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The isoflavonoid phytoalexin pathway: From enzymes to genes to transcription factors

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The pterocarpan phytoalexins of the Leguminosae are synthesized from L-phenylalanine via a minimum of 11 enzymatic steps involving the central phenylpropanoid pathway, three reactions of flavonoid biosynthesis, and the isoflavonoid branch pathway. The extractable activities of all these enyzmes, and of enzymes supplying precursors from primary metabolism, increase in response to fungal infection or exposure of plant cells to elicitor macromolecules isolated from the cell walls of yeast or plant pathogenic fungi. The involvement of reductases and cytochrome P450 hydroxylases places a high demand for NADPH on elicited cells. The NADPH is most likely supplied by activation of the pentose phosphate pathway. Genes or cDNAs encoding 7 of the enzymes involved in the synthesis of the phytoalexin medicarpin have been cloned from alfalfa and/or other species. Induction of enzyme activity results from transcriptional activation of the corresponding genes, leading to increased steady state levels of translatable mRNAs. This transcriptional activation is programmed through the interaction of sets of elicitor/infection-modulated transcription factors with their cognate *cis* elements in the promoters of the phytoalexin biosynthetic genes. Gene activation occurs through generation of intracellular signals which lead to modulation of transcription factor activity, through either increased synthesis of the factor(s), activation via reversible post-translational modification (e.g. phosphorylation/dephosphorylation), translocation of factors from cytoplasm to nucleus, or combinations of these. Coordinated induction of the enzymes of phytoalexin synthesis may involve multiple signals and factors for transcriptional activation, as well as feedback and feed-forward fine controls at both transcriptional and post-transcriptional levels. In beneficial mycorrhizal interactions, induction of early pathway genes is uncoupled from that of later, phytoalexin-specific genes.

Key words – Alfalfa, mycorrhizal associations, plant-pathogen interactions, pterocarpan phytoalexins, secondary metabolism, signal transduction, transcriptional activation.

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Introduction

Antimicrobial isoflavonoids are produced primarily in the Leguminosae. In the most studied species of agronomic importance, namely soybean, pea, alfalfa, chickpea and green bean, pterocarpan phytoalexins accumulate in most organs of the plant in response to fungal infection, whereas isoflavones or isoflavone and pterocarpan glycosides may accumulate constitutively, particularly in roots (Graham 1991, Harrison and Dixon 1993). The phytoa-

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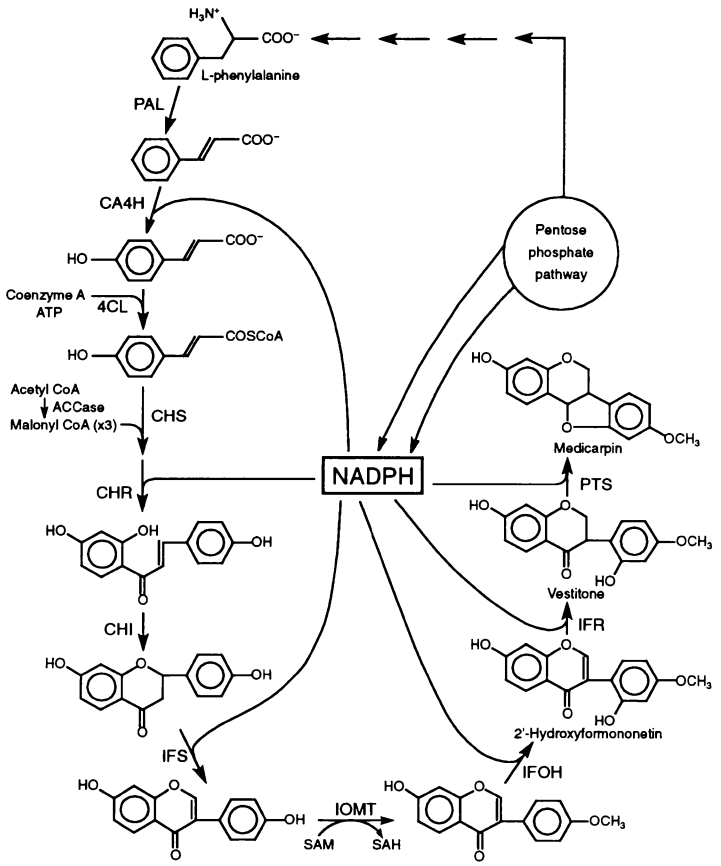

