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Molecules of Interest

Transcription factors controlling plant secondary metabolism: what regulates the regulators?

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Abstract

Plants produce secondary metabolites, among others, to protect themselves against microbial and herbivore attack or UV irradiation. Certain metabolite classes also function in beneficial interactions with other organisms. For example, anthocyanin pigments and terpenoid essential oils have key roles in attraction of flower pollinators. Secondary metabolites also have direct uses for man. Flavonoids and terpenoids for example have health-promoting activities as food ingredients, and several alkaloids have pharmacological activities. Controlled transcription of biosynthetic genes is one major mechanism regulating secondary metabolite production in plant cells. Several transcription factors involved in the regulation of metabolic pathway genes have been isolated and studied. There are indications that transcription factor activity itself is regulated by internal or external signals leading to controlled responses. The aim of this review is to discuss the regulation of transcription factors involved in secondary metabolism in plants at gene and protein levels, using phenylpropanoid and terpenoid indole alkaloid pathways as two well-studied examples.

Keywords: Secondary metabolism; Transcription factors; Alkaloids; Flavonoids; Jasmonic acid; MYB; MYC; ORCA

1. Introduction

Coordinate transcriptional control of biosynthetic genes emerges as a major mechanism dictating the final levels of secondary metabolites in plant cells. This regulation of biosynthetic pathways is achieved by specific transcription factors. Transcription factors are sequence-specific DNA-binding proteins that interact with the promoter regions of target genes, and modulate the rate of initiation of mRNA synthesis by RNA polymerase II. These proteins regulate gene transcription depending on tissue type and/or in response to internal signals, for example plant hormones, and to external signals such as microbial elicitors or UV light. External signals may induce production of internal signals. For several plant species, it has been shown that elicitor-induced accumulation of secondary metabolites is mediated by the stress hormone jasmonic acid (JA; Memelink et al., 2001). Transcription factors have been isolated and characterized for two plant metabolic pathways, leading to biosynthesis of flavonoids and of terpenoid indole alkaloids (TIA), respectively.

End products of the flavonoid biosynthesis pathway include the anthocyanin pigments. In various plant species it has been shown that tissue-specific regulation of the structural genes involved in anthocyanin biosynthesis is directly controlled by a combination of two distinct transcription factor families with homology to the protein encoded by the vertebrate proto-oncogene c-*MYB*, and the vertebrate basic-Helix-Loop-Helix (bHLH) protein encoded by the proto-oncogene c-*MYC*, respectively (Mol et al., 1998).

In the plant species *Catharanthus roseus* (Madagascar periwinkle), methyl jasmonate (MeJA) induces TIA production. ORCA (Octadecanoid-Responsive Catharanthus AP2/ERF-domain) transcription factors have been shown to regulate the JA-responsive activation of several TIA biosynthesis genes (van der Fits and Memelink, 2000; Memelink et al., 2001).

The role and characterization of these transcription factors involved in secondary metabolism have been reviewed previously (Memelink et al., 2001; Mol et al.,

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1998), and will only briefly be reviewed here. External and/or internal signals appear to modulate the levels and activities of these regulators, leading to appropriate responses to stress or development. The main aim of this contribution is to review and speculate on the regulation of the regulators at gene and protein levels.

2. MYB and bHLH proteins in flavonoid metabolism

Most plant MYB proteins contain two related helix-turn-helix motifs, the R2 and R3 repeats responsible for binding to target DNA sequences. They are encoded by large gene families, for example *Arabidopsis thaliana* has 125 *R2R3-MYB* genes. Specific MYB family members are involved in the regulation of the flavonoid pathway in combination with specific bHLH protein partners. Certain MYB and bHLH proteins can physically interact with each other (Mol et al., 1998). MYB/bHLH proteins have been mainly studied in petunia, snapdragon and maize as regulators of anthocyanin biosynthesis, and more recently in *Arabidopsis* as regulators of anthocyanin and seed coat tannin biosynthesis.

