METABOLIC ENGINEERING AND BIOSYNTHESIS OF INDOLE ALKALOID IN *Catharantus roseus* (L) G.DON

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ABSTRACT

Terpenoid indole alkaloids (TIAs) is the most important alkaloid for pharmaceutical interest. *Catharanthus roseus* (L.) G. Don (*Apocynaceae*) is the commercial source of three such alkaloids, which have pharmaceutical applications and intensively inversigated. Ajmalicine is used to improve cerebral circulation, and the dimeric alkaloids vinblastine and vincristine are important anticancer drugs. The biosynthesis of these alkaloids has been described quite extensively. Elicitor-induced defense responses include the biosynthesis of secondary metabolites accumulation, gene expression, have been studying for their effect on alkaloid biosynthesis. The effects of environmental such as light and abiotik stress on alkaloid accumulation have also been studied.

INTRODUCTION

Any biochemical pathway can be subjected to metabolic engineering. In practice efforts to modify the biosynthesis of a variety of plant primary and secondary metabolites have been confounded by the very metabolic flexibility that researchers are trying to exploit. Although these studies may uncover novel information about a pathway and its regulation that will inform subsequent attempts to manipulate it, such difficulties must be overcome if a genetically engineered .plant is to become commercially viable fact, regulatory genes are becoming valuable tools for metabolic engineering.

There are four major variables that appear to affect the outcome of metabolic engineering experiments. First, it is not always possible to predict the effects that directed perturbations (i.e., changing the level or activity of a single biosynthetic enzyme) may have on the entire pathway—unidentified endogenous feedback and/or feedforward controls may constrain metabolic pathways in manners that are poorly defined. Second, manipulating primary metabolic pathways can have pleiotropic (and often detrimental) effects on plant growth and development. Third, no matter how well a transgenic plant is characterized in an experimental setting, crop plants in the field will be subjected to environmental perturbations that may adversely affect accumulation of the engineered product. Fourth, because many applications of genetic engineering demand that the engineered products get to the right place in the plant at the right time, it is critical to ensure that these products are appropriately targeted within and/or between cells in the transgenic plant. Despite the fact that they are dispensable, many secondary metabolites play important roles in plant biology. For example, specific secondary metabolites have been reported to function in defending plants from pathogens, as insecticidal compounds, and as UV protectants[1,2]. Secondary metabolites are also the source of many components in the pharmaceutical arsenal against human pathogens and diseases[3]. Others are food colorings, and still others contribute to the scent and color of cut flowers.

For these reasons, secondary metabolic pathways have been subjected to intense genetic and biochemical investigation over 20 years. In fact, the biosynthetic pathways of many plant secondary metabolites are generally well characterized and, in some cases, a number of genes that could be used as targets for metabolic engineering strategies have been cloned in catharantus roseus. Using cultured plant cells as metabolic factories instead of whole plants can circumvent problems associated with inappropriate targeting and with the vagaries in metabolite accumulation that can occur in field-grown material. Growing cell cultures in defined media may also bypass a number of other potential problems, such as tissue-specific or

seedlings. Light-dependent enhancement of nicotine biosynthesis was also observed in 6-week-old plants, in which a correlation between photoperiod length and nicotine accumulation was found. Phytochrome seems to be involved in this process, since a red-light pulse given at the end of the day promoted a further nicotine accumulation, whereas a similar far-red-light treatment reversed these effects .

Early studies have shown that the pattern of alkaloids extracted from *C. roseus* seedlings was greatly affected by development and light[12]. Etiolated seedlings contained high levels of the late vindoline precursor tabersonine, which upon illumination was transformed stoichiometrically into vindoline[13]. In contrast, catharanthine, which accumulated to high levels in etiolated seedlings, was hardly affected by the light regime[12]. These studies suggested that light is a major limiting factor in the conversion of tabersonine to vindoline and in the formation of dimeric indole alkaloids[13].

