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Stress-Induced Phenylpropanoid Metabolism

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INTRODUCTION

Phenylpropanoid compounds encompass a wide range of structural classes and biological functions. Limiting discussion to stress-induced phenylpropanoids eliminates few of the structural classes, because many compounds that are constitutive in one plant species or tissue can be induced by various stresses in another species or in another tissue of the same plant (Beggs et al., 1987; Christie et al., 1994).

Biosynthesis of Stress-Induced Phenylpropanoids

All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL), the branch point enzyme between primary (shikimate pathway; see Herrmann, 1995, this issue) and secondary (phenylpropanoid) metabolism (Harborne, 1988; Hahlbrock and Scheel, 1989; Lewis and Yamamoto, 1990; Dixon et al., 1992). The biosynthetic relationships among many of the classes of phenylpropanoids are outlined in Figure 1; representative structures are shown, but the substituents can vary widely among plant species.

Several simple phenylpropanoids (with the basic C6-C3 carbon skeleton of phenylalanine) are produced from cinnamate via a series of hydroxylation, methylation, and dehydration reactions; these include *p*-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins (Figure 1). The free acids rarely accumulate to high levels inside plant cells; instead, they are usually conjugated to sugars (e.g., salicylate–glucose conjugates), cell wall carbohydrates (e.g., ferulate esters), or organic acids (e.g., sinapate esters, chlorogenic acid). Salicylic, benzoic, and *p*-hydroxybenzoic acids, although not strictly phenylpropanoids themselves because they lack the three-carbon side chain, originate from the phenylpropanoids cinnamate and *p*-coumarate (Schnitzler et al., 1992; León et al., 1993; Yalpani et al., 1993). Lignin and suberin are complex polymers formed from a mixture of simple phenylpropanoids; their composition varies from species to species (Lewis and Yamamoto, 1990; Bernards and Lewis, 1992; see Whetten and Sederoff, 1995, this issue).

A large number of stress-induced phenylpropanoids are derived from the C15 flavonoid skeleton, which is synthesized via the chalcone synthase (CHS)–catalyzed condensation of *p*-coumaroyl–coenzyme A (CoA) and three molecules of malonyl-CoA (Harborne, 1988). In most plant families, the initial product of CHS is a tetrahydroxychalcone, which is further converted to other flavonoid classes, such as flavones, flavanones, flavanols, anthocyanins (see Holton and Cornish, 1995, this issue), and 3-deoxyanthocyanidins. In legumes, which possess chalcone reductase (CHR) as well as CHS, a trihydroxychalcone may be formed (Welle and Grisebach, 1989). In a number of species, including pine, grapevine, and peanut, the condensation of *p*-coumaroyl–CoA or cinnamoyl-CoA with three malonyl-CoA molecules can also give rise to stilbenes by the action of stilbene synthase (SS) (Schröder et al., 1988). In legumes, isoflavone synthase (IFS) rearranges the flavonoid carbon skeleton, leading to the accumulation of a wide range of simple isoflavonoids, coumestans, pterocarpans, and isoflavans. Structural diversity among the phenylpropanoids is brought about by a variety of modifications, including regiospecific hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation.

Before molecular biology techniques became available, the sequence of reactions in many of the induced phenylpropanoid biosynthetic pathways was determined using a combination of enzyme purification and labeled precursor feeding approaches. In the case of anthocyanin biosynthesis, the isolation by breeders of mutants altered in flower color was of major assistance. Classical biochemical approaches, in combination with improved methods of chemical structure analysis, will continue to advance our knowledge of the pathways of phenylpropanoid formation, as witnessed by recent developments in lignan and suberin biosynthesis (Davin and Lewis, 1992; Bernards et al., 1995). However, molecular and genetic approaches have given us new insights into the enzymology of phenylpropanoid synthesis, the cellular and subcellular sites of synthesis, and the role(s) or significance of phenylpropanoid compounds in the life of the plant as well as providing information on the molecular genetic control of induction of the phenylpropanoid pathway. It is these advances that are discussed in this review.