In maize, the majority of the structural genes encoding enzymes committed to anthocyanin biosynthesis are thought to be regulated coordinately by the bHLH protein-encoding gene *R* and the *MYB* gene *C1* in the aleurone layer of maize kernels and by other gene family members in other plant parts (Mol et al., 1998). Ectopic expression of *R* and *C1* in normally unpigmented maize cells cultured in vitro caused biosynthesis and accumulation of anthocyanins due to the coordinate expression of most of the structural genes (Fig. 1; Grotewold et al., 1998; Bruce et al., 2000). Production of 3-deoxy flavonoids, which have been associated with insecticidal or antifungal activity, in maize requires P, a MYB-type transcriptional regulator that acts independently of R and C1. Ectopic expression of P in maize cells induced coordinate expression of a subset of biosynthesis genes (Fig. 1) leading to accumulation of flavonoids different from those regulated by C1/R (Grotewold et al., 1998; Bruce et al., 2000).

Ectopic expression of the MYB transcription factor production of anthocyanin pigment1 (PAP1) from *Arabidopsis* resulted in strongly enhanced expression of flavonoid biosynthesis genes (Fig. 1) and to intense purple pigmentation of most plant organs (Borevitz et al., 2000). The sequence similarity with *C1* suggests that *PAP1* and a related gene, *PAP2*, may be *C1* orthologs from *Arabidopsis*. The fact that the PAP1-mediated phenotype does not require co-expression of a bHLH protein partner indicates that ectopically expressed PAP1 either functions independently of bHLH transcription factors, or alternatively and more likely relies on bHLH proteins which are constitutively present.

Proanthocyanidins, also called condensed tannins, are another class of flavonoids, which accumulate in *Arabidopsis* developing seeds. Knockout of the *transparent testa2 (TT2)* gene encoding an R2R3 MYB protein specifically affected seed pigmentation due to a decrease in the content of brown tannins in the seed endothelium. Furthermore, it dramatically reduced the expression of several structural genes involved in tannin metabolism. Gain-of-function experiments demonstrated that TT2 induced the ectopic expression of *banyuls (BAN)*, which is thought to encode a leucoanthocyanidin reductase (LAR), the first enzyme committed to proanthocyanidin biosynthesis. The ectopic activation of flavonoid late biosynthetic genes such as *dihydroflavonol-4-reductase (DFR)* and *BAN* by TT2 requires TT8, a bHLH protein (Fig. 1; Nesi et al., 2001). TT2 and TT8 proteins may

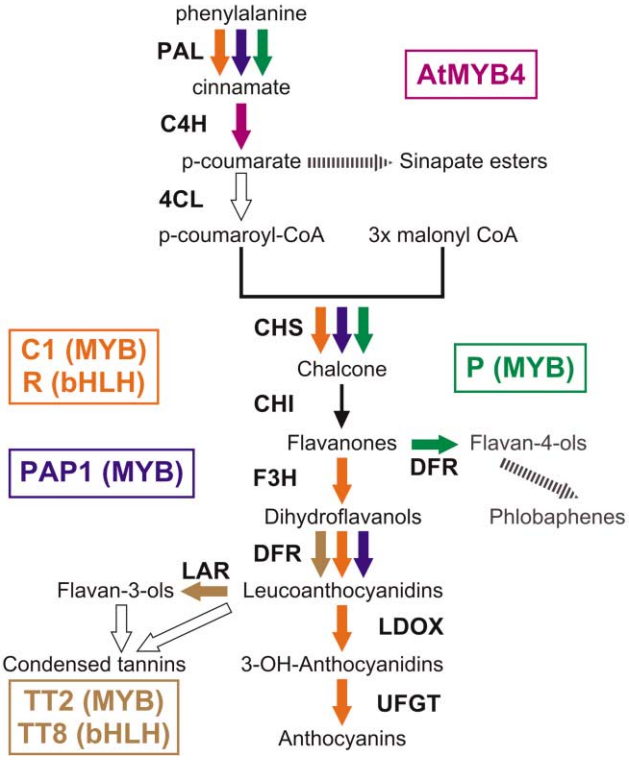

Fig. 1. Simplified diagram of the flavonoid biosynthetic pathway. Enzymes that catalyze the reactions are shown in capital letters. Unbroken arrows indicate single enzymatic conversions and broken arrows indicate multiple enzymatic steps. The color of the arrow corresponds to the color of the transcription factor(s), which regulate(s) the enzyme-encoding gene. Transcription factors C1, R and P are from maize, whereas MYB4, PAP1, TT2 and TT8 are from *Arabidopsis*. The black arrow for the CHI step indicates that this reaction can occur spontaneously without enzymatic intervention. Enzyme abbreviations: PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydroxylase, 4CL: 4-coumaroyl-coenzyme A ligase, CHS: chalcone synthase, CHI: chalcone-flavanone isomerase; F3H: flavanone 3-hydroxylase, DFR: dihydroflavonol 4-reductase, LAR: leucoanthocyanidin reductase, LDOX: leucoanthocyanidin dioxygenase, UFGT: UDP glucose-flavonol glucosyl transferase. Other abbreviations: At: *Arabidopsis thaliana*, bHLH: basic Helix-Loop-Helix, PAP1: production of anthocyanin pigment1, TT: transparent testa.

