Genetic Modification of Plant Secondary Metabolite Pathways Using Transcriptional Regulators

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Plant secondary metabolism is the source of many natural products with diverse applications, including pharmaceuticals, food colors, dyes and fragrances. Functions in plants include attraction of pollinating insects and protection against pests and pathogens. An important regulatory step in secondary metabolism is transcription of the biosynthetic genes. The aim of this chapter is to discuss results and opportunities concerning modification of secondary metabolism using transcriptional regulators. The transcriptional regulation of two well-studied secondary pathways, the phenylpropanoid pathway and its flavonoid branch, and the terpenoid indole alkaloid biosynthetic pathway, are reviewed. Some examples of successful engineering of these pathways via transcriptional regulators are discussed.

Keywords. Anthocyanins, AP2-domain, Jasmonate, Terpenoid indole alkaloids, Transcription factor

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Abbreviations

AP2	APETALA2
AP2/ERF	APETALA2/ethylene-responsive factor
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
Ca	calcium
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
JA	jasmonic acid
JERE	jasmonate-and elicitor-responsive element
MADS	MCM1-AGAMOUS-DEFICIENS-SRF
MAPK	mitogen-activated protein kinase
MeJA	methyl jasmonate
4-mT	4-methyltryptophan
ODA	octadecanoid
ORCA	octadecanoid-responsive *Catharanthus* AP2-domain
STR	strictosidine synthase
TDC	tryptophan decarboxylase
T-DNA	transferred DNA
TIA	terpenoid indole alkaloid
UV	ultraviolet
VP1	VIVIPAROUS-1

1 Introduction

Prospects to modify secondary metabolite production in plants or plant cells via genetic engineering of a single or a few enzymatic steps have been reviewed previously [1–3]. The aim of this chapter is to discuss opportunities to modify secondary metabolism using transcriptional regulators. Transcriptional regulation is largely mediated by sequence-specific DNA-binding proteins that recognize *cis*-acting DNA elements located in the promoter and enhancer regions of the corresponding genes. In general, these *cis*-acting elements are concentrated in a relatively small region of a few hundred to a few thousand nucleotides upstream of the transcriptional start site. In most genes, this start site is determined by the presence of a TATA box around 30 bp upstream. On the TATA box the basal transcription machinery is assembled. Together with RNA polymerase II and a large number of other proteins, the preinitiation complex is formed. During initiation of transcription, the preinitiation complex shifts from the closed to the open configuration. Transcription factors regulate gene transcription in response to developmental, tissue-specific or environmental signals by binding with their so-called 'DNA-binding domains' to specific DNA sequences in the gene promoters. Transcription factors may modify the rate of transcription initiation by controlling the rate of recruitment of components of the preinititation complex. Alternatively, they can regulate the rate of transition of the preinitiation complex from the closed to the open configuration, or via other

mechanisms (reviewed in [4]). Transcription factors may affect these processes by direct interactions between their so-called 'activation-domains' and components of the basal transcription machinery, or by indirect interactions via adapter or co-activator proteins (reviewed in [5]).

Depending on the epigenetic make-up of the target cell, single transcription factors can switch on complex pathways involving numerous target genes. A notable example is muscle differentiation in animals, where either one of a set of myogenic bHLH transcription factors (MyoD, myogenin, Myf5, MRF4) in combination with the MADS-domain transcription factor MEF2 induces muscle cell differentiation and switches on numerous muscle-specific genes in skin fibroblasts and certain other cell types [6]. Other examples include single homeodomain transcription factors in the fruit fly, which regulate complex pathways resulting in the determination of segment identity [7, 8]. In *Arabidopsis thaliana*, overexpression of the transcription factor CBF-1 results in coordinate upregulation of a set of cold-regulated genes, and in increased freezing tolerance [9]. These examples illustrate that single transcription factors can act as master regulators of complex gene expression programs, and can control the expression of a large number of target genes in a coordinate fashion. A general characteristic of secondary metabolic pathways is that the expression of the structural genes is coordinately regulated depending on cell type or in response to environmental stimuli. This coordinate control is most likely due to master regulators, and one can envisage that secondary metabolism can be modified by engineering the activity of such master switches. The following sections will review some aspects of the transcriptional regulation of two well-studied secondary metabolic pathways, the phenylpropanoid pathway and its flavonoid branch, and the terpenoid indole alkaloid biosynthetic pathway. Some examples of successful engineering of these pathways via transcriptional master regulators will be discussed.

2 Regulation of the Phenylpropanoid and Flavonoid Pathways

The phenylpropanoid pathway is the source of a large number of compounds that are derived from the amino acid phenylalanine (Fig. 1). The flavonoid branch is responsible for the production of anthocyanin pigments, UV-absorbing flavones and flavonols, and antimicrobial phytoalexins. Other branches produce lignin precursors and soluble phenolics such as the signaling compound salicylic acid. A large number of structural genes have been cloned from many plant species [10]. The best-studied aspect of transcriptional regulation regards the formation of the anthocyanin pigments, due to the fact that they provide a convenient visible marker. Another well-studied aspect is the stress- and pathogen-induced biosynthesis of phytoalexins. Both classes of end products share a large part of the biosynthetic pathway. The structural flavonoid genes are subject to different regulatory mechanisms within a single plant depending on the cell type and environmental conditions.

Tissue-specific expression of the flavonoid structural genes is controlled by a combination of two distinct transcription factor species, one of which has

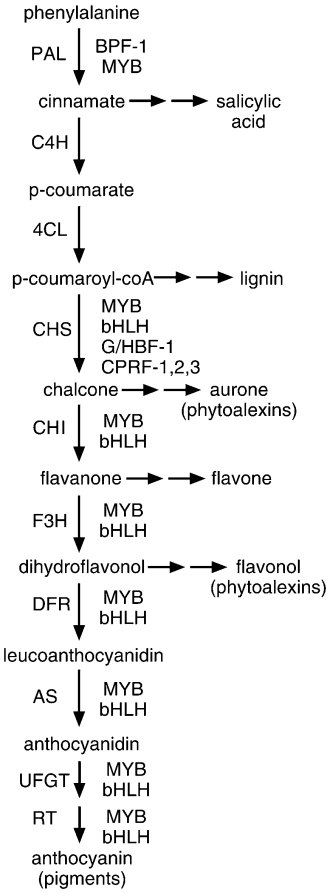

Fig. 1. Simplified diagram of the phenylpropanoid and flavonoid biosynthetic pathways. Enzymes that catalyze the reactions are placed on the left-hand side, and transcription factors on the right-hand side of the arrows. Both transcription factors for which their control over the enzymatic steps has been genetically proven, as well as transcription factors that have been shown to interact with promoters of the structural genes, are shown. *PAL* Phenylalanine ammonia lyase; *C4H* cinnamate 4-hydroxylase; *4CL* 4-coumaroyl-coenzyme A ligase; *CHS* chalcone synthase; *CHI* chalcone-flavanone isomerase; *F3H* flavanone 3β-hydroxylase; *DFR* dihydroflavonol 4-reductase; *AS* anthocyanin synthase; *UFGT* UDP glucose-flavonol glucosyl transferase; *RT* anthocyanin rhamnosyl transferase

