural genes encoding enzymes catabolizing certain steps, but also transport has a major regulatory function. This should lead to further insight in the possible role of various transporters in the regulation of the biosynthesis of these alkaloids (Roytrakul et al., 2007). Up to date, there is relatively little information about transport mechanism of TIAs in *C. roseus*. A multidrug-resistance protein-type ABC (ATP-binding cassette) transporter has recently been demonstrated in the transport of isoquinoline alkaloid berberine at the plasma membrane of *Coptis japonica* (Goossens et al., 2003). *CjMDR1*, an ABC transporter gene originally isolated from *C. japonica*, was expressed in *C. roseus* cell cultures, which displayed that the endogenous

alkaloids ajmalicine and tetrahydroalstonine were accumulated significantly more in *C. roseus* cells expressing CjMDR1 in comparison with control lines after feeding these alkaloids (Barbora et al., 2009). Meanwhile, it was found that CrMDR1 is a MDR-like ABC transporter protein that may be involved in the transport and accumulation of TIAs in *C. roseus* (Jin et al., 2007). However, the latest studies suggested that berberine was transported across the tonoplast via an H^+/berberine antiporter which is a proton gradient formed by V-ATPase and V-PPase (Otani et al., 2005). In addition to the H^+-antiporter, ABC transporters have been suggested to be involved in the vacuolar transport of several TIAs in *C. roseus*, including strictosidine, ajmalicine, catharanthine and vindoline (Roytrakul and Verpoorte, 2007). The characterization of the transport systems of TIA pathway will provide a new angle to solve the neck of TIAs metabolic engineering in *C. roseus*.

CONCLUDING REMARKS

In this review, we have summarized how some kinds of small molecules regulate TIA biosynthesis pathway based on the current progress made in *C. roseus*. Although extensive efforts have been conducted in the latest few years, there would not be a great stride towards the industrialization of improving TIAs contents in *C. roseus* cultures. Part of the explanation is that the DNA, RNA and protein levels of the regulation mechanism are still unclear in TIAs pathway in *C. roseus*. The complexity of genetic and catalytic processes and those of transport of the TIA biosynthesis is one of the most stimulating intellectual challenges in the medicinal plant, *C. roseus*. How to move ahead? Based on previous reviews and research results, some perspectives on the molecular mechanism of TIAs pathway are provided to promote the elucidation of these intricate networks and fine-tuned regulation in *C. roseus*, and in particular with regard to the following:

(a) Phytohormones are involved in many aspects of plant growth and development, including TIAs biosynthesis pathway in *C. roseus*. Still, there is ambiguity on how to regulate the molecular mechanism of phytohormones in TIA pathway. Forward and reverse genetic strategies should be made to identify important related molecular components in plant hormone perception, signaling and transport involved in TIA pathway in *C. roseus*. For example, auxin is probably the most investigated plant hormone. It was demonstrated that auxin down-regulated the transcription level of the STR gene in *C. roseus* cell cultures (Goddijn et al., 1992). Auxin is perceived by auxin receptors, represented by members of the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) family, which results in the proteolysis of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins, thereby releasing their inhibitory effect on AUXIN RESPONSE FACTORS (ARFs) and transcription factors that regulate auxin responsive gene expression (Quint and Gray, 2006; Benjamins and Scheres, 2008). This signaling pathway would help us to explore the relationship between plant hormone and TIAs. Meanwhile, we could improve the contents of the endogenous plant hormone by trans-formed related key genes in *C. roseus* culture systems. More detailed picture should be drawn about the relationship between phytohormone and TIAs pathway in *C. roseus*.

(b) It is now widely accepted that calcium is a ubiquitous secondary messenger in animal and plant cell responses to various extracellular stimuli. The specificity of calcium signalling is controlled by changes in cytosolic free calcium concentration as a function of specific spatial and temporal calcium signature characteristics (Ng and McAinsh, 2003). Calcium signalling components from plant and animal systems are highly similar. In the case of *C. roseus* cells, previous works have suggested the involvement of calcium in the regulation of monoterpenoid alkaloid accumulation (Lee-Parsons and Ertürk, 2005).