Functions of Stress-Induced Phenylpropanoids

Until recently, postulated functions for stress-induced phenylpropanoids had been based almost solely on correlative

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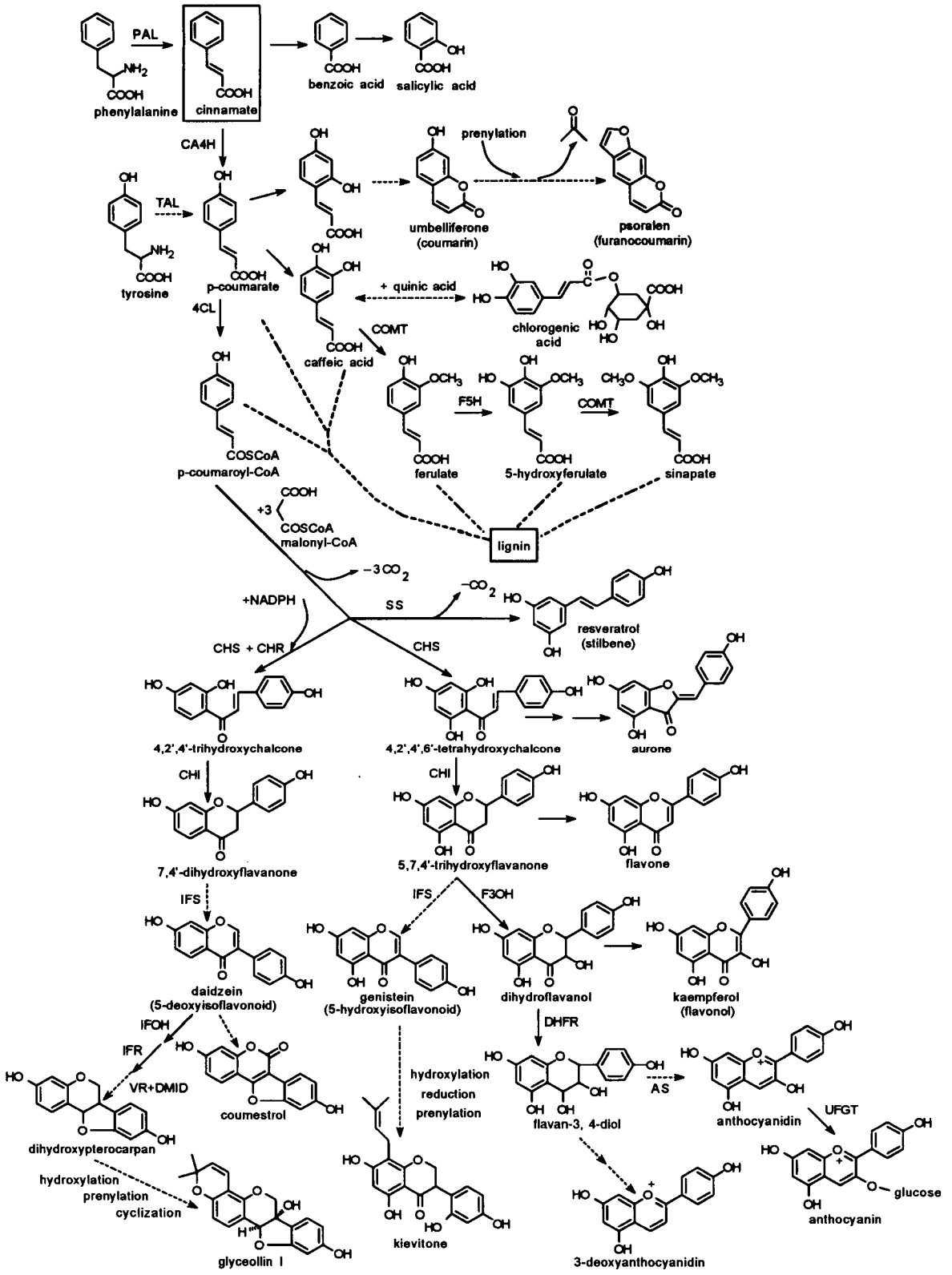

Figure 1. Biosynthetic Relationships among Stress-Induced Phenylpropanoids.