homology to the protein encoded by the vertebrate proto-oncogene c-*Myb*, and the other to the vertebrate basic helix-loop-helix (bHLH) protein encoded by the proto-oncogene c-*Myc*. These transcription factors bind to specific sequences in the promoters of the target genes [11–13]. The DNA-binding domain of plant MYB proteins consists of two, or for some one or three, imperfect repeats [14, 15]. The DNA-binding specificity of plant MYB proteins varies considerably [14]. The bHLH proteins recognize variants of the sequence CANNTG. In particular, the G-box (CACGTG) has been reported to interact

with bHLH factors [16, 17]. The G-box is also a target for basic leucine zipper (bZIP) transcription factors [18]. Plant MYB and bHLH proteins can physically interact with each other [19]. In maize, the entire set of genes encoding enzymes in anthocyanin biosynthesis is thought to be regulated coordinately by the bHLH protein-encoding gene *R* and the *Myb* gene *C1* in the aleurone (epidermal layer of the kernel endosperm), and by homologous genes in other parts of the plant [10, 20] (Fig. 1). In dicotyledonous plants, anthocyanin control appears to be more complex. Although all the structural genes in the anthocyanin branch of the pathway are coordinately regulated during flower development, the earlier and later parts of the biosynthetic pathway are thought to be controlled independently [20]. Nevertheless, introduction of the maize *R* and *C1* regulators in *Arabidopsis* intensifies pigmentation in normally pigmented tissues and induces pigmentation in plant tissues that are normally unpigmented [21]. Overexpression of the maize *Lc* gene, which encodes a bHLH regulatory protein, in petunia upregulated the whole flavonoid biosynthetic pathway starting from *Chs* and including the earlier and later genes, resulting, among others, in intensely pigmented leaves [22].

The expression of the MYB and bHLH protein-encoding genes coincides with that of the structural genes that they regulate [20, 23]. The mere presence of a MYB and a bHLH protein in a cell appears to switch on the expression of the anthocyanin structural genes. Although the DNA-binding affinity of an *Antirrhinum* MYB protein was negatively affected by phosphorylation [12], evidence that the activity of MYB and bHLH proteins is regulated by cell-type-specific mechanisms involving protein modification in vivo is lacking. Additional regulators of anthocyanin biosynthesis have been scarcely identified. Notable examples are the petunia *AN11* gene [24] and the *TTG1* gene from *Arabidopsis* [25]. The expression of the *Dfr* anthocyanin structural gene in the petunia *an11* mutant can be rescued by expression of the MYB protein AN2, indicating that the AN11 protein acts upstream of MYB. Since the *An*2 mRNA level is normal in the *an11* mutant, it has been suggested that the AN11 protein regulates MYB protein activity [24]. In contrast to the *Myb* gene *An*2, *An*11 is ubiquitously expressed. The *Arabidopsis ttg1* mutant has many aberrant phenotypes, including lack of anthocyanin biosynthesis. The *ttg1* mutant phenotype can be rescued by expression of the maize *R* gene [21], indicating that the TTG1 protein regulates the activity of a bHLH-type protein. AN11 and TTG1 proteins are highly similar, and both contain four WD repeat motifs, which are also found in the β subunits of heterotrimeric G proteins and a number of other regulatory proteins. Given their high similarity, it seems unlikely that AN11 and TTG1 have different functions, and they probably regulate the activity of MYB-bHLH complexes. Another example of an additional regulator of anthocyanin biosynthesis is the VP1 protein. The maize *viviparous1* mutant does not produce anthocyanin in seeds, while other plant parts are normally pigmented. The *vp1* mutation also affects seed maturation, resulting in shoots and roots that emerge from the seeds while the kernel is still attached to the ear. Certain *vp1* alleles prevent anthocyanin biosynthesis but produce normal, nonviviparous seed, suggesting that control of the anthocyanin pathway is at least partially separable from regulation of embryo maturation. The *vp1-R* mutant

fails to express the *C1* gene in developing seed tissues [26], and the VP1 protein can transactivate a reporter gene driven by the *C1* promoter [27] by specifically binding to the Sph sequence [28]. Therefore, VP1 is a transcription factor that acts upstream of the MYB protein *C1* by regulating its expression via direct binding to the promoter.

The fact that so few mutants affected in regulation of MYB and bHLH activity have been found in plants is surprising in view of the multitude of protein modifications and protein–protein interactions reported for their mammalian counterparts. Mammalian MYC is active only when complexed with the bHLH protein MAX [29]. The potential of MYC to activate transcription is suppressed by association with p107, an Rb-related tumor suppressor [30]. Phosphorylation stimulates transactivation by MYC [31]. Phosphorylation [31, 32] or reversible oxidation [33] of MYB reduces its DNA-binding affinity. The interaction of MYB with the general co-activator CREB binding protein (CBP) is required for transactivation [34, 35]. It can be expected that similar interactions and modifications play a role in the regulation of the activity of plant bHLH and MYC proteins. The fact that no mutants have yet been found in these regulatory processes may be due to the fact that the genes involved are redundant or absolutely essential.

Many phenylpropanoid compounds that are constitutively present in certain cell types and/or during development can be produced in other tissues in response to various stress-related signals [36]. Bean *Pal* and *Chs* genes are rapidly and coordinately induced by elicitors [36]. UV light induces the transcription of the parsley *Chs* gene [37]. In the promoters of phenylpropanoid biosynthetic genes from various plant species common sequence motifs can be identified. The H-box [(T/A)CT(C/A)ACCTA(C/A)C(C/A)] and box P [CCA(C/A)C(A/T)AAC(C/T)CC] are present in the promoters of *Chs*, *Pal* and *4-cl* genes in different plant species, and have been associated with regulation of gene activation by stress stimuli such as UV light or elicitors [37–40]. In the *Chs* gene from parsley [37, 41] and *Arabidopsis* [42], a light-responsive unit (LRU) has been identified, which is necessary and sufficient to confer gene expression upon UV-B and UV-A/blue light illumination. The LRU is composed of a H-box, also called a MYB recognition element (MRE) and an ACGT element. In the parsley *Chs* gene the ACGT element forms part of a G-box (CACGTG), which is a conserved element in plant promoters [18]. The ACGT element has been found to interact with the parsley bZIP class proteins CPRF-1, 2 and 3 [41]. CPRF-1 transcripts are induced by UV light with much faster kinetics than *Chs* mRNA, suggesting that CPRF-1 is responsible for UV-responsive expression of the *Chs* gene. The *Arabidopsis* bZIP protein HY5 also interacts with the G-box in the LRU [43]. The H- (or MRE) box has been found to interact with DNA-binding proteins of the MYB class [44, 45]. The parsley MYB protein *Pc*MYB1 transactivates expression of an MRE-containing reporter construct [45]. In contrast to *Chs* (and the *CPRF-1* gene), *PcMyb*1 expression is not light-regulated. In addition, the H- (or MRE) and G-boxes in the bean *Chs* gene were shown to interact with a bZIP class protein with relaxed DNA-binding specificity called G/HBF-1 [46]. While G/HBF-1 transcript and protein levels do not increase during the induction of phenylpropanoid biosynthetic genes, the DNA-binding affinity of the G/HBF-1

protein is enhanced by elicitor-induced phosphorylation, suggesting that G/HBF-1 is responsible for elicitor-responsive expression of the bean *Chs* gene. However, there are no in vivo expression studies that prove that the H- and G-boxes are involved in elicitor-responsive expression of the bean *Chs* gene. The P-box in the parsley *Pal* gene was shown to interact with BPF-1, a protein with some similarity to MYB transcription factors [47]. Expression of the gene encoding BPF-1 is induced by fungal elicitor with slightly faster kinetics than those of *Pal* expression. More recent studies have shown that box P is a relatively low-affinity binding site for BPF-1 [48], indicating that the in vivo relevance of the box P/BPF-1 interaction is questionable.