interact with each other for the regulation of target genes. TT2 overexpression induced ectopic expression of the *BAN* gene, thus suggesting that TT2 is the limiting factor for *BAN* expression. A crucial issue to be investigated now is how the temporal regulation of the *TT2* gene during seed development is determined.

The MYB and bHLH transcriptional activators discussed above appear to be responsible for tissue-specific gene expression. Despite considerable research efforts, transcription factors responsible for UV-, pathogen-, or stress-responsive activation of flavonoid biosynthesis genes have not been identified unequivocally.

MYB transcription factors can also have repressing effects on genes involved in phenylpropanoid biosynthesis. *AmMYB308* and *AmMYB330* genes from snapdragon, when expressed in tobacco, caused an inhibition of hydroxycinnamic acid and monolignol accumulation by reducing the expression of genes encoding the corresponding biosynthetic enzymes (Tamagnone et al., 1998). It was hypothesized that *AmMYB308* and *AmMYB330* may encode weak activators/repressors that compete with other stronger MYB-related activators to control target gene expression. An *Arabidopsis* knockout mutant of *AtMYB4* showed an increase in sinapate ester accumulation, which resulted in enhanced UV-B irradiation tolerance (Jin et al., 2000). *AtMYB4* is the *Arabidopsis* ortholog of *AmMYB308*. Overexpression in tobacco of FaMYB1, a transcription factor isolated from red strawberry fruit, resulted in suppression of anthocyanin and flavonol accumulation (Aharoni et al., 2001). All these MYB factors involved in repression contain in their C-terminal region the conserved motif pdLNL$^{D}/_{E}$Lxi$^{G}/_{S}$. Deletion and mutational analysis of AtMYB4 showed that this motif forms part of the region involved in the repression of transcription (Jin et al., 2000). It is also interesting to note that this motif has similarities to a characterized repressor motif called ERF-associated amphiphilic repression (EAR) motif, present in class II AP2/ERF transcriptional repressors and also in zinc finger proteins which function as repressors (Ohta et al., 2001). The fact that these repressors have this EAR-like domain indicates that they function as active repressors in addition to a possible passive mechanism via competition with activators for binding.

The picture that emerges is that various branches of phenylpropanoid metabolism are regulated by an interplay between branch-specific activating and repressing MYB transcription factors, some of which depend on specific bHLH protein partners (Fig. 1).

3. ORCA proteins in alkaloid metabolism

Another well-studied secondary metabolic pathway leads to the biosynthesis of terpenoid indole alkaloids (Fig. 2). Methyl jasmonate (MeJA) stimulates TIA biosynthesis by coordinately inducing the expression of the biosynthetic genes (Collu et al., 2001; Menke et al., 1999a; van der Fits and Memelink, 2000). A promoter element involved in jasmonate- and elicitor-responsive gene expression (JERE) was identified in the TIA biosynthetic gene *strictosidine synthase* (*STR*; Menke et al., 1999b). Yeast one-hybrid screening with the JERE as bait identified ORCA2, a transcription factor of the AP2/ERF (APETALA2/ethylene responsive factor)-domain class (Menke et al., 1999b). The AP2/ERF transcription factors form large families unique to plants, and are characterized by the AP2/ERF DNA-binding