It was demonstrated that auxin-dependent monoterpenoid indole alkaloids biosynthesis is differentially regulated by two distinct calcium release components from internal stores in *C. roseus* showing pharmacological profiles similar to those displayed by animal RyR and IP3 channels. Monoterpenoid indole alkaloids biosynthesis is stimulated by caffeine (Ca^{2+}-release activator through RyR channels) and by heparin and TMB8 (Ca^{2+}-release inhibitors of IP3 channels), whereas monoterpenoid indole alkaloids biosynthesis is inhibited by mastoparan (Ca^{2+}-release activator of IP3 channels) and by ruthenium red and DHBP [Ca^{2+}-release inhibitors of RyR channels: 2, 5-Dimethyl-2, 5-di (tert-butylperoxy) hexane] (Poutrain et al., 2009). Using the model plant, *Medicago truncatula*, Arimura and his co-worker compared the ethylene-insensitive mutant *sickle* (*skl*) with wild-type plants and found that the ethylene signaling cascade modulates the intracellular Ca^{2+}-level and interacts with JA signaling to generate a specific blend of terpenoids in response to the feeding beet armyworm larvae (Gen-ichiro et al., 2008). Consequently, noval strategies would be provided to identify the calcium signaling components that are involved in TIAs pathway in *C. roseus*.

(c) There would be important impacts of recent advances in genomics and postgenomics over the production of plant secondary metabolites. The use of some new related techniques such as reverse-genetics approaches, gene-to-metabolite networks, metabolomics analysis, expressed sequence tags, and transcriptome and proteome analysis, would deepen our understanding of the interrelationships between biosynthetic pathways and developmental regulation (Oksman-Caldentey and Inzé, 2004). The method of metabolomic analysis from the analytical methods back to the preanalytical phase and the biological experiment will be used. For example, the applications of NMR-based metabolomics would be used

to study the plant secondary metabolism (Verpoorte et al., 2008). Collaboration with bioinformatics will be crucial to better plan experiments and to extract all possible information from the huge amount of data that is produced by the analysis of plant metabolomes (Verpoorte et al., 2008). Prenylated proteins are a large class of molecules involved in almost all signal transduction pathways; however, only little information is acquired in the recent years. The concrete function of prenylated proteins is still unclear; therefore, further studies should be made in the future. Other global key transcription factors could not be got, partly due to the limitation of related technologies; perhaps, new creative methods could resolve this problem.

(d) *C. roseus* produces a variety of TIAs to protect themselves from pathogens and herbivores and/or to influence the growth of neighbouring plants. Some of these metabolites are toxic to the producing cells when their target sites are present in the producing organisms. Compounds, such as vinblastine, vincristine and 3', 4'-anhydrovinblastine, can bind to tubulin and prevent the formation of the microtubules which lead to an accumulation of cells in the M and G2 phase and the effect is lethal in the S phase (Jordan et al., 1991). Therefore, a specific self-resistance mechanism must exist in *C. roseus*, including extracellular excretion, vacuolar sequestration, vesicle transport, extracellular biosynthesis and accumulation of the metabolite in a non-toxic form (Sirikantaramas et al., 2008). For example, sanguinarine, which is a toxic benzophenanthridine alkaloid, exhibits cytotoxic activity by interfering with the functions of DNA, tubulin and enzymes such as Na^+/K^+ ATPase (Scheiner-Bobis, 2001). The transport of vacuolar-accumulated sanguinarine has been reported to be associated with the ER via a vesicle and/or recycling mechanism (Alcantara et al., 2005; Weiss et al., 2006). However, a new angle would be provided about investigating and clarifying the entire molecular mechanism of the translocation of TIAs in the medicinal plant, *C. roseus*.

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