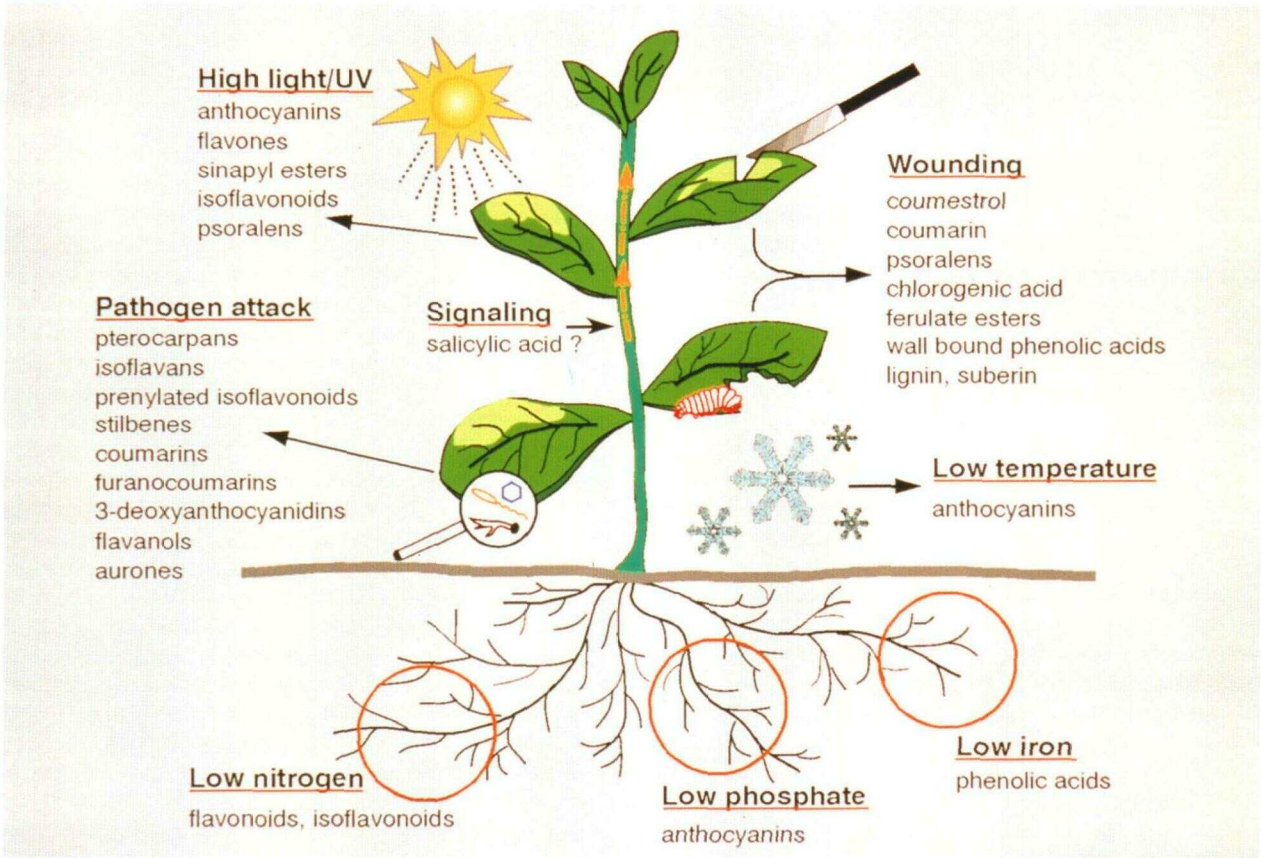

Figure 2. Examples of Stress-Induced Phenylpropanoids.

evidence. In few cases had these functions been rigorously tested by, for example, isolating mutants that no longer produce the metabolite and examining whether sensitivity to stress is increased.

Figure 2 summarizes the types of phenylpropanoid compounds induced in plants by various biotic and abiotic stresses. Many stress-induced phenylpropanoids are classified as phytoalexins. These are antimicrobial compounds synthesized in response to pathogen attack. They include pterocarpans (e.g., glyceollin), isoflavans, prenylated isoflavonoids (e.g., kievitone), stilbenes, psoralens, coumarins, 3-deoxyanthocyanidins, flavonols (e.g., quercetin, kaempferol), and aurones (Bailey and Mansfield, 1982; Dixon et al., 1995). The levels of these compounds increase greatly around the site of infection to concentrations toxic to pathogens in in vitro bioassays. Salicylic acid levels increase in tobacco, cucumber, and Arabidopsis in response to infection and exposure to UV light and ozone (Rasmussen et al., 1991; Yalpani et al., 1994), but rather than salicylic acid having antimicrobial activity per se, it is believed to be part of a signaling process that results in systemic

Figure 1. (continued).