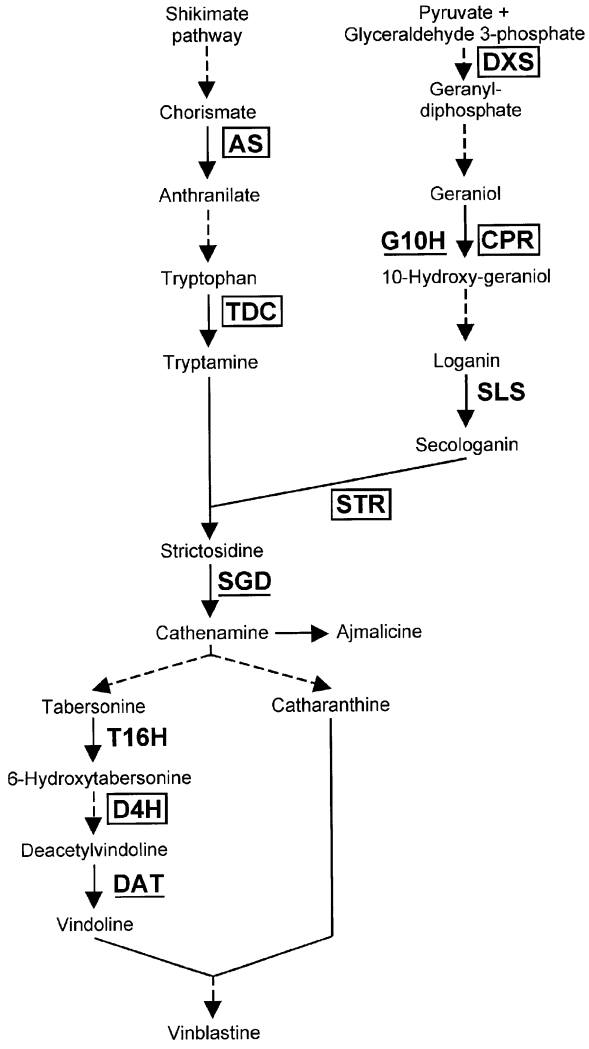

Fig. 2. Biosynthetic pathway for terpenoid indole alkaloids in periwinkle. Unbroken arrows indicate single enzymatic conversions and broken arrows indicate multiple enzymatic steps. Enzymes for which the corresponding genes have been cloned are indicated. Underlining indicates that the corresponding genes have been tested for being an ORCA3 target gene. Enzyme-encoding genes regulated by ORCA3 are boxed. AS: anthranilate synthase, CPR: cytochrome P450 reductase, D4H: desacetoxyvindoline 4-hydroxylase, DAT: acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase, DXS: D-1-deoxyxylulose 5-phosphate synthase, G10H: geraniol 10-hydroxylase, SGD: strictosidine β-D-glucosidase, SLS: secologanin synthase, STR: strictosidine synthase, TDC: tryptophan decarboxylase, T16H: tabersonine 16-hydroxylase.

domain (Memelink et al., 2001). *ORCA2* gene expression was induced by MeJA and elicitor. Furthermore, the ORCA2 protein transactivated *STR* promoter activity by sequence-specific binding to the JERE (Menke et al., 1999b).

ORCA3, another member of the AP2/ERF-domain transcription factor family in *C. roseus*, was isolated by T-DNA activation tagging (van der Fits and Memelink, 2000). ORCA3 shows a high similarity to ORCA2 in the AP2/ERF DNA-binding domain, but amino acid identity elsewhere was not observed. The expression of the *ORCA3* gene was induced by MeJA with similar kinetics as *ORCA2* expression (van der Fits and Memelink, 2001). The ORCA3 protein bound the promoter regions of the TIA biosynthetic genes *STR*, *tryptophan decarboxylase* (*TDC*) and *cytochrome P450 reductase* (*CPR*), and increased their transcriptional activity in a transient assay (van der Fits and Memelink, 2000). Activation of the *STR* promoter depended on a specific interaction of ORCA3 with the JERE (van der Fits and Memelink, 2001). Overexpression of *ORCA3* in transgenic *C. roseus* cell suspension cultures induced several genes in primary and secondary metabolism leading to TIA biosynthesis, including *STR*, *TDC* and *CPR* (Fig. 2), and resulted in increased TIA production upon feeding of the secologanin precursor loganin (van der Fits and Memelink, 2000). Evidence indicates that ORCA proteins may be activated in response to JA to regulate TIA biosynthetic genes (van der Fits and Memelink, 2001). An important issue to be investigated now is how jasmonates activate these AP2/ERF-domain proteins and genes.