Fig. 1. Biosynthesis of medicarpin from L-phenylalanine. See list of abbreviations for enzymes. SAH, S-adenosyl L-homocysteine.

lexin response seen in intact tissues can be quantitatively reproduced in cell suspension cultures exposed to fungal elicitor macromolecules (Dixon 1980, Kessmann and Barz 1987).

The isoflavonoids are the best understood class of phytoalexins in terms of their biosynthesis, enzymology and molecular biology. Furthermore, their role in disease resistance has been addressed through many studies correlating the timing and localization of their appearance with inhibition of microbial ingress (Rhodes 1985), and there is supportive, albeit indirect, genetic evidence for a role in resistance based on the lack of virulence of fungal progeny unable to detoxify isoflavonoid phytoalexins (Van Etten et al. 1989). The recent cloning of genes encoding enzymes specific for late stages of isoflavonoid phytoalexin biosynthesis now makes it possible to address phytoalexin function directly by reverse genetics.

Isoflavonoids are synthesized from L-phenylalanine via a series of enzymes that can be grouped into 3 subpathways: the core phenylpropanoid pathway from phenylalanine to 4-coumaroyl coenzyme A, (CoA); reactions of flavonoid synthesis utilizing 4-coumaroyl CoA and malonyl CoA to yield a flavanone intermediate; and the isoflavonoid-specific branch pathway (Fig. 1). These three sub-pathways are under complex, and often differential, developmental and environmental control, but their respective enzymes are coordinately induced in response to infection or elicitation (Daniel et al. 1988). The pathway therefore serves as an excellent model for dissecting the molecular mechanisms underlying coordinate gene expression in plant secondary metabolism. It is also an important potential target for genetic manipulation, and strategies for metabolite engineering to yield more effective antimicrobial defenses have recently been reviewed (Lamb et al. 1992).

The forage legume alfalfa is a good model for studies of isoflavonoid phytoalexin biosynthesis, regulation and genetic manipulation in view of its economic importance, its biochemistry (the alfalfa phytoalexin medicarpin is structurally the simplest of the pterocarpans), and its ease of genetic transformation compared to other legumes such as soybean and bean. The biochemistry and molecular biology of the alfalfa phytoalexin response has been reviewed (Dixon et al. 1992). This article will concentrate on more recent results and important outstanding questions.

Abbreviations – ACCase, acetyl coenzyme A carboxylase (EC 6.4.1.2); CA4H, cinnamic acid 4-hydroxylase (EC 1.14.13.11); CHI, chalcone isomerase (EC 5.5.1.6); CHR, chalcone reductase (EC 1.1.1.–); CHS, chalcone synthase (EC 2.3.1.74); 4CL, 4-

Tab. 1. Genes of isoflavonoid biosynthesis in alfalfa. ND, not determined. *Determined by nuclear transcript run-on analysis.

Gene encoding	Number of genes	Size of open reading frame (amino acids)	Timing of maximimum transcriptional activity (h post-elicitation)*	Localization of gene product
PAL	>3	725	4	Cytoplasm
CA4H	2	506	6	ER
ACCase	2	2 257	ND	Cytoplasm
CHS	>7	389	9	Cytoplasm
CHR	>2	312	1.5	Cytoplasm
CHI	ND	incomplete	3	Cytoplasm
IFR	1	318	9	Cytoplasm

coumarate:CoA ligase (EC 6.2.1.12); IFOH, isoflavone 2'-hydroxylase (EC 1.14.13.52); IFR, isoflavone reductase (EC 1.3.1.45); IFS, isoflavone synthase (EC 5.4.99.–); IOMT, isoflavone 4'-O-methyltransferase; PAL, L-phenylalanine ammonia-lyase (EC 4.3.1.5); PTS, pterocarpan synthase (EC 1.1.1.246); SAM, S-adenosyl L-methionine.