The functional identification of these transcription factors is based on more or less specific recognition of stress-responsive elements in the promoters of the structural genes, and sometimes their stress-induced expression. Definitive proof of their function awaits (reverse) genetic evidence. Although the conservation of the binding sites in different structural genes suggests that some of these stress-related transcription factors coordinate the expression of several genes, experimental evidence is lacking. It is not known how stress-induced expression of phenylpropanoid biosynthetic genes ties in with their tissue-specific control by MYB and bHLH proteins. Since a MYB and a bHLH protein can induce pigmentation in otherwise unpigmented tissues without stress signals, it appears that they overrule stress-signaling pathways. On the other hand, the structural genes can be induced by stress signals in tissues that appear to lack the appropriate tissue-specific MYB and/or bHLH proteins. Whether there is crosstalk between tissue-specific MYB/bHLH regulation and stress-induced signaling remains an open question.

3 Modification of Flavonoid Metabolism Using Transcription Factors

Myb and bHLH protein-encoding genes have been ectopically expressed in the plant species from which they originated, as well as in heterologous plant species. All studies described here used the cauliflower mosaic virus (CaMV) 35S RNA promoter, which is highly active in most tissues of most plant species, to express the transcription factors.

Introduction of the *Delila* (*del*) gene from *Antirrhinum*, encoding a bHLH transcription factor, in tomato strongly increased pigmentation in vegetative tissues [49]. In tobacco, *del* only intensified pigmentation of flowers, whereas in *Arabidopsis*, *del* had no phenotypic effects. This indicates that DELILA is active in some, but not all, heterologous plant species, and recognizes its orthologous target genes. Overexpression of the maize *Lc* gene encoding a bHLH regulatory protein in petunia upregulated the whole flavonoid biosynthetic pathway starting from *Chs* and including the earlier and later genes, resulting among others in intensely pigmented leaves [22]. The expression of the general phenylpropanoid genes *Pal* and *C4h* was not affected by *Lc* overexpression, indicating that *Lc* only regulates structural genes in the flavonoid branch. Ectopic expression of the bHLH transcription factors DELILA and *Lc* led to increased pigmentation only in tissues that are normally pigmented, or that can become pigmented

under stress conditions. The ectopically expressed bHLH proteins probably rely on endogenous MYB proteins to activate their target genes.

Introduction of the maize *R* and *C1* regulators in *Arabidopsis* intensified pigmentation in normally pigmented tissues and induced pigmentation in plant tissues that are normally unpigmented [21]. In maize cell suspension, ectopic expression of *C1* and *R* led to the accumulation of anthocyanins and a number of other related 3-hydroxyflavonoids [50]. In addition, six anthocyanin structural genes that are targets for *C1*/*R* were expressed at high levels in the transgenic cell line.

These experiments demonstrate that the ectopic expression of transcription factors is a viable method for engineering secondary metabolism in plants and plant cells.

4 Jasmonates as Stress Signals in Secondary Metabolism

Jasmonic acid (JA) and its volatile derivative methyl jasmonate (MeJA), collectively called jasmonates, play key regulatory roles in stress-induced secondary metabolism in plants.

Jasmonates are fatty acid derivatives with a 12-carbon backbone, which are synthesized from 18-carbon intermediates via the so-called octadecanoid (ODA) pathway (Fig. 2; [51, 52]). Farmer and Ryan [53] have proposed that a lipase generates α-linolenic acid, which is the first precursor in the ODA pathway. α-Linolenic acid is then converted by a lipoxygenase, an allene oxide synthase and an allene oxide cyclase into the intermediate 12-oxophytodienoic acid (12-oxo-PDA). This compound is converted into JA through the action of a reductase and three rounds of β-oxidation. (Me)JA and some of its octadecanoid precursors play key roles as intermediate signals in elicitor-induced secondary metabolite accumulation [54–57]. A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite accumulation was shown in *Eschscholtzia californica* cells [55] and in rice cells [57]. In parsley cells, phenylpropanoid biosynthetic genes are induced by octadecanoids, and elicitor-induced gene expression is blocked by a lipoxygenase inhibitor [56]. For induction of secondary metabolite accumulation, 12-oxo-PDA may also play a major role. Structural analogues of 12-oxo-PDA that cannot be converted into JA are effective in the induction of alkaloid accumulation [58]. Furthermore, some plant species accumulate more 12-oxo-PDA than JA upon elicitor treatment [59], in support of the hypothesis that 12-oxo-PDA acts as the predominant ODA signal in certain defense responses. Experiments using the *Bryonia dioica* tendril-coiling bioassay for jasmonate also suggest that, in that system, 12-oxo-PDA, rather than JA, has to be considered as the endogenous signal transducer [60].

How elicitors affect JA biosynthesis and how the JA signal is transduced to affect gene expression is largely unknown. Several reports have implicated the activation of a wound-responsive MAPK cascade upstream of JA biosynthesis [61–63]. Downstream of the ODA pathway there are also one or more protein kinases involved in transduction of the JA signal [64]. A protein kinase (cascade) ultimately changes the activity of transcription factors, which regulate the