Solid arrows represent well-characterized reactions catalyzed by single enzymes, many of which have been cloned. Dashed lines represent transformations that require multiple enzymes, that are less characterized, or that vary among plant species. The enzymes are CA4H, cinnamic acid 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; 4CL, 4-coumarate:coenzyme A ligase; COMT, caffeic acid *O*-methyltransferase; DHFR, dihydroflavonol reductase; DMID, 7, 2′-dihydroxy-4′-methoxyisoflavanol dehydratase; F3OH, flavanone 3-hydroxylase; F5H, ferulic acid 5-hydroxylase; IFR, isoflavone reductase; IFS, isoflavone synthase; PAL, L-phenylalanine ammonia-lyase; SS, stilbene (or resveratrol) synthase; TAL, tyrosine ammonia-lyase; UFGT, UDP-glucose flavonol 3-*O*-glucosyl transferase; VR, vestitone reductase. The reaction to form pterocarpans was formerly thought to be carried out by a single enzyme, pterocarpan synthase. It has recently been shown in alfalfa that two enzymes, a reductase (VR) and a dehydratase (DMID), act together to catalyze this reaction (Guo et al., 1994). Similarly, the steps labeled IFS and AS each appear to be catalyzed by two enzymes, the second in each case being a dehydratase, neither of which has been fully characterized (Kochs and Grisebach, 1986; Heller and Forkmann, 1988). Although TAL activity is commonly observed in the grasses, it may result from PAL enzyme with nonstringent substrate specificity.

acquired resistance (SAR) (Rasmussen et al., 1991; Delaney et al., 1994). It is not known whether other phenolic compounds play similar signaling roles in plants.

Many phenylpropanoid compounds are induced in response to wounding or to feeding by herbivores. Increased levels of coumestrol and coumarin are toxic to potential herbivores, causing estrogenic and anticoagulant effects, and psoralens can cause photo-induced blistering (Smith, 1982). Wound-induced chlorogenic acid, alkyl ferulate esters, and cell wall–bound phenolic esters may act directly as defense compounds or may serve as precursors for the synthesis of lignin, suberin, and other wound-induced polyphenolic barriers (Hahlbrock and Scheel, 1989; Bernards and Lewis, 1992). The accumulation of flavonols such as kaempferol and its glycosides is induced by both wounding and pollination in petunia stigmas and appears to be required for normal pollen development (Mo et al., 1992; van der Meer et al., 1992; Vogt et al., 1994); these flavonoids may also serve to prevent microbial infection in an otherwise nutrient-rich environment.

Anthocyanins and flavones increase in response to high visible light levels, and it is thought that these compounds help attenuate the amount of light reaching the photosynthetic cells (Beggs et al., 1987). UV irradiation induces flavonoids (particularly kaempferol derivatives) and sinapate esters in Arabidopsis and isoflavonoids and psoralens in other species (Hahlbrock, 1981; Beggs et al., 1985; Li et al., 1993; Lois, 1994). These UV-absorbing compounds are thought to provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage.

Other stresses that induce phenylpropanoids have been less well studied. Levels of anthocyanins increase following cold stress (Christie et al., 1994) and nutritional stress (notably phosphate limitation), but the reasons for this increase are unclear. Other nutritional stresses cause increases in the concentrations of phenylpropanoids in roots or root exudates; for example, low nitrogen induces flavonoid and isoflavonoid *nod* gene inducers and chemoattractants for nitrogen-fixing symbionts (Graham, 1991; Wojtaszek et al., 1993), whereas low iron levels can cause increased release of phenolic acids, presumably to help solubilize metals and thereby facilitate their uptake (Marschner, 1991).

MOLECULAR AND GENETIC APPROACHES TO UNDERSTANDING THE ENZYMOLOGY OF STRESS-INDUCED PHENYLPROPANOID BIOSYNTHESIS

Classical biochemical techniques have been used to isolate and purify many of the enzymes of phenylpropanoid biosynthesis and to analyze isoform patterns. Work on flavonol sulfotransferases has revealed the presence of several similarly sized enzymes that can be resolved only by isoelectric focusing, each catalyzing the sulfation of a specific flavonol hydroxyl (Varin, 1992). Similarly, by examining product ratios in a series of lupin tissue extracts, it became apparent that several highly regio-specific isoflavonoid prenyltransferases are present in this plant (Laflamme et al., 1993). Information of this type is often a prerequisite for meaningful molecular studies.