Since the biosynthesis of many secondary metabolites is induced by jasmonates, the identification of an AP2/ERF-domain protein as a regulator of multiple genes involved in JA-responsive metabolism uncovers a control mechanism that may be operative in other stress-responsive plant metabolic pathway as well. If so, AP2/ERF-domain proteins may form powerful tools to increase the production of valuable JA-inducible secondary metabolites such as taxol and related taxoids and diverse types of alkaloids in plants or plant cell cultures.

4. Regulation of transcription factor mRNA abundance

Activity of transcriptional regulators can be controlled via several mechanisms. The most direct way to regulate the abundance of a factor is by adjusting the production of the encoding mRNA. A well-studied example is represented by the MYB protein C1. C1 is involved in regulation of anthocyanin production in the developing maize seed (Mol et al., 1998). Maize *viviparous1* (*vp1*) mutants have multiple seed maturation defects, including the lack of *C1* gene expression in developing seed tissue. This results in seeds that do not contain anthocyanins. The VP1 protein can transactivate a reporter gene driven by the *C1* promoter. A conserved B3 domain of VP1, also present in abscisic acid-insensitive 3 (ABI3) factor, which is believed to be orthologous to VP1 and has similar functions in dicotyledonous seed development, has a highly cooperative, sequence-specific DNA-binding activity. The specificity of this protein-DNA interaction is in excellent agreement with sequences identified as being functionally important for VP1 activation of the *C1* gene in maize cells (Suzuki et al., 1997 and references therein). Therefore, VP1 is a transcription factor that acts upstream of the MYB protein C1 by regulating its expression in developing seed via direct binding to the promoter. VP1 also controls other changes in gene expression associated with seed maturation, as discussed below.

Since many plant secondary metabolite biosynthesis genes are responsive to various stress conditions including elicitation by microbial signal molecules, the regulation of these genes is likely to be identical or similar to the regulation of genes that are generally regarded as pathogen-responsive (PR). Functional dissection of *PR* gene promoter regions resulted in the identification of a number of common *cis*-acting elements and, in some cases, their cognate binding factors. The GCC box is found in the promoter region of several *PR* genes. This *cis*-acting element was first shown to function as an ethylene-responsive element (Stepanova and Ecker, 2000). DNA-binding proteins that specifically interact with the ethylene-responsive GCC-box are EREBPs (ethylene responsive element binding proteins) from tobacco and ERFs (ethylene responsive factors) from *Arabidopsis* (Stepanova and Ecker, 2000). They belong to the AP2/ERF domain family of transcription factors. Ethylene-insensitive3 (EIN3) is a sequence-specific DNA binding protein that was shown to be necessary and sufficient for the activation of *ERF1* transcription, and as a consequence promoted transcription of ethylene-regulated downstream target genes (Stepanova and Ecker, 2000). Thus, the transcription factors EIN3 and ERF1 act sequentially in a cascade of transcriptional regulation initiated by ethylene gas.

As an alternative to a transcriptional cascade, the expression level of a transcription factor gene can be controlled by auto-regulation. Parsley WRKY1 protein is a member of a family of plant-specific zinc-finger type factors implicated in the regulation of genes associated with pathogen defense. In vitro WRKY1 bound specifically to functionally defined TGAC-containing W box promoter elements within the parsley *PR1* genes. Transient expression studies in parsley protoplasts showed that a specific arrangement of W box elements in the promoter of the *WRKY1* gene itself is necessary and sufficient for early elicitor-responsive activation and that WRKY1 binds to such elements (Eulgem et al., 1999),

indicating that WRKY1 activates its own expression in response to elicitation.

ORCA mRNA accumulation is rapidly and transiently induced by MeJA and the peaks of induced *ORCA* expression preceded the maximal induction of target genes like *TDC* and *STR*, suggesting the existence of a transcriptional cascade (Fig. 3). Surprisingly, however, the protein synthesis inhibitor cycloheximide did not inhibit MeJA-induced *TDC* and *STR* expression (van der Fits and Memelink, 2001), indicating that de novo synthesis of transcription factors is not necessary. Therefore, MeJA does not induce TIA gene expression simply by increasing ORCA protein abundance via a transcriptional cascade including ORCA or by ORCA auto-regulation, but instead appears to activate pre-existing ORCA protein (Fig. 3).