The transformation of tabersonine to vindoline involves six strictly ordered enzyme reactions. aromatic hydroxylation, O-methylation, hydration of the 2,3-double bond, N(1)-methylation, hydroxylation at position 4, and 4-O-acetylation[13]. The first of these reactions is catalyzed by tabersonine 16-hydroxylase, a Cyt P450-dependent monooxygenase associated with microsomal cell fractions, whereas the next reaction is catalyzed by a cytosolic S-adenosyl-L-Met, 16 hydroxytabersonine O-methyltransferase. The enzyme involved in the hydration of the double bond of the 16-methoxy compound has yet to be characterized, but the product from this hydroxylase is N-methylated by a thylakoid-associated S-adenosyl-L-Met, S-adenosyl-L-Met: 2,3-dihydro-3-hydroxytabersonine-N-methyltransferase, which forms desacetoxyvin-doline[14,15]. The second-to-the-last reaction involves the 4-hydroxylation of desacetoxyvindoline and is catalyzed by a cytosolic 2-oxoglutarate-dependent dioxygenase known as D4H[15,16]. Final O-acetylation of deacetylvindoline to yield vindoline is catalyzed by a cytosolic DAT[15,17]. In addition, these studies revealed that expression of tabersonine 16-hydroxylase, D4H, and DAT in developing *C. roseus* seedlings is light regulated. However, although D4H and DAT activities are detected exclusively under conditions resulting in vindoline biosynthesis, expression of tabersonine 16-hydroxylase occurs at low levels in *C. roseus* cell cultures that do not accumulate vindoline[18].

Some of the early steps leading to strictosidine, the key intermediate to all TIAs and quinoline alkaloids, have been studied in detail. Strictosidine is formed by the coupling of secologanin to tryptamine in a reaction catalyzed by the enzyme strictosidine synthase (EC 4.3.3.2). This enzyme is located in the vacuole. Tryptamine is formed from the amino acid tryptophan by the action of the cytosolic enzyme tryptophan decarboxylase (EC 4.1.1.28), and secologanin is a glucoiridoid formed from geraniol. The expression of the Str and Tdc genes is induced by the plant stress signaling hormone MeJA. Furthermore, MeJA increases alkaloid production in cell suspension cultures and seedlings of *C. roseus*, showing that TIA-producing plants respond to abiotik stress such as osmotik stress by PEG 10%, by increasing their alkaloid content, among others, through increased gene expression[8].

It is unknown how strictosidine is channeled into various pathways that lead to the different types of TIA skeletons. The first step after strictosidine formation is removal of the glucose moiety, a reaction that is catalyzed by the enzyme strictosidine D-glucosidase (SGD; EC 3.2.1.105). Removal of glucose results in a highly reactive dialdehyde. Depending on the conditions (e.g. solvent, pH), various products can be formed from this dialdehyde. Under mildly acidic incubation conditions, the major product of SGD from *C. roseus* is cathenamine. Better understanding of this step may lead to the development of strategies to channel the flux toward the desired type of alkaloid.

Apart from its role in the biosynthesis of TIAs, which have putative functions in plant defense, SGD may also have a more direct role in defense. In young tissue of *C. roseus*, where strictosidine is the major TIA, SGD in combination with strictosidine is thought to be part of a damage-inducible biochemical defense system. Strictosidine is stored in the vacuole, whereas SGD is thought to be outside the vacuole. Upon cell damage, SGD will rapidly convert strictosidine into the aglucon, which was shown to have antimicrobial activity. This proposed defense mechanism shows similarity to cyanogenesis occurring as a result of the

damage-induced interaction between cyanogenic glucosides and their specific glucosidases .

Vindoline biosynthesis in *Catharanthus roseus* also appears to be under this type of developmental control. In the leaves of *C. roseus*, vindoline is enzymatically coupled with catharanthine to produce the powerful cytotoxic dimeric alkaloids vinblastine and vincristine. Vindoline as well as the dimeric alkaloids are restricted to leaves and stems, whereas catharanthine is distributed equally throughout the aboveground and underground tissues. The developmental regulation of vindoline biosynthesis has been well documented in *C. roseus* seedlings, in which it is light inducible. This is in contrast to catharanthine, which also accumulates in etiolated. Furthermore, cell cultures that accumulate catharanthine but not vindoline. Recover this ability upon redifferentiation of shoots. These observations suggest that the biosynthesis of catharanthine and vindoline is differentially regulated and that vindoline biosynthesis is under more rigid tissue development, and environment-specific control than is that of catharanthine.