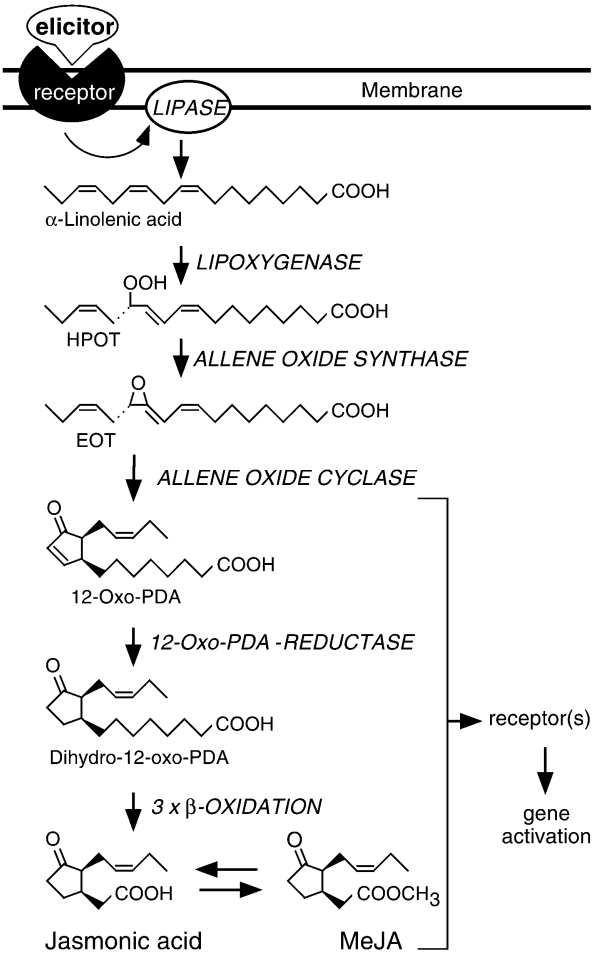

Fig. 2. Schematic representation of the jasmonate biosynthetic pathway: *12-oxo-PDA* 12-Oxophytodienoic acid; *HPOT* 13(*S*)-hydroperoxyoctadecatrienoic acid; *EOT* 12,13(*S*)-epoxyoctadecatrienoic acid; *MeJA* methyl jasmonate. Chiral centers have been excluded from the names for clarity. The figure is adapted from Mueller [52]

expression of genes via recognition of specific sequences in the promoter regions. Sequences involved in jasmonate-responsive expression have been identified in the strictosidine synthase gene promoter as discussed below.

5 Regulation of the Terpenoid Indole Alkaloid Biosynthetic Pathway

Research on the terpenoid indole alkaloids (TIAs) is mainly primed by the pharmaceutical applications of several of the compounds. The monomeric alkaloids serpentine and ajmalicine are used as a tranquilizer and to reduce hy-

pertension, respectively. The dimeric alkaloids vincristine and vinblastine are potent antitumor drugs. In plants, TIAs are thought to be involved in defence responses. Several reports show that physiological concentrations of TIAs can have antifeeding activity or can delay growth of insect larvae, fungi and microbes [65–67]. Terpenoid indole alkaloids are found in a limited number of plant species belonging to the plant families Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae. The best progress on molecular characterization of the pathway has been made with *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle), a member of the Apocynaceae family. *C. roseus* cells have the genetic potential to synthesize over 100 terpenoid indole alkaloids.

The initial step in TIA biosynthesis is the condensation of tryptamine with the iridoid glucoside secologanin (Fig. 3). This condensation is performed by the enzyme strictosidine synthase (STR) and results in the synthesis of 3α(S)-strictosidine. Strictosidine is the universal precursor of a wide range of different TIAs in various plant species. Additional species-specific enzymes determine the types of TIAs that are formed. In *C. roseus*, strictosidine is deglucosylated by strictosidine β-D-glucosidase (SGD). Further enzymatic and spontaneous conversions result in the biosynthesis of numerous TIAs. Tryptamine, providing the indole moiety of TIAs, is formed by decarboxylation of tryptophan by the enzyme tryptophan decarboxylase (TDC). The shikimate pathway provides chorismate, which is the precursor of aromatic amino acids. Anthranilate synthase (AS) catalyzes the first step in the conversion of chorismate into tryptophan (Fig. 3). Secologanin, providing the terpenoid part of the TIAs, is synthesized via multiple enzymatic conversions from geraniol. The terpenoid precursors that constitute the backbone of geraniol are produced via the MEP (2-*C*-methyl-D-erythritol 4-phosphate) pathway [68] (formerly called the deoxyxylulose, GAP/pyruvate or Rohmer pathway; [69]). Most steps involved in the conversion of geraniol to secologanin are unknown. Geraniol 10-hydroxylase (G10H), an enzyme of the cytochrome P450 family, catalyzes the first committed step in the formation of secologanin by 10-hydroxylation of geraniol. The enzyme NADPH:cytochrome P450 reductase (CPR) is essential for the activity of G10H and other cytochrome P450 monooxygenases, since it functions in electron transfer to cytochrome P450 proteins. Dimeric alkaloids are formed by peroxidase-catalyzed condensation of vindoline and catharanthine (Fig. 3). Many monomeric TIAs are found in all plant organs, but vindoline and vindoline-derived dimeric alkaloids are only found in chloroplast-containing plant tissues [70]. Vindoline is derived via a number of steps from tabersonine. The first step is catalyzed by the P450 enzyme tabersonine 16-hydroxylase. The two final steps are catalyzed by acetyl CoA:deacetylvindoline 4-*O*-acetyltransferase (DAT) and the 2-oxoglutarate-dependent dioxygenase desacetoxyvindoline-4-hydroxylase (D4H) (Fig. 3).

The biosynthesis of TIAs is highly regulated, and depends on tissue-specific factors as well as environmental signals. Developmental control of TIA biosynthesis and gene expression has recently been reviewed [71]. Here, we will restrict ourselves to stress signals that regulate TIA biosynthesis and gene expression. Inducing effects were observed for fungal elicitors [72], jasmonates [73, 74], auxin starvation [74] and UV-B light [75].

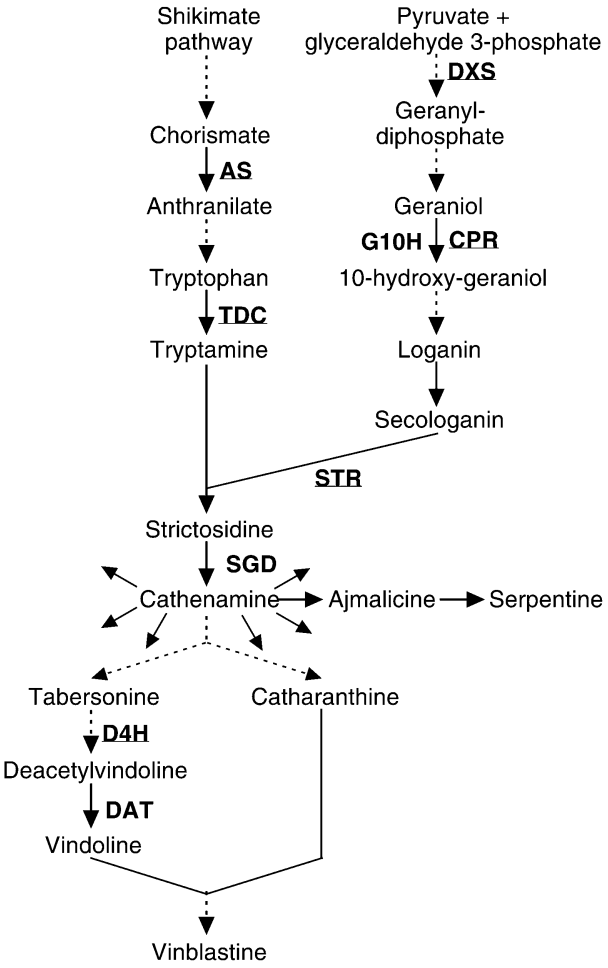

Fig. 3. Biosynthesis of TIAs in *C. roseus*. *Solid arrows* indicate single enzymatic conversions, whereas *dashed arrows* indicate multiple enzymatic conversions. *AS* Anthranilate synthase, *DXS* D-1-deoxyxylulose 5-phosphate synthase; *G10H* geraniol 10-hydroxylase; *CPR* cytochrome P450 reductase; *TDC* tryptophan decarboxylase; *STR* strictosidine synthase; *SGD* strictosidine β-D-glucosidase; *D4H* desacetoxyvindoline 4-hydroxylase; *DAT* acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyl transferase. Genes regulated by ORCA3 are *underlined*. Reprinted with permission from [91]. Copyright (2000) American Association for the Advancement of Science