5. Regulation of transcription factor protein activity

In addition to regulation of cellular concentrations of regulatory proteins by transcriptional or posttranscriptional control, transcription factor activity can be regulated by posttranslational modifications and/or interactions with other proteins. Proteins can be modified posttranslationally among others by phosphorylation, acetylation, hydroxylation, nitrosylation, gluthathiolation, intra- and intermolecular S–S bridge formation between cysteines, myristoylation, farnesylation, ubiquitination, or glycosylation. Transcription factor modifications may alter protein conformation, allow interaction with other regulatory proteins, or affect subcellular localization. These changes in turn can affect DNA binding affinity, activation potential, nuclear localization and/or protein stability.

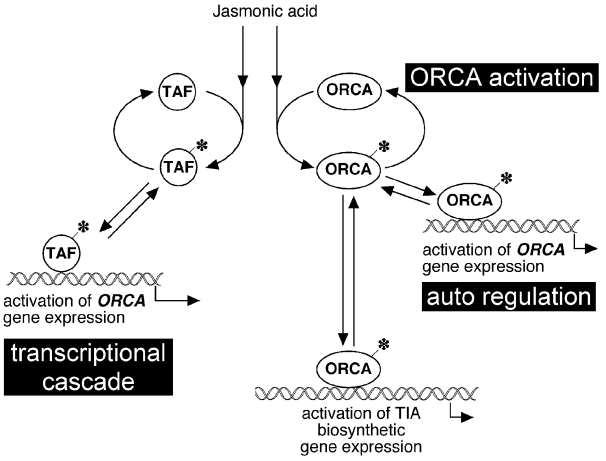

Fig. 3. Model for JA-induced gene expression mediated by ORCA transcription factors. Jasmonic acid activates ORCA transcription factors by posttranslational modification and/or protein interaction indicated with a star. Active ORCA stimulates TIA biosynthetic gene expression by direct binding to their promoters. Active ORCA may also be able to induce its own expression. Alternatively, jasmonic acid may activate an upstream transcription factor, which then induces orca gene expression. ORCA: Octadecanoid-Responsive Catharanthus AP2/ERF-domain protein, TAF: transcription activating factor, TIA: terpenoid indole alkaloid.

A well-known mechanism to regulate transcription factor activity in animals is by phosphorylation/dephosphorylation events. Several reports suggest that the activity of plant transcription factors can also be modulated by phosphorylation. One example of possible transcription factor phosphorylation in plants is provided by the AP2/ERF protein Pto-interactor 4 (Pti4), which was first identified on the basis of its interaction with the receptor kinase Pto, involved in disease resistance in tomato. Pti4 was phosphorylated by Pto in vitro, and this phosphorylation enhanced in vitro binding of Pti4 to the GCC box (Gu et al., 2000). If these observations reflect the in vivo situation, phosphorylation presumably results in enhanced ability of Pti4 to activate expression of GCC-box-containing *PR* genes in tomato.

Direct evidence that the activity of plant MYB proteins in vivo is regulated by mechanisms involving protein modification is lacking. However, the in vitro DNA-binding affinity of a snapdragon AmMYB340 protein, implicated in controlling flavonoid metabolism, was shown to be negatively affected by phosphorylation (Moyano et al., 1996). AmMYB340 phosphorylation in vivo may provide further fine-tuning of transcription control in addition to antagonistic and synergistic interactions with other MYB family members.

In the case of the ORCA transcription factors, since cycloheximide did not inhibit MeJA-induced target gene expression, it is likely that preexisting ORCA is activated somehow by posttranslational modification and/or protein-protein interaction. JA-responsive *STR* and *TDC* expression is sensitive to protein kinase inhibitors (Menke et al., 1999a) suggesting that phosphorylation plays a role in the activation of TIA genes. It is possible that JA activates ORCAs via phosphorylation, or that ORCAs have to be phosphorylated to be active.

Protein-protein interactions can also regulate transcription factor activity. An example already discussed here is the interaction between MYB and bHLH transcription factors. Interactions between transcription factors may stabilize their interaction with target promoters and/or may have synergistic effects on transcription rate.