Induced enzymes and genes of medicarpin synthesis

The pathway of medicarpin synthesis (Fig. 1) was initially elucidated by radiolabeled precursor feeding experiments (Dewick and Martin 1979, Martin and Dewick 1980) and has been confirmed, in its essentials, by characterization of most of the enzymes involved. The 6 reactions requiring NADPH comprise 3 reductases (chalcone reductase [CHR], isoflavone reductase [IFR] and pterocarpan synthase [PTS]) and 3 cytochrome P450s (cinnamic acid 4-hydroxylase [CA4H], isoflavone synthase [IFS] and isoflavone 2'-hydroxylase [IFOH]). Apart from the ER-associated P450s, all the activities are believed to be located in the soluble cytoplasm. Molecular properties of phenylalanine ammonia-lyase (PAL), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI) have been reviewed elsewhere (Dixon and Harrison 1990). Information on isoflavonoid pathway genes cloned from alfalfa is given in Tab. 1.

Although the activities of the 3 P450 enzymes have been demonstrated in vitro, CA4H is the only P450 of the phytoalexin pathway to have been characterized at the molecular level, having recently been cloned from Jerusalem artichoke (Teutsch et al. 1993), mung bean (Mizutani et al. 1993) and alfalfa (Fahrendorf and Dixon 1993). Unfortunately, sequence homologies among different classes of plant P450 are very low; for example, alfalfa CA4H is less than 60% identical at the nucleotide level and 31% identical at the amino acid level to the first reported plant P450 sequence, that of avocado P450 CYP71A1. Therefore, it not possible to clone other P450s by screening cDNA libraries with CA4H sequences. However, with an increasing number of plant P450 sequences appearing in the databases, it has become possible to design polymerase chain reaction (PCR) primer strategies for cloning these genes, and this is now the method of choice in view of the extreme difficulty in purifying these membrane-bound enzymes to homogeneity. Confirmation of identity can be obtained through expression studies in yeast, which, in the case of alfalfa CA4H, supports correct processing (addition of heme), membrane targeting and accessibility to cytochrome P450 reductase (Fahrendorf and Dixon 1993). An elicitor-induced P450 which is distinct from CA4H has recently been isolated by this strategy (T. Fahrendorf, unpublished data).

It is is often forgotten that malonyl CoA is as important a precursor as phenylalanine for synthesis of flavonoids and isoflavonoids. The activity of acetyl CoA carboxylase (ACCase), which produces malonyl CoA from acetyl CoA, is induced approximately 3-fold in elicited alfalfa cells. An alfalfa ACCase has recently been cloned (Shorrosh et al. 1994). On the basis of lack of a chloroplast transit peptide, this would appear to represent a cytoplasmic form of the enzyme involved in the provision of malonyl CoA for flavonoid synthesis and long chain fatty acid elongation. It should be noted that most malonyl CoA is destined for de novo fatty acid synthesis and is formed by a chloroplastic ACCase (Post-Beittenmiller et al. 1992). The 7 194 bp ACCase transcript from alfalfa encodes a polypeptide of 2 257 amino acids with a deduced M_r of 252 039. This polypeptide has a functional architecture similar to that of enzymes from mammals and yeast, containing biotin carboxylase, biotin carboxyl carrier protein and carboxyltransferase domains on the same polypeptide. Availability of cloned ACCase sequences should allow reverse genetic approaches for investigating how distribution of the flux of malonyl CoA between primary (fatty acid) and secondary (flavonoid) metabolism is regulated in infected or elicited cells.

Most isoflavonoid phytoalexins are of the 5-deoxy class, lacking one of the 3 A-ring hydroxyls that are a consequence of the construction of the A-ring by head-to-tail condensation of 3 malonyl groups (Kreuzaler and Hahlbrock 1975). The mechanism of loss of the 5-hydroxyl group was puzzling for many years, until the demonstration of the existence of a CHR, which co-acts with CHS to reduce a keto group on the polyketide intermediate, with subsequent loss of H_2O, prior to ring