Because stress induction of phenylpropanoid biosynthesis is likely to involve increases in steady state transcript levels for the various biosynthetic enzymes, strategies such as differential hybridization, subtraction library screening, or differential mRNA display would seem to be attractive approaches for the isolation of cDNAs corresponding to stress-induced phenylpropanoid biosynthetic enzymes. However, to date the most common method for cloning phenylpropanoid pathway genes has been purification of the enzyme to homogeneity followed by either antibody generation and immunoscreening (e.g., Paiva et al., 1991) or by partial amino acid sequence determination and subsequent screening with oligonucleotides, either directly or using the polymerase chain reaction (e.g., Maxwell et al., 1993).

Polymerase chain reaction techniques have been successfully applied to isolate cDNA clones encoding novel types of plant cytochrome P-450 enzymes (Meijer et al., 1993; T. Fahrendorf and R.A. Dixon, unpublished results), using degenerate primers based on conserved regions in mammalian and bacterial P-450s. Although the functions of these P-450s have yet to be determined, this approach may be useful in obtaining clones for several of the as yet uncharacterized phenylpropanoid pathway P-450 genes, such as those encoding the low-abundance and unstable enzymes IFS and isoflavone 2′-hydroxylase (IFOH). Such clones can be identified by expression of P-450 activity in yeast (Fahrendorf and Dixon, 1993; Pierrel et al., 1994). With the dramatic improvements in cloning and expression systems for *Escherichia coli* and yeast and the increases in assay sensitivity and automation, initial identification of clones by direct enzyme assay may become increasingly common.

Recently, some genes of phenylpropanoid biosynthesis have been cloned by genetic strategies involving identification of mutants with defects in this process. For example, T-DNA tagging in Arabidopsis has been used to isolate the gene encoding ferulate 5-hydroxylase (F5H), an enzyme involved in the synthesis of lignin monomers and wall-esterified phenolics (Chapple, 1994). Tagging with the *Ac* transposable element was used in maize to clone UDP glucose-flavonol glucosyltransferase (UFGT) (Fedoroff et al., 1984). Development of similar genetic approaches for other species, particularly those that contain pathways not found in maize and Arabidopsis (such as the legumes *Medicago truncatula* or *Lotus corniculatus*, which have potential as model systems for the isoflavonoid pathway), would facilitate the isolation of those stress-induced phenylpropanoid-related genes whose products are difficult to purify (e.g., P-450s) or are low in abundance (e.g., regulatory transcription factors).

Genome and random cDNA sequencing projects have identified clones with high homology to genes previously isolated from other species (Newman et al., 1994), but the assignment of function must be confirmed by careful biochemical or genetic

complementation evidence. For example, isoflavone reductase (IFR) is an enzyme specific to isoflavonoid biosynthesis, a pathway so far restricted to legumes. IFR has been cloned from alfalfa and pea, and the protein expressed in *E. coli* has been shown to have the same substrate and product specificity as the purified native plant enzyme (Paiva et al., 1991, 1994). Recently, differential screening approaches have resulted in the isolation of IFR-like cDNAs from tobacco, a plant that does not accumulate isoflavonoids (Drews et al., 1992; Hibi et al., 1994). One tobacco IFR-like protein was 58% identical and 79% similar to the alfalfa protein, but it showed no IFR activity when expressed in *E. coli*, indicating that homology data can be misleading.

Antisense and sense suppression strategies are also useful for confirming the identity of cloned genes of phenylpropanoid metabolism, even though a molecular understanding of these phenomena is still lacking (van Blokland et al., 1994). Antisense suppression of caffeic acid/5-hydroxyferulic acid *O*-methyl transferase has recently been shown to modify lignin content and composition in transgenic tobacco (Dwivedi et al., 1994; Ni et al., 1994), confirming the role of this enzyme in lignin biosynthesis. Negative results should be interpreted with caution, however, because whereas antisense suppression of CHS and dihydroflavonol reductase (DFR) resulted in reduced levels of anthocyanins and flavonols in petunia and of condensed tannins in transgenic hairy roots of *Lotus*, antisense suppression of chalcone isomerase (CHI) did not cause the expected phenotype in these species (van Tunen and Mol, 1991; Carron et al., 1994).