ELICITOR INDUCE SECONDARY METABOLITE

The initiation of a plant defense response requires the perception of pathogen-derived (exogenous) or plant-derived (endogenous) signal molecules, collectively referred to as elicitors. Elicitor-induced defense responses include the biosynthesis of secondary metabolites and proteinase inhibitors. Protein phosphorylation is an essential component of elicitor-induced signal transduction. The lipid-based octadecanoid pathway leading to JA has also been implicated as an integral part of the signal transduction pathway leading to the activation of defense responses. The octadecanoid pathway was first implicated in wounding-induced biosynthesis of proteinase inhibitors. Upon wounding, the octadecanoid pathway is activated by the polypeptide systemin and by oligouronides, resulting in elevated levels of JA and its octadecanoid precursors activate the synthesis of wounding-inducible proteinase inhibitors. Induction of the synthesis of proteinase inhibitors by wounding, systemin, and oligouronides is blocked by several inhibitors of the jasmonate biosynthetic pathway. Furthermore, identified a MeJA-responsive element in the promoter of a proteinase inhibitor II gene, indicating that this gene is transcriptionally regulated in response to MeJA.

How elicitors affect JA biosynthesis and how the JA signal is transduced to effect gene expression is largely unknown. Much more is known about the JA biosynthetic pathway itself. Have proposed that a lipase generates.-linolenic acid, the first precursor in the octadecanoid pathway. .Linolenic acid is then converted by a lipoxygenase, an allene oxide synthase, and an allene oxide cyclase into the intermediate 12-oxo-phytodienoic acid. This compound is converted into JA through the action of a reductase and three rounds of oxidation.

JA and its octadecanoid precursors have also been implicated as intermediate signals in elicitor-induced secondary metabolite accumulation. A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite accumulation was shown in cells of California poppy. These reports indicate that in elicitor-induced secondary metabolism JA plays a role that is similar to its role in the accumulation of wound-induced proteinase inhibitors, for which it has been elegantly demonstrated that jasmonates are intermediate signals that transcriptionally activate proteinase inhibitor genes. However, the studies that have been done on various metabolic pathways in different plant species using diverse elicitors lack the integrated approach of measuring jasmonate biosynthesis and studying the effect of JA and octadecanoid pathway inhibitors on gene expression. In general, metabolite accumulation has been studied instead of gene expression. Therefore, in most cases it remains unclear on what regulatory level jasmonates exert their effect on secondary metabolism. In *Catharanthus roseus* cell suspensions, the expression of two TIA biosynthetic genes, Tdc and Str, encoding Trp decarboxylase and strictosidine synthase, respectively, is coordinately induced by fungal elicitors such as YE[10]. The enzymes encoded by these genes are important in the TIA biosynthetic pathway. Both genes are present as single copies in the haploid C. roseus genome[10].

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LIGHT INDUCE ALKALOID IN SEEDLING

In addition to morphogenesis, developmental processes result in biochemical specialization of cells for the biosynthesis and/or accumulation of secondary metabolites, such as phenylpropanoids, monoterpenoids and alkaloids. Studies with germinating seedlings have suggested that alkaloid biosynthesis and accumulation are associated with seedling development[13]. Studies with mature plants also reveal this type of developmental control. Furthermore, alkaloid biosynthesis in cell suspension cultures appears to be coordinated with cytodifferentiation.

Developmental studies with etiolated seedlings confirmed that the D4H gene is expressed in the dark[5,19]. The appearance of D4H protein followed closely the levels of hydroxylase transcripts in etiolated seedlings but these produced only low-D4H enzyme activities throughout the time course. Treatment of etiolated seedlings with light did activate D4H enzyme activity, but this depended on the age at which seedlings were exposed .. Five-day-old seedlings appeared to be optimally primed to respond to light treatment, producing the highest D4H activities ., which also correlated with the most appropriate developmental stage for vindoline accumulation[13]. In contrast, younger seedlings did not respond well to light treatment, and older seedlings were only capable of a more limited response, producing maximal D4H activities directly related to their developmental stage of growth. The importance of seedling development in the light response was corroborated when 9- and 11-d-old etiolated seedlings were treated with light, and the D4H activities reached only those of later stages of development of continuously illuminated seedlings. The light treatment, therefore, appears to activate processes already triggered and controlled by seedling development.