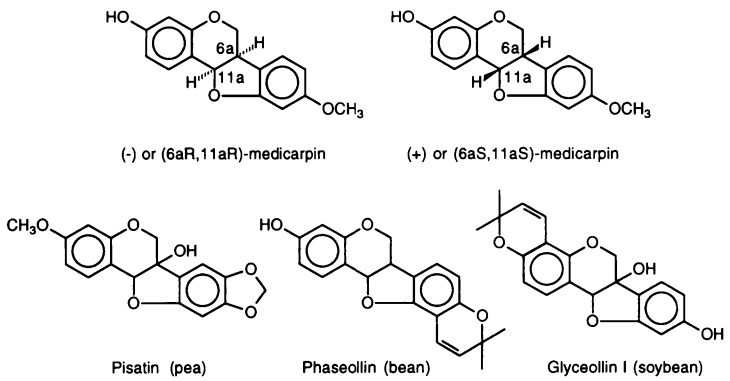

Fig. 2. Structures of pterocarpan phytoalexins.

closure to yield the chalcone (Welle and Grisebach 1988). This NADPH-dependent reductase has been cloned from soybean (Welle et al. 1991) and alfalfa (M. Ballance and R. A. Dixon, unpublished data). The 5-deoxy class of flavonoid/isoflavonoid, the synthesis of which it initiates, is involved primarily in interactions of plants with microorganisms, in contrast to the 5-hydroxy series which includes anthocyanin flower pigments and UV protective flavonoids. Induction of CHR is highly coordinated with that of CHS in elicited soybean and alfalfa cell suspension cultures (Welle and Grisebach 1989, M. Ballance, unpublished data).

Isoflavone synthase catalyzes the first reaction specific for isoflavonoid biosynthesis. Although the mechanism of aryl migration is not fully understood, it is believed to involve a two-step reaction initiated by a P450-dependent hydroxylation of the 2 or 3 position of the flavanone substrate (Kochs and Grisebach 1986). Early precursor labelling supported a model in which methylation of the 4'-hydroxyl was an integral part of the aryl migration step of the isoflavone synthase reaction (Dewick and Martin 1979), although the nature of the substrate and product of in vitro assay of isoflavone synthase clearly suggests that this is not the case. However, the introduction of the 4'-methoxy group on the isoflavone B-ring is still not understood at the enzymological level. An isoflavone O-methyltransferase activity is strongly induced in elicitor-treated alfalfa cells but, surprisingly, this methylates the A-ring rather than the B-ring hydroxyl, at least in vitro (Edwards and Dixon 1991). A similar situation holds in chickpea (W. Barz, personal communication).

The pterocarpan phytoalexins of the Leguminosae can exist in two possible stereoisomeric forms, (+) or (−) (6aS, 11aS or 6aR, 11aR, respectively, in the case of the medicarpins; Fig. 2). Generally, one species produces only one isomer, and this is more commonly the (−) isomer (e.g. in alfalfa, chickpea, bean and soybean). However, pea produces (+) pisatin, and peanut (+) medicarpin. The first of the two chiral centers is introduced by IFR. This enzyme has been characterized biochemically from chickpea and pea and cloned from chickpea (Tiemann et al. 1991), pea (Paiva et al. 1994) and alfalfa (Paiva et al. 1991). The alfalfa IFR expressed in *E. coli* produces (−) vestitone from 2'-hydroxyformononetin, consistent with the accumulation of (−) medicarpin in alfalfa. Surprisingly, the cloned pea IFR also produces a (−) isoflavanone product (Paiva et al. 1994), contradictory to a previous report of the activity found in pea cell extracts (Sun et al. 1991). It now appears possible that an epimerase is involved in the conversion of (−) isoflavanone to (+) isoflavanone in those species producing (+) pterocarpan phytoalexins. This is consistent with the stereochemical requirements of the pterocarpan synthases in legumes producing (−) or (+) pterocarpans (N.L. Paiva, unpublished data). Genetic manipulation of pterocarpan chirality is a potential strategy for improving disease resistance, as many pathogenic fungi are more sensitive to the stereoisomer that is not produced by their host plant than to the isomer they generally meet in nature and are often able to detoxify (VanEtten et al. 1989).