As more cloned sequences and three-dimensional protein structural information become available, a combination of molecular modeling and site-directed mutagenesis may help provide clues about the nature of enzyme active sites and determinants of substrate or product specificity. For example, although the sequence requirements for ATP/NADPH binding sites were deduced years ago, it is still unclear what characterizes a flavonoid binding site. Such structure–function analyses should lead eventually to a better understanding of how phenylpropanoid biosynthesis has evolved and to the potential construction of novel "designer enzymes" for the synthesis of biologically active molecules.

TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL CONTROL OF DEFENSE-RELATED PHENYLPROPANOID METABOLISM

In most systems studied, induction of phenylpropanoid synthesis under stress conditions is the result of increased transcription of genes encoding the corresponding biosynthetic enzymes. One notable exception concerns a hydroxylase that converts coumaroyl CoA to caffeoyl CoA in parsley cells. This enzyme has a very narrow pH optimum and is presumed to be inactive at the normal cellular pH; exposure of cells to fungal elicitor results in a rapid decrease in intracellular pH, leading to increased enzyme activity and to the production of caffeoyl and feruloyl esters (Kneusel et al., 1989).

Increased transcription rates for enzymes of both the central phenylpropanoid pathway and specific branch pathways of isoflavonoid or furanocoumarin synthesis are observed at the onset of the phytoalexin response in elicitor-treated cell supensions of alfalfa and parsley, respectively (Oommen et al., 1994; Hahlbrock et al., 1995; W. Ni and R.A. Dixon, unpublished results). The kinetics of these transcriptional changes have implications for the signal transduction mechanisms involved. For example, transcription of PAL and CHS genes in bean and alfalfa is extremely rapid and coordinated (Lawton and Lamb, 1987; W. Ni and R.A. Dixon, unpublished results), whereas transcription of some branch pathway enzymes, such as the bergaptol *O*-methyltransferase of furanocoumarin synthesis in parsley, may be delayed (Hahlbrock et al., 1995), implying the involvement of multiple signals for activation of the pathway as a whole. Consistent with this picture is the existence of common sequence motifs in the promoters of PAL and CHS genes from a number of sources (Dixon and Harrison, 1990), whereas genes encoding later branch pathway enzymes, such as the IFR of pterocarpan phytoalexin biosynthesis in alfalfa (Oommen et al., 1994), may lack exact copies of these motifs. This complex regulatory architecture is presumably necessary to allow for flexible deployment of various biosynthetic alternatives in response to different environmental and developmental cues.

In spite of technical advances for the cloning of transcriptional regulators, progress in identifying the factors involved in orchestrating the stress-induced transcriptional regulation of phenylpropanoid pathway genes has been relatively slow. Using DNA ligand screening of a cDNA expression library, a factor, BPF-1, that recognizes a conserved element (Box P) found in a number of PAL and 4CL genes was cloned from parsley (da Costa e Silva et al., 1993). BPF-1 is a member of a novel class of transcription factors that lacks both leucine repeat and zinc finger motifs. It is itself transcriptionally activated in elicited parsley cell cultures, although somewhat less rapidly than its target gene PAL, suggesting that increased levels of BPF-1 may be necessary to support the massive increase in PAL transcription observed in elicited cells (da Costa e Silva et al., 1993). Similarly, transcripts encoding three bZIP factors (CPRF-1, CPRF-2, and CPRF-3) that bind to a functional *cis*-element that confers light responsiveness on the parsley *CHS* promoter are induced on exposure of dark-grown parsley cells to UV light (Weisshaar et al., 1991). The H-box ($CCTACCN_7CT$) has been implicated in both stress and developmental expression of the bean *CHS15* gene (Loake et al., 1992). Two factors binding to the H-box (KAP-1 and KAP-2) have been isolated by classical biochemical approaches (Yu et al., 1993); preliminary evidence suggests that they may be located in the cytosol and are translocated to the nucleus on elicitation. KAP-1 also binds with high affinity to the G-box element (K. Lindsay, C.J. Lamb, and R.A. Dixon, unpublished results), which was implicated previously in the expression of a range of light- and hormonally regulated genes. It is therefore probable

that overlapping signal pathways exist for the developmental and environmental regulation of stress-inducible genes.