Interactions of transcription factors with regulatory proteins such as 14-3-3 have also been reported. The tobacco basic-leucine-ZIPPER (bZIP) transcriptional activator repression of shoot growth (RSG), which is involved in the regulation of endogenous amounts of gibberellins, interacts in the yeast two-hybrid system and in tobacco plant cells with 14-3-3 proteins. Moreover, evidence suggests that 14-3-3 proteins negatively modulate RSG activity by excluding RSG from the nucleus (Igarashi et al., 2001). 14-3-3 proteins bind to conserved amino acid motifs containing phosphoserine

residues. A serine within a conserved 14-3-3 binding motif present in RSG was essential for binding to 14-3-3, and its mutation enhanced the transcriptional activity of RSG in tobacco protoplasts (Igarashi et al., 2001). This indicates that the subcellular localization of RSG is regulated via phosphorylation-dependent interaction with 14-3-3. 14-3-3 proteins may also play regulatory roles as part of transcriptional complexes within the nucleus. Anti-14-3-3 antibodies detected 14-3-3 proteins in rice nuclear extracts, in complexes with proteins, which were able to bind to Em1a, an abscisic acid-VP1 response element in the promoter of the *Em* (*embryonic maturation*) gene (Schultz et al., 1998). The rice 14-3-3 protein GF14b interacted with both the Em1a-binding bZIP protein EmBP1 and VP1 in vitro. Because a *vp1* mutant that cannot bind DNA still promotes *Em* transcription, and because 14-3-3 proteins cannot bind DNA, 14-3-3 proteins may provide a structural link between VP1 and Em1a-binding proteins forming a DNA-bound complex in the nucleus.

Apart from the *MYB* and *bHLH* transcription factor genes, surprisingly few genes regulating the expression of flavonoid biosynthesis genes have been identified. Two of such regulators are the petunia AN11 protein (de Vetten et al., 1997) and *Arabidopsis* transparent testa glabra1 (TTG1; Walker et al., 1999). AN11 and TTG1 are highly similar, and both contain four WD repeat motifs (conserved amino acid sequences usually ending in Trp-Asp), which are also found in the β subunits of heterotrimeric G proteins and a number of other regulatory proteins. The expression of the *DFR* gene in the petunia *an11* mutant can be rescued by expression of the MYB protein AN2, indicating that AN11 acts upstream of MYB. Since the *AN2* mRNA level is normal in the *an11* mutant, it has been suggested that the AN11 protein regulates MYB protein activity (de Vetten et al., 1997). The *Arabidopsis ttg1* mutant has several pleiotropic defects including lack of anthocyanin biosynthesis and trichomes. The *ttg1* mutant phenotype can be rescued completely by expression of the maize bHLH protein R and partially by the *Arabidopsis* bHLH protein glabra3 (GL3; Payne et al., 2000 and references therein) indicating that TTG1 acts upstream of one or more bHLH proteins. GL3 overexpression suppresses the *ttg1–1* mutant phenotype including lack of anthocyanin biosynthesis, but does not give a wild-type complement of trichomes. Overexpression of the MYB transcription factor glabra1 (GL1) does not suppress the *ttg1–1* mutation. In F1 hybrid plants overexpressing GL1 and GL3 in a *ttg1–1* background, trichome numbers and distribution came closer to wild type (Payne et al., 2000). This finding indicates that GL1 and GL3 can interact synergistically in *Arabidopsis* plants, even in the absence of TTG1. However, trichome number was still somewhat lower than in wild-type, suggesting that TTG1 is required for full function of the putative GL3:GL1 complex. Also, physical interactions between GL3 and both GL1 and TTG1 were observed in a yeast two-hybrid assay (Payne et al., 2000). GL3:TTG1 interaction suggests that TTG1 activates, modifies, stabilizes, or in some other fashion has a positive effect on the capacity of the GL3 protein to activate gene transcription. It seems likely that AN11 and TTG1 have similar functions, and they probably regulate the activity of MYB-bHLH complexes.

6. Regulation of transcription factor protein abundance

In addition to regulation of cellular concentrations by transcriptional control, transcription factor abundance can be regulated by adjustment of protein turnover rate. Protein stability is often regulated via covalent modifications such as phosphorylation and/or ubiquitination, and/or via interaction with other proteins.