cDNA clones encoding strictosidine synthase from *Rauwolfia serpentina* [76] and from *C. roseus* [77, 78] and genomic sequences from *R.serpentina* and *R. mannii* [79] and *C. roseus* [80] have been isolated. A *Tdc* cDNA [81] and corresponding genomic clone [82] have been isolated from *C. roseus*. *Str* and *Tdc* mRNA accumulate in suspension-cultured cells after auxin starvation [78, 83], and exposure to fungal elicitors [78, 84] or (methyl) jasmonate [64]. Elicitor-re-

sponsive expression of *Tdc* and *Str* depends on jasmonate as a secondary signal [64]. Elicitor-induced jasmonate biosynthesis requires protein kinase activity [64] and an elevation of cytosolic calcium concentration [85] (Fig. 5). *Tdc* and *Str* in leaves are induced by a UV-B light pulse [75]. As TDC and STR are encoded by single-copy genes in *C. roseus* [78, 82], all these signals act on the same promoter regions. The Chinese tree *Camptotheca acuminata*, on the other hand, contains at least two distinct *Tdc* genes, which are differentially controlled [86]. Accumulation of *Tdc*1 mRNA is developmentally regulated, whereas *Tdc*2 mRNA is induced by fungal elicitor and methyl jasmonate.

In addition, cDNA clones for G10H [87], CPR [88], which is essential for the G10H-catalyzed reaction, and SGD [89] have been isolated. *Cpr* mRNA accumulation is rapidly induced by fungal elicitor [88] and the *Cpr* promoter is elicitor-responsive in transgenic tobacco [90]. All three genes are induced by MeJA in *C. roseus* cell cultures [89, 91].

From the vindoline pathway, cDNA clones for tabersonine 16-hydroxylase [92], DAT [93], and D4H [94] have been isolated. The expression of the corresponding genes is light-regulated in seedlings. In addition, *D4h* and *Dat* have been shown to be induced by MeJA in cell cultures [91].

The observations that *Tdc* and *Str* mRNAs coordinately accumulate in response to fungal elicitors, jasmonates, UV light, and auxin starvation, clearly indicate that the *Tdc* and *Str* genes are controlled by common regulators. Since all the TIA biosynthetic genes tested were induced by MeJA [91], this suggests that a common jasmonate-responsive regulator controls many and possibly all TIA structural genes. Such regulators could prove to be useful to engineer TIA biosynthesis, and the following sections discuss two strategies for their isolation.

6 Isolation of Regulatory Genes via Yeast One-Hybrid Screening

The yeast one-hybrid system is a genetic screening method for the isolation of proteins binding to characterized *cis*-acting promoter elements (Fig. 4). In this approach, a yeast strain is constructed carrying a reporter gene driven by an artificial promoter, with a bait composed of multimers of the promoter element of interest fused to a yeast TATA box region. Upon transformation of a cDNA library, cloned as a translational fusion with the heterologous GAL4 activation domain, yeast cells are selected for reporter gene activation. Since the resulting clones encode fusion proteins consisting of the cDNA-encoded protein and the GAL4 activation domain, cDNAs are solely selected for binding of the corresponding proteins to the promoter element of interest. This system has been used successfully to clone several transacting factors from mammalian systems and plants [48, 95].

The *Str* promoter has been extensively studied to identify elicitor- and jasmonate-responsive sequences [48, 80, 95]. Two regions were identified that dictated elicitor- and jasmonate-responsive reporter gene activation, the so-called BA region (Fig. 5; [48]), and a region close to the TATA box called jasmonate- and elicitor-responsive element (JERE; [95]). An *Str* promoter derivative with a deletion of the BA region is still active and responsive to stress signals, but at a

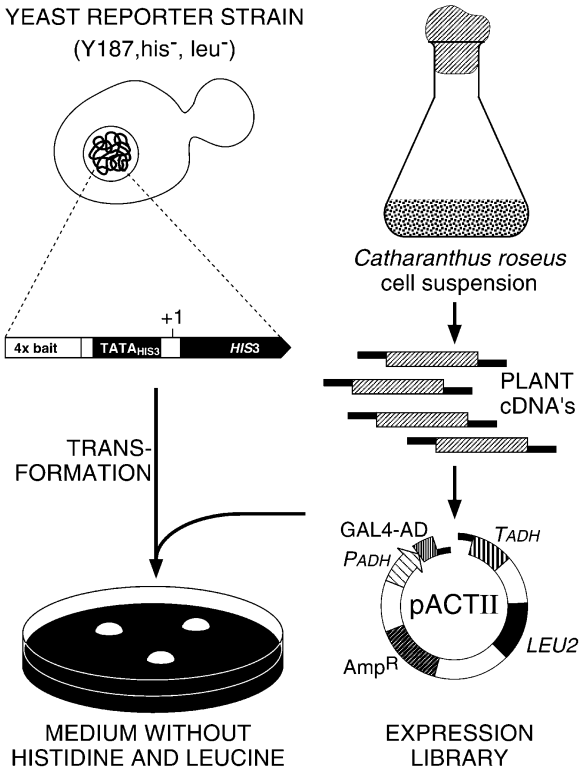

Fig. 4. Schematic representation of yeast one-hybrid transcription factor screening. *C. roseus* cDNAs were cloned in a fusion with the GAL4-activation domain (GAL4-AD) in yeast/*E.coli* shuttle vector pACTII. Yeast reporter strains carrying a tetramer of the *cis*-acting elements of interest (indicated in the figure with 4xbait) were transformed with the cDNA library and selected for transformation and reporter gene activation on medium lacking leucine and histidine, respectively

lower level. Mutation or removal of the JERE results in an inactive and unresponsive *Str* promoter derivative [95].

Yeast one-hybrid screening with the JERE as a bait identified two transcription factors of the AP2/ERF-domain family that were called ORCA1 and ORCA2 (*O*ctadecanoid-*R*esponsive *Catharanthus A*P2; [95]). The AP2/ERF transcription factors are unique to plants, and are characterized by the AP2/ERF DNA-binding domain [96, 97]. *Orca2* gene expression was induced by MeJA and elicitor. Furthermore, the ORCA2 protein showed sequence-specific binding to and transactivated *Str* gene expression via the JERE [95]. In contrast to *Orca2*, *Orca1* gene expression was not induced by MeJA and elicitor, indicating that ORCA1 is not involved in regulation of JA- and elicitor-induced *Str* expression.