Prenyl, methylene-dioxy or 6a-hydroxy substitutents are found on the pterocarpan phytoalexins of pea, bean and soybean (Fig. 2). Isoflavonoid prenyltransferases have been partially characterized from bean, soybean and lupin (Biggs et al. 1987, Welle and Grisebach 1991, Laflamme et al. 1993). Surprisingly, the origin of the 6a-hydroxyl group on the pea phytoalexin pisatin and the soybean phytoalexin glyceollin appears to be different, arising from molecular oxygen (via a cytochrome P450 reaction) in soybean (Kochs and Grisebach 1989) and from H_2O in pea (Matthews et al. 1987). More work is clearly needed to elucidate the enzymology of the final stages of pterocarpan phytoalexin biosynthesis.

Coordinated transcriptional activation of phytoalexin biosynthetic genes

Genes and/or cDNAs encoding 7 of the enzymes of medicarpin biosynthesis have been cloned from alfalfa (Tab. 1), and PAL, CA4H, 4CL, CHS and CHI genes have been isolated from other legumes (Dixon and Harrison 1990). Several are encoded by multigene families, the largest number of genes (>7 in alfalfa) being observed for CHS (Junghans et al. 1993). Elicitation results in in-

Tab. 2. *Cis*-elements in the promoters of elicitor-inducible genes of isoflavonoid phytoalexin synthesis. Core consensus sequences are in bold. In parsley, the phytoalexins are furanocoumarins.

Gene	Sequence	Name of element
Bean *chs15*	5'-TTAA**AGTTAA**AAAC	SBF-I Box I
Bean *chs15*	5'-TATT**GGTTAC**TAAA	SBF-I Box III
Bean *chs 15*	5'-**CCTACC**TCACGAA**CT**	H-box I
Bean *chs15*	5'-**CCTACC**CTACTTC**CT**	H-box III
Alfalfa *chs2*	5'-**CCTACC**AAACTCCAA	H-box
Bean *chs15*	5'-GTTG**CACGTG**ATAC	G-box
Parsley *pal-1*	5'-TTAAT**CTCCAACAAACCCCT**	Box P
Bean *pal-2*	5'-TATTT**CTCCACCAACCCCCT**	Box P
Alfalfa *chs2*	5'-**CTCCAACA**AGGTCAA	Box P
Alfalfa *ifr*	5'-**TACCACCTAAC**ATCA	Box P

creases in steady state transcript levels for enzymes encoding each of the steps of the pathway. Use of gene-specific probes has demonstrated that transcripts encoding at least 5 different CHS genes are induced in elicited or infected alfalfa cells (Junghans et al. 1993). Similarly, induction of multiple PAL and CHS genes has been demonstrated in other legume species (Dixon and Harrison 1990). Nuclear transcript run-on analysis has shown that elicitor-induced increases in steady state transcript levels are a result of increased transcriptional activity of the corresponding genes (Chappell and Hahlbrock 1984, Lawton and Lamb 1987, Somssich et al. 1989). Futhermore, this transcriptional activation is closely coordinated, with near identical induction kinetics being observed for PAL, CHS and IFR transcription over the first 3 h post-elicitation in alfalfa cells (W. Ni and R.A. Dixon, unpublished data). Increased transcription can be measured as early as 5 min post-elicitation (Lawton and Lamb 1987).

Elicitor-induced transcriptional activation is not limited to genes encoding enzymes of the phytoalexin pathway. The 6 molecules of NADPH required per molecule of medicarpin synthesized most probably arise through the activity of the pentose phosphate pathway (Fig. 1). In alfalfa, increased activities of two key enzymes of this pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are preceeded by increased transcription of their corresponding genes in response to elicitor (T. Fahrendorf and R.A. Dixon, unpublished data). A similar situation holds for SAM synthetase (Gowri et al. 1991), which provides the methyl group donor for the isoflavone O-methyltransferase reaction. It is not known whether these genes are directly responsive to the initial elicitation signal, or are induced in response to a secondary metabolic event such as a change in metabolite pools or redox-state.