Biosynthesis of indole glucosinolates in *Arabidopsis* is strongly induced by MeJA and elicitor-induced biosynthesis of these secondary metabolites requires a functional *coronatin-insensitive1* (*COI1*) gene (Brader et al., 2001). The *COI1* gene is also required for jasmonate-dependent defense and fertility in *Arabidopsis* (Xie et al., 1998). Analysis of the *COI1* sequence indicates that it codes for an F-box protein. F-box proteins are part of SCF (SKP1, Cullin and F-box) complexes and are responsible for the specific recognition and targeting of proteins designated for ubiquitin-mediated proteolysis. The *Arabidopsis* F-box protein transport inhibitor response 1 (TIR1) is thought to regulate auxin responses by recruiting the AUX/IAA repressors for ubiquitin-mediated proteolysis (Gray et al., 2001). AUX/IAA proteins are thought to repress the auxin-response pathway by negatively regulating the auxin response transcription factors (ARF). The proposed model is that auxin promotes the binding of AUX/IAA proteins to the SCF^{TIR1} ubiquitin ligase leading to their ubiquitination and subsequent proteasome-mediated degradation, which results in activation of the ARF transcription factors and derepression of the auxin response pathway (Gray et al., 2001). By analogy, COI1 could be involved in degradation of a negative regulator of JA-responsive AP2/ERF-domain transcription factors.

An example of regulation by specific proteolysis combined with phosphorylation is given by the bZIP transcription factor long hypocotyl 5 (HY5), which promotes photomorphogenesis in *Arabidopsis*. HY5 binds to a conserved G box sequence found in the promoters of light-inducible genes such as *chalcone synthase* (*CHS*), and plays a positive role in mediating light-activated *CHS* gene expression and anthocyanin accumulation during seedling development (Hardtke and Deng, 2000). HY5 protein accumulates to about 20-fold

higher levels after shifting seedlings from darkness into light, while *HY5* mRNA levels show only a 2- to 3-fold difference between dark and light, indicating that protein stability plays a role in regulating HY5 abundance. HY5 interacts with constitutive photomorphogenesis1 (COP1; a RING-finger protein with WD repeats), which is a negative regulator of photomorphogenesis. There is evidence that RING-finger proteins can act as E3 ubiquitin-protein ligases to target proteins for degradation (Hardtke and Deng, 2000). HY5 exists in two different phosphorylation states and it is constitutively present in the nucleus. In darkness, COP1 is enriched in the nucleus, presumably targeting the unphosphorylated HY5 isoform for degradation (Hardtke and Deng, 2000). At the same time, casein kinase II (CKII) activity ensures maintenance of a small pool of phosphorylated HY5, which is less susceptible to degradation but physiologically inactive. Upon light stimulus, COP1 is excluded from the nucleus and at the same time HY5-related CKII activity is reduced (Hardtke and Deng, 2000). This results in an increase of physiologically more active unphosphorylated HY5 in the light, and activation of its light-inducible target genes. Together, the parallel regulation of HY5 by nuclear COP1 levels and phosphorylation extends the range for the fine-tuning and the magnitude of HY5 activity.

7. Conclusions

Production of secondary metabolites is under strict regulation in plant cells due to coordinate control of the biosynthetic genes by transcription factors. Here we discussed a few examples of how the activity of such regulators can be regulated. The most direct way is the regulation of transcription factor abundance at the gene expression level. This can be achieved either via a transcriptional cascade, as exemplified by EIN3/ERF1 in the ethylene response and by VP1/C1 in seed maturation, or via auto-regulation, as illustrated by WRKY1 transcription factor gene expression in pathogen defense. Protein modifications and/or protein interactions appear to play an important role in the regulation of several transcription factors. Protein phosphorylation is the best-studied example in plants. Protein interactions can depend on protein phosphorylation state, as illustrated for RSG/14–3-3 interaction in gibberellin biosynthesis. Regulation of protein abundance via targeted degradation of specific regulators, as in photomorphogenesis and in the auxin response, also seems to be an important mechanism. Degradation can also depend on the protein phosphorylation state, as shown for HY5.

It is likely that tissue-specific secondary metabolism is regulated mainly via tissue-specific control of transcription factor mRNA abundance possibly in combination with tissue-specific control of protein abundance. On the other hand, protein interaction and/or modification is the best way for the cell to strictly regulate the activity of a transcription factor in signal transduction pathways that require rapid gene induction. However, it seems to be a rule in plants that genes encoding transcription factors involved in rapid response pathways are themselves also rapidly induced by the same signal (e.g. the WRKYs and the ORCAs). More knowledge on protein interactions and covalent modifications of transcription factors involved in secondary metabolism will facilitate the understanding of their regulation and consequently can provide tools for genetic engineering of secondary metabolite production.

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