Use of the BA region in a yeast one-hybrid screen resulted in the isolation of a periwinkle homologue of the MYB-like factor BPF-1 from parsley [48]. Like its parsley counterpart [47], transcription of periwinkle *CrBPF-1* is induced by

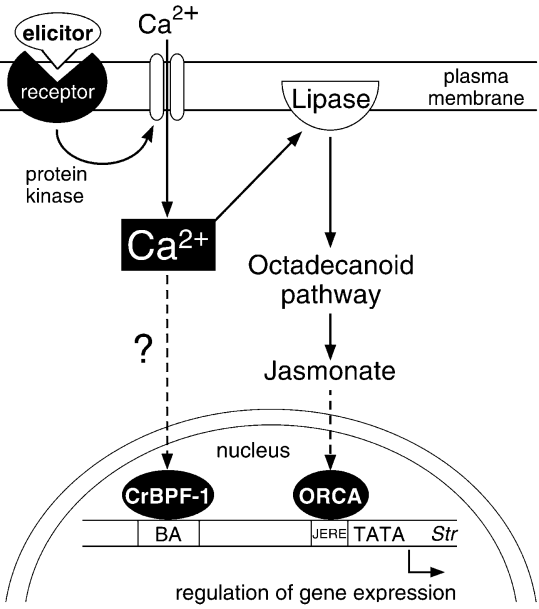

Fig. 5. Model for elicitor signal transduction leading to *Str* expression. The model shows the positions of CrBPF-1 in a JA-independent, and ORCAs in a JA-dependent, elicitor signal transduction pathway. Protein phosphorylation and calcium influx are required for elicitor-induced jasmonate biosynthesis, as well as for the induction of *CrBPF-1*, *ORCA2*, and *ORCA3*. ORCA activation additionally depends on jasmonate biosynthesis. The positions of the TATA box, the BA region, and the jasmonate- and elicitor-responsive element (JERE) within the *Str* promoter are indicated

fungal elicitor. *CrBPF1* elicitation depends on protein kinase activity and a rise in cytosolic calcium concentration, but is independent of jasmonate biosynthesis (Fig. 5).

Figure 5 shows a model that summarizes activation of *Str* gene expression by elicitor. Upon perception of the elicitor, a protein phosphorylation step is required to induce a calcium influx. This transient increase in cytosolic [Ca^{2+}] is necessary for activation of the ODA pathway [85]. Jasmonate induces *Orca2* gene expression [95]. ORCA2 then activates *Str* expression via interaction with the JERE. In addition, calcium influx is required for JA-independent induction of *CrBPF-1* mRNA accumulation, putatively involved in regulation of *Str* via interaction with the jasmonate- and elicitor-responsive BA region. Deletion of the BA fragment from the *Str* promoter did not abolish elicitor and jasmonate responsiveness of this promoter, whereas deletion or mutation of the JERE within the –339 promoter context resulted in a promoter derivative that is inactive and lacks elicitor and JA responsiveness [95]. Therefore, CrBPF-1 cannot be sufficient for elicitor-induced expression of *Str*. It can be speculated that the JERE of the *Str* promoter forms a switch for elicitor and jasmonate responsiveness, and that other *cis*-elements with their cognate binding factors (such as BA/CrBPF-1) are important for enhancing the elicitor and/or jasmonate response.

7
Isolation of Regulatory Genes via T-DNA Activation Tagging

One of the most direct ways to find regulators of metabolism is the generation and analysis of genetic mutants. Mutations resulting from chemical mutagenesis or tagging with transposons or T-DNAs usually cause loss of function, and are therefore recessive. Consequently, the mutant phenotype can only be visualized following selfing of the mutated plants. This demands a substantial amount of effort, and is not possible for all plant species. Another drawback of classical mutagenesis is that mutation of functionally redundant genes does not result in phenotypically altered plants. In contrast, mutants generated by T-DNA activation tagging are dominant, allowing direct selection of the desired phenotype in the primary transformants. Furthermore, a phenotype can result from T-DNA activation tagging of a functionally redundant gene, allowing its cloning and functional analysis. The T-DNA activation tagging approach consists of transformation of plants or plant cells with a T-DNA, which carries promoter elements reading towards one of its borders. Upon random integration in the plant genome, flanking plant sequences can be transcribed, which can result in a dominant mutation (Fig. 6). T-DNA activation tagging has been used to isolate genes involved in the timing of flowering [98, 99], or in responses to abscisic acid [100] or cytokinin [101].

This strategy was successfully applied to isolate a regulator of TIA biosynthesis. *C. roseus* cells transformed with a T-DNA tag were selected on a toxic level of 4-methyltryptophan. This amino acid analogue can be converted by the TDC enzyme into its nontoxic derivative 4-methyltryptamine [102]. This selection strategy therefore will select T-DNA tagged cells with elevated levels of TDC enzyme activity (Fig. 6). In addition, cells can survive 4-mT selection by producing more tryptophan, by reduced 4-mT uptake, and many other possible mechanisms. To select for regulators of *Tdc* gene expression, 4-mT resistant cell

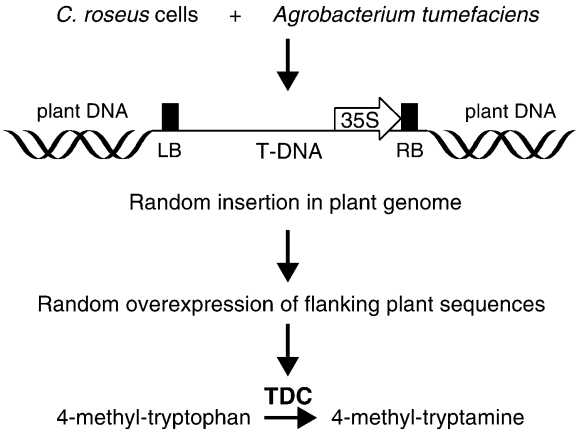

Fig. 6. Schematic representation of the T-DNA tagging approach to identify regulators of TIA biosynthetic genes in *C. roseus*. For explanation, see the text

lines were screened for high *Tdc* mRNA levels via Northern blot hybridization. To select for cell lines in which the T-DNA tag activated a master regulator of TIA biosynthetic gene expression, cell lines with high *Tdc* mRNA levels were subsequently screened for high *Str* mRNA levels (Fig. 7). In total 400,000 to 500,000 independent cells, stably transformed with a T-DNA construct carrying CaMV 35S enhancer sequences reading towards the right border sequence, were selected for 4-mT resistance. The haploid genome size of *C. roseus* is 8×10^8 bp according to a recent estimation (Frank van Iren, personal communication). This is 2.5-fold smaller than a previous estimation [103]. Thus, by generation of 400,000 to 500,000 independent transformants with on average two T-DNA copies per genome [104], the probability of finding a T-DNA insert in a given 2 kb DNA region is around 90% using the formula $P = 1 - (1 - [x/800.000])^n$ (where P = probability; x = length of DNA region in kb; n = number of T-DNA insertions in population; and T-DNA integration is assumed to be random). Since T-DNA