The differential effects of light on the expression of D4H transcripts, protein, and enzyme activity at various stages of seedling development suggest that multiple levels of control may be involved in the regulation of D4H, show that even though D4H transcripts and protein appear in dark-grown seedlings, light is required for the appearance of significant hydroxylase activity. The modulation by light of these three parameters appears to vary with seedling development and decreases progressively with the age of etiolated seedlings. A possible explanation of these results may involve several levels of control in which light modulates development-related transcription, translation, and undetermined posttranslational modifications . that would activate or inactivate the enzyme.

The occurrence of posttranslational modifications in D4H protein has been suggested by previous studies involving the purification of this protein to homogeneity from C. roseus leaves[7] and by the fact that D4H exists as a single-copy gene. The purified protein could be resolved by IEF and SDS-PAGE into three 45-kD isoforms with pI values of 4.6, 4.7, and 4.8. The results presented in this paper suggest that the pI-4.7 isoform, which also occurs in dark-grown seedlings may be inactive, and that light treatment may convert this isoform into an active, more acidic isoform by an undetermined posttranslational modification . In this context, it is interesting to note that DAT, which is involved in the last step of vindoline biosynthesis, also appears to exist as isoforms with various specific activities.

The phytochrome receptor may be involved in the transcriptional, translational, and posttranslational regulation of D4H. Other light-regulated plant proteins displaying this behavior are usually enzymes involved in basic metabolic activities, such as nitrate reductase, the small subunit of Rubisco, and starch phosphorylase[18]. This report suggests that these mechanisms may regulate alkaloid biosynthesis for an undetermined but important reason. Developmental studies have shown that the complete pathway leading to catharanthine biosynthesis occurs in etiolated seedlings, whereas several of the terminal steps in vindoline biosynthesis appear only upon light stimulation.

Chemical inducers of vindoline biosynthesis such as methyl jasmonate appear to be effective only if light is applied and only within a specific developmental time frame, suggesting an intimate association between the light activation of vindoline biosynthesis and light-dependent developmental processes. In vitro experiments have shown that enzymatic coupling of vindoline and catharanthine can be carried out by rather nonspecific peroxidase preparations[5]. It is reasonable, therefore, to suggest that the combined presence of catharanthine and

vindoline in the cell would lead to the production of the antimitotic dimers vinblastine and vincristine. In this way, light activation of the terminal steps in vindoline biosynthesis may be coupled with essential and undetermined ontogenetic processes required to sequester cytotoxic vinblastine and vincristine dimers, which would otherwise kill the plant. Specialized laticifers and idioblasts have been shown to exist in *C. roseus* but their potential roles in alkaloid biosynthesis and accumulation remain to be shown. Strictosidine is the precursor for both the Iboga (catharanthine) and Aspidosperma (tabersonine and vindoline) types of alkaloids According to this biosynthetic scheme, further enzymatic reactions would transform tabersonine into vindoline, including hydroxylation at C-16, 16-O-methylation, hydration of the 2,3-double bond, N-methylation, hydroxylation at C-4, and 4-O-acetylation[13,14]. The first pair of reactions are catalyzed by tabersonine 16-hydroxylase and S-adenosyl-L-methionine: 16-hydroxytabersonine-O-methyltransferase, respectively[28]. The third to the last reaction is catalyzed by an S-adenosyl-L-methionine:2,3-dihydro-3-hydroxytabersonine-N-methyltransferase[7,20], whereas 4-hydroxylation is catalyzed by desacetoxyvindoline 4-hydroxylase (D4H), a 2-oxoglutarate-dependent dioxygenase[15,16]. The final reaction is catalyzed by acetyl coenzyme A (CoA):deacetylvindoline 4-O-acetyltransferase[14]. Some of these enzymes are not expressed in cell cultures or in tissues unable to produce vindoline[13].

CONCLUSION

Several inversigaters. represent a promising results in the metabolite engineering of indole alkaloids as secondary metabolite biosynthesis in *catharantus roseus* plant cell cultures. However, manipulating metabolic pathways and biosynthesis study in cultured cell lines to produce commercial anti-cancer indole alkaloid vincristine and vinblastine still in progress. The expense of production and maintenance, and whether it will becoming economically preferable to use cell cultures as biological factories remain to be continues evaluating in the future.

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