Isoflavonoid metabolism in mycorrhizal interactions

The establishment of a symbiotic interaction between roots of the alfalfa relative *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme* is characterized by induction of PAL and CHS transcript levels but suppression of IFR transcripts, corresponding to a small increase in the levels of some flavonoid derivatives, including 7,4'-dihydroxyflavone, daidzein and formononetin malonyl glycoside, but a decrease in isoflavonoid phytoalexins (Harrison and Dixon 1993). In situ hybridization has indicated that the increase in PAL and CHS transcripts is localized specifically to cells containing fungal arbuscules (the site of nutrient and ion transport between host and symbiont), whereas IFR transcripts are reduced throughout the root cortex, suggesting responsiveness of IFR transcription or mRNA stability to a diffusible signal of plant or fungal origin (Harrison and Dixon 1994). The evolution of beneficial fungal interactions clearly necessitates mechanisms for differential regulation of the components of a biosynthetic pathway which are coordinately regulated in response to fungal pathogens.

Cis-elements and *trans* acting factors for isoflavonoid pathway gene expression

In order to address the molecular mechanisms underlying coordinated transcriptional activation of elicitor-responsive genes, it is first necessary to know which sequence elements in the upstream promoter regions of these genes are needed for elicitor- or infection-responsive expression. Such elements may be essential for transcriptional activation or may quantitatively modulate expression by acting as enhancers or suppressors. Deletional and mutational analysis of PAL, 4CL and CHS promoter-reporter gene fusions in stably transformed plants or in protoplast transient expression systems has defined a number of *cis*-elements which appear important for elicitation (Dangl 1992; Tab. 2). These elements have been shown, by gel-retardation and in vitro or in vivo footprinting techniques, to bind nuclear proteins in a sequence-specific manner. Thus, the SBF-1 elements present in bean and alfalfa CHS promoters have been implicated as silencer or enhancer elements depending on the cells in which the promoter-reporter construct is being expressed (Harrison et al. 1991a). They bind a 95 kDa protein with an essential requirement for the two G residues of the 5'-GGTTAA consensus binding site. This protein is present equally in nuclear extracts from elicited or unelicited cells, but loses DNA binding activity on dephosphoryla-

tion (Harrison et al. 1991b), suggesting a mechanism for its regulation.

The H-box and G-box elements of the bean CHS15 promoter (also present in an alfalfa CHS promoter) have been implicated together in transcriptional activation in response to elicitor (Loake et al. 1992, Arias et al. 1993). The H-box (5'-CCTACC[N_7]CT) specifically binds two factors, KAP-1 (97 kDa) and KAP-2 (76 and 56 kDa) which retain DNA binding activity upon dephosphorylation. Both factors have an absolute requirement for the cytosine pairs of the 5'-CCTACC core consensus sequence. Although the total cellular concentrations of KAP-1 and KAP-2 do not appear to change upon elicitation, there is evidence for movement of both factors from cytoplasm to nucleus at the onset of CHS transcription (Yu et al. 1993). This suggests a model whereby post-translational modification (such as phosphorylation) of CHS transcription factors in the cytoplasm as an early event in elicitor-mediated signal transduction leads to rapid nuclear localization of an active transcription factor or factor complex, by analogy with activation of interferon-stimulated genes in mammalian cells (Schindler et al. 1992). Such a direct pathway of signal transduction would be consistent with the rapid rate of gene activation in elicited cells.

Box P is an element in the parsley PAL promoter shown by in vivo footprinting to bind a factor in response to elicitation (Lois et al. 1989). This factor, BPF-1, has been cloned, and is a member of a novel class of transcription factors, containing acidic, basic and α-helical domains (da Costa e Silva et al. 1993). Elicitation results in a rapid increase in BPF-1 transcripts, accompanying, but not preceeding, PAL transcription. It is possible that the increase in BPF-1 levels is necessary to support continued PAL transcription rather than to initiate transcriptional activation. It should be noted that parsley produces furanocoumarin rather than flavonoid-derived phytoalexins. However, elements similar to box P are found in CHS and IFR promoters (Tab. 2).