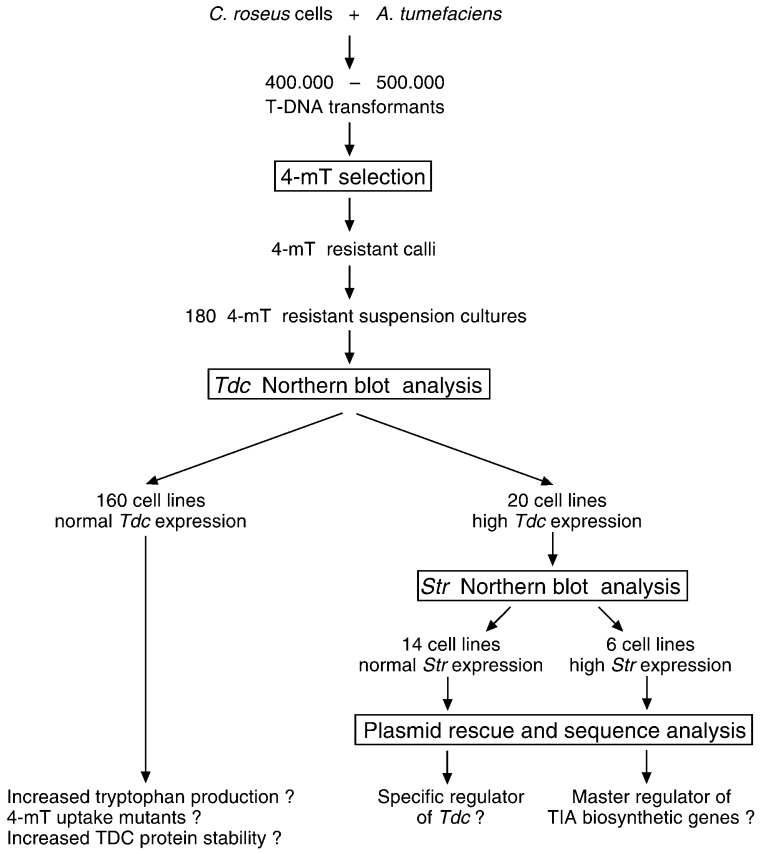

Fig. 7. Schematic representation of the selection procedure and the numbers of transformants and mutants that were generated. Possible mechanisms for passing the specific selection steps are indicated at the bottom of the figure

preferentially inserts into transcribed regions of the plant genome, T-DNA density near or into genes may even be higher. The CaMV 35S promoter has been shown to activate gene expression over a distance of at least 3.6 kb in an activation tagging approach [99]. Based on these calculations, it can be concluded that T-DNA activation tagging of the *C. roseus* genome by construct Tag-2B4A1 was close to saturation. After several weeks of selection, 4-mT resistant calli were obtained. To generate sufficient biomass for subsequent analysis, suspension cultures were generated from 180 calli. Further screening of 4-mT resistant cell lines for high *Tdc* expression by Northern blot analysis identified 20 lines with increased *Tdc* mRNA accumulation. Thus, only approximately 10% of the 4-mT resistant lines showed the desired phenotype. The other 90% of the lines were 4-mT resistant via a mechanism different from increased *Tdc* expression; for example, by increased tryptophan production, by impaired 4-mT uptake, or by increased TDC protein stability.

The 4-mT resistant cell lines were further checked for increased expression of *Str*, a TIA biosynthetic gene that was not used as selectable marker in the initial selection. Only six lines showed, in addition to increased *Tdc* expression, enhanced *Str* mRNA accumulation, suggesting that a component of a shared signal transduction pathway leading to regulation of several, and possibly all, TIA biosynthetic genes was tagged in these lines. Thus, the frequency of tagging central signaling components was low. Production of secondary metabolites and active cell proliferation appear to be mutually exclusive processes. Since the T-DNA activation tagging approach selected for actively growing cells, it can be speculated that there was a negative selection pressure against cells with increased expression of the complete TIA biosynthetic pathway and consequent production of secondary metabolites. Alternatively, the number of target genes that act as master regulators for multiple genes operating in the TIA biosynthetic pathway may be limited.

For one of the six lines with increased expression of *Tdc* and *Str* (line 46), the isolation of the T-DNA-flanking plant DNA by plasmid rescue was successful. Subsequent characterization of the rescued DNA showed that the T-DNA tag activated a gene encoding another AP2/ERF-domain transcription factor, which was called ORCA3 [91]. ORCA3 shows a high similarity to ORCA2 in the AP2/ERF DNA-binding domain, but no amino acid identity elsewhere was observed. The expression of the *Orca3* gene was induced by MeJA with similar kinetics as *Orca2* expression [105]. The ORCA3 protein bound the *Str*, *Tdc* and *Cpr* promoters, and increased their transcriptional activity in a transient assay [91]. The activation of the *Str* promoter depended on a specific interaction of ORCA3 with the JERE [105]. The effect of ORCA3 on metabolism is described in the next section.

8 Modification of TIA Metabolism Using the Transcription Factor ORCA3

In line 46, overexpressing ORCA3 due to the inserted T-DNA activation tag, expression of TIA biosynthetic genes *Tdc*, *Str*, *Sgd, Cpr* and *D4h* was increased (schematically depicted in Fig. 3). TIA biosynthetic genes *G10h* and *Dat* were

not induced, suggesting that these genes are not controlled by ORCA3. Genes encoding the α subunit of AS (ASα) and DXS, enzymes involved in primary metabolism leading to TIA precursor synthesis, were induced by *Orca3* overexpression, whereas two other primary metabolic genes not involved in production of TIA precursors (*Ggpps* and *Ics)* were not regulated by ORCA3. Gene expression patterns observed in cell lines that were independently transformed by particle bombardment with the rescued *Orca3* gene under control of CaMV 35S promoter elements were identical to that of line 46. These results indicate that ORCA3 is a regulator of primary as well as secondary metabolite biosynthetic genes involved in TIA biosynthesis. Although previous studies report differential regulation of genes involved in early (i.e. *Tdc* and *Str*) and late (i.e. *D4h*) steps in vindoline biosynthesis in periwinkle plants [71, 106], genes of both classes are controlled by *Orca3* in suspension-cultured cells.

Plant cells must regulate their primary metabolic pathways to accommodate the biosynthesis of secondary metabolites. Previously, a strong correlation between induction of tryptophan biosynthetic gene expression and indolic phytoalexin accumulation was observed in *Arabidopsis* plants [107], indicating coordinate regulation of these processes. In *C. roseus* genes involved in both primary and secondary metabolic pathways can be regulated by a single AP2/ERF-domain transcription factor, ORCA3. Thus, ORCA3 acts as a central regulator to direct metabolic fluxes into the TIA biosynthetic pathway by regulation of expression of genes involved in primary as well as secondary metabolism.

All primary and secondary metabolite biosynthetic genes that were tested, together with *Orca3* itself, were induced by MeJA [91]. However, not all MeJA-induced genes were regulated by *Orca3*. For example, expression of *G10h* was strongly induced by MeJA, whereas *G10h* expression was not detected in *Orca3* overexpressing cell lines, suggesting that additional jasmonate-responsive transcription factors are involved in regulation of this gene. A possible candidate for this regulation is ORCA2.

Cell cultures overexpressing *Orca3* accumulated significantly increased amounts of tryptamine, providing the indole moiety of TIAs. Since no TIAs were detected in these cultures, it was concluded that the terpenoid part was limiting for TIA production, and that this limitation could not be overcome by *Orca3* overexpression. A possible bottleneck was *G10h*, since *G10h* expression was not observed in the *Orca3* overexpressing cell cultures. When the terpenoid precursor loganin was added to the cell cultures, *Orca3* overexpression caused an increase in TIA production [91]. A significant increase in TIA biosynthesis has never been conclusively shown upon overexpression of *Tdc*, *Str* or both genes, even after feeding with terpenoid precursors [108, 109]. This clearly demonstrates the benefit of using central regulators for metabolic engineering of plant cells.

9 Conclusions

The examples discussed here show that for two secondary metabolite pathways, metabolic engineering using transcription factors was successful. Modification of the phenylpropanoid/flavonoid pathway made use of the tissue-specific MYB

and bHLH transcription factors, whereas for modulation of the TIA biosynthetic pathway, a jasmonate-responsive AP2/ERF-domain transcription factor was used.

Overexpression experiments with the MYB and bHLH protein-encoding genes have demonstrated that flavonoid biosynthesis can be engineered using transcription factors. The MYB and bHLH factors are active over species borders and faithfully recognize their orthologous target genes in heterologous species. In this respect the MYB and bHLH proteins behave as master regulators of the flavonoid pathway, although maybe not in all plant species to a similar extent. Ectopic expression of *C1* and *R* constitutes a viable strategy for engineering anthocyanin production in plant cell cultures [50].

Overexpression of the jasmonate-responsive AP2/ERF-domain transcription factor ORCA3 resulted in elevated levels of tryptophan and tryptamine, and, upon feeding of a terpenoid precursor, elevated levels of certain TIAs [91].

AP2/ERF-domain transcription factors occupy central positions in regulation of plant stress responses [96]. ERF1, for example, is an ethylene-responsive AP2/ERF-domain protein (Fig. 8). Its overexpression resulted in induction of several ethylene-responsive defense genes in *Arabidopsis thaliana* [110]. In tomato, Pto is a receptor kinase that is involved in the recognition of *Pseudomonas* avirulence gene product AvrPto [111]. Yeast two-hybrid screening identified three Pto-interacting (Pti4, 5 and 6) tomato proteins, each of which contained an AP2/ERF domain [112]. Pti4, 5 and 6 are thought to be involved in elicitor-induced activation of defense genes in tomato (Fig. 8). Another family of AP2/ERF-domain proteins, represented by DREB2A and DREB2B, is involved in drought-responsive gene expression [113], whereas the CBF/DREB1 family includes central regulators of cold-regulated gene expression in *Arabidopsis* [9, 113] (Fig. 8). Upon overexpression of the AP2/ERF-domain protein CBF1, the

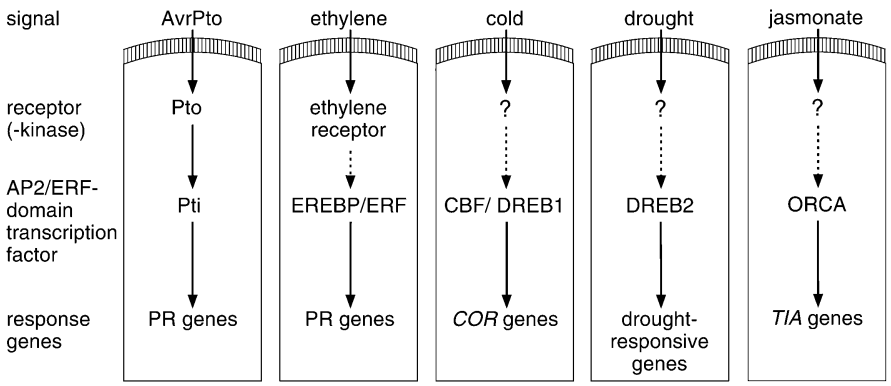

Fig. 8. Models showing the involvement of AP2/ERF-domain transcription factors in regulation of stress and defense gene expression in plants. The stress signal is perceived by interaction with a receptor (-kinase). Induction of de novo synthesis and/or modulation of the preexisting AP2/ERF-domain transcription factor activate gene expression. Unidentified signal transduction components are indicated with a *question mark*. *Dashed arrows* indicate the possible involvement of multiple signalling steps

expression of a set of cold-induced genes was enhanced, thereby increasing freezing tolerance [9]. In *C. roseus* the expression of TIA biosynthetic genes is coordinately induced by jasmonates [64, 89, 91]. A considerable number of these genes are controlled by ORCA3 [91]. Therefore, the spectrum of defense genes regulated by AP2/ERF-domain transcription factors includes genes involved in jasmonate-responsive secondary metabolism (Fig. 8).

Since several plant secondary metabolic pathways are regulated by jasmonate, it is tempting to speculate that these pathways in other plant species are controlled by ORCA-like AP2/ERF-domain transcription factors. Therefore, identification and isolation of *Orca* homologues might offer opportunities for genetic engineering of JA-responsive metabolism in plants in general. Overexpression of maize MYB and bHLH regulatory proteins in heterologous plant species resulted in increased production of secondary metabolites [21], demonstrating the possibilities for interspecies exchange of regulatory proteins. Expression of *C. roseus* ORCA proteins in other plant species might result in induction of jasmonate-responsive metabolic pathways, and, subsequently, in increased metabolite production.

Although ORCA3 regulates multiple genes in primary and secondary metabolism, not all genes in TIA metabolism are controlled by ORCA3. Possibly, other transcription factors are involved in controlling these genes. A good candidate is ORCA2, another jasmonate-responsive AP2/ERF-domain transcription factor from *C. roseus*. Preliminary results indicate that ORCA2 and ORCA3 have overlapping, but distinct, sets of target genes. This indicates that these ORCA proteins have different functions, and that both are required for the full spectrum of jasmonate-induced metabolic changes. Future experiments using a combination of ORCA2 and ORCA3 should establish to what extent they regulate the TIA biosynthetic pathway, and whether additional transcription factors may be involved.

In using central regulators to engineer secondary metabolism, the use of inducible promoters deserves attention, because constitutive expression of a pleiotropic regulator, or a high constitutive level of certain secondary metabolites, may not be compatible with cell viability. In addition, transcriptional regulators may be engineered to make them dominant and independent of the normal control of their activity. This may be especially important for central regulators that are activated by stress signals. The two approaches may be combined by constructing hybrid transcription factors, that consist of the DNA-binding domain to direct them to their target genes, a strong transactivation domain, and an inducible domain that silences the activation domain in the uninduced state. Such an approach was used to produce anthocyanins in the *Arabidopsis ttg* mutant that lacks anthocyanins. Expression of a fusion between the DNA-binding domain of the maize bHLH protein *R* and the steroid-binding domain of the rat glucocorticoid receptor restored anthocyanin production in a steroid hormone-dependent manner [114]. In addition, inducible expression of transcription factors provides a valuable tool for the identification of their target genes [115].

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