The simplest model to account for the coordinated transcriptional activation of several genes encoding enzymes of a complex metabolic pathway would be one in which each gene promoter contained one or more copies of a common stimulus-response element, as occurs with the heat-shock or anaerobic response elements found in other groups of co-regulated genes (Nagao et al. 1990, Walker et al. 1987). Inspection of available elicitor-/infection-responsive promoter sequences suggests that the picture is not so simple. For example, the alfalfa IFR gene is transcriptionally activated in cell cultures with kinetics identical to CHS, but contains neither SBF-1, G-box nor H-box elements. The element in the IFR promoter most closely resembling the parsley box P can be deleted with no loss in elicitor-responsiveness (A. Oommen, R.A. Dixon and N.L. Paiva, unpublished data). The regulatory architecture of isoflavonoid-pathway genes may have first evolved to satisfy developmental requirements, in which case different transcriptional activation mechanisms could have been in place to flexibly regulate different sectors or branches of the pathway prior to evolutionary selection of isoflavonoids as antimicrobial agents. The molecular components of such mechanisms may be an integral part of the defense responsive transcriptional machinery.

The exact nature of the signals which link elicitor perception to defense gene activation is still not known. The demonstration that the *Pto* gene, which confers resistance of tomato to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, is a serine-threonine protein kinase with homologs in many plant species (Martin et al. 1993) adds to the increasing evidence implicating reversible phosphorylation/dephosphorylation (along with rapid changes in oxidative metabolism such as production of active oxygen species and changes in cellular redox state) as key signal transduction components (reviewed by Dixon et al. 1994, Lamb 1994). The biochemical characterization of isoflavonoid pathway transcription factors and their functional modification is central to establishing the chain of events initiated by the biological recognition of an avirulent pathogen by a resistant host legume.

Although several genes in the medicarpin pathway are activated coordinately, the overall kinetics of the transient increases in transcription rate, transcription levels and enzymatic activity differ, suggesting that specific controls operate for turnover of transcripts and enzyme activity. Cinnamic acid, the product of the PAL reaction, and 4-coumaric acid, the product of CA4H, have been shown to regulate (negatively and positively, respectively) transcription of the bean CHS15 promoter in electroporated alfalfa protoplasts (Loake et al. 1991, 1992), although it is difficult to show a correlation between the pool sizes of these pathway intermediates and changes in PAL or CHS transcript levels (Orr et al. 1993). Treatment of bean cells with L-α-aminooxy-β-phenylpropionic acid, a potent inhibitor of PAL activity, results in superinduction of PAL and CHS transcripts and transcription rate (Bolwell et al. 1988), consistent with involvement of pathway intermediates as negative transcriptional regulators. The decline in PAL activity subsequent to attainment of maximum levels post-elicitation requires protein synthesis, and evidence has been presented for the involvement of a proteinaceous PAL inhibitor which may require cinnamic acid for its activity (Bolwell et al. 1986). Pathway intermediates could also regulate mRNA stability. An elicitor-induced redox perturbation has been proposed to activate an RNA-binding protein which recognizes an element in the 3'-untranslated region of an elicitor down-regulated proline-rich protein gene (Zhang and Mehdy 1994). A similar mechanism could operate to remove phytoalexin biosynthetic gene transcripts post-elicitation. Clearly, our understanding of post-transcriptional and post-translational events in defense gene regulation is significantly less than our still incomplete understanding of transcriptional activation mechanisms.

Concluding remarks

Work on the isoflavonoid pathway is now moving from a descriptive phase (in which metabolites and enzymes were characterized) to a broader focus utilizing the newest techniques of molecular and structural biology. Transgenic strategies can now be used for metabolite engineering to modify isoflavonoid compounds, qualitatively and quantitatively, thereby addressing their true functions in the plant and providing possible strategies for improving disease resistance. Studies of protein structure:function relationships within the isoflavonoid and related pathways can provide an information base for protein engineering to modify enzyme stereo- and regio-specificity. Finally, the isoflavonoid pathway has emerged as an excellent model system for studying coordinate gene expression and signal transduction within a complex plant biosynthetic pathway. Development of mutant screens with genetically tractable species such as *Medicago truncatula* (Dixon et al. 1994) will be a powerful complement to the biochemical and molecular biological approaches currently being taken to dissect the signal pathways for isoflavonoid pathway gene activation.

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