The signal molecules linking elicitor or stress perception with transcription of downstream response genes remain to be defined unequivocally, although the use of inhibitors and other pharmacological approaches has led to the proposed involvement of active oxygen species, changes in inorganic ion fluxes, and reversible phosphorylation/dephosphorylation (Dixon et al., 1994; Hahlbrock et al., 1995). Profitable new approaches to provide more direct evidence for the involvement of specific signal molecules include mutational analysis in genetically tractable species, reconstitution of pathways using in vitro transcription assays (Arias et al., 1993), and the use of promoter–reporter gene fusions as assay systems for purification of signal molecules affecting transcription of specific genes.

Post-transcriptional control of phenylpropanoid synthesis has received little recent attention, in spite of considerable evidence in the early literature that inactivation is a key component in the post-induction regulation of PAL activity (Creasy, 1987) and more recent evidence that phosphorylation of PAL is associated with turnover of the enzyme (Bolwell, 1992). Cinnamic acid, the product of the PAL reaction, strongly inhibits PAL transcription as well as the activities of a subset of phenylpropanoid biosynthetic enzymes (Mavandad et al., 1990; Barz and Mackenbrock, 1994). In addition, exogenous application of cinnamic acid may induce a proteinaceous inactivator of PAL (Bolwell et al., 1986). Inhibition of cinnamate synthesis in vivo with potent and apparently specific inhibitors of PAL activity leads to superinduction of extractable PAL activity and transcript levels, as would be predicted if this molecule acts as an endogenous regulator of PAL synthesis and activity. It has, however, proven difficult to correlate changes in endogenous cinnamate pools with PAL expression under physiological conditions (Orr et al., 1993). This problem can now be reevaluated using transgenic approaches to modify steady state cinnamate levels.

SPATIAL ORGANIZATION OF INDUCED PHENYLPROPANOID BIOSYNTHESIS

Cellular Sites of Synthesis

Early phytochemical analyses defined overall relationships between stress and phenylpropanoid synthesis but rarely identified the cells in which synthesis takes place. By subdividing tissues, it was shown that phytoalexins generally accumulate only near the site of infection. Similarly, UV-induced anthocyanins and other flavonoids were shown to accumulate specifically in upper epidermal cells. However, it was not known whether the compounds were synthesized in these cells themselves or whether they were transported there from adjacent cells. Use of in situ hybridization and immunolocalization techniques has elegantly demonstrated that flavonoids, CHS protein, and CHS, PAL, and 4CL transcripts all accumulate in the same epidermal cells, following UV irradiation (Schmelzer et al., 1988; Wu and Hahlbrock, 1992). Likewise, furanocoumarin phytoalexins and PAL, 4CL, and bergaptol *O*-methyltransferase proteins and mRNAs accumulate in a distinct set of cells around the sites of infection of parsley leaves by the nonpathogen *Phytophthora megasperma* f sp *glycinea* (Jahnen and Hahlbrock, 1988; Schmelzer et al., 1989).

These and similar studies therefore indicate that stress-induced phenylpropanoids usually accumulate in the cells in which they are synthesized. Although in situ hybridization and immunolocalization techniques are of general use for investigating the location of gene products, many phenylpropanoid metabolites are more difficult to localize than the strongly fluorescing and UV-absorbing parsley flavonoids and furanocoumarins. Development of new techniques for the cellular localization of metabolic end products is therefore urgently needed.

Subcellular Sites of Synthesis

The subcellular sites of phenylpropanoid biosynthesis are still a matter of debate. Many phenylpropanoids have been shown to accumulate in the vacuole, usually as glycosides or other conjugates. The final conjugation reactions may be directly involved in transport into the vacuole; for example, the glucosyl and malonyl transferases of pterocarpan conjugate synthesis are associated with the tonoplast membrane (Mackenbrock et al., 1992; Barz and Mackenbrock, 1994). In contrast, the enzyme that produces sinapoyl malate esters from sinapoyl glucose (1-sinapoylglucose:L-malate sinapoyltransferase) appears to be located exclusively inside the vacuole (Sharma and Strack, 1985). Studies on the transport of anthocyanins and flavonoids into vacuoles have indicated active transport via specific carrier proteins; only certain acylated forms are transported within a species, and vacuoles isolated from parsley and cotton do not take up carrot anthocyanins (Hopp and Seitz, 1987). Such evidence argues against the vacuole being a "passive dumping ground" that accumulates metabolites solely due to pH gradients and ionization.

In a number of species, anthocyanins accumulate in vesicles that form from the vacuole and eventually coalesce into one large membrane-bound "anthocyanoplast." This structure may gradually degrade, yielding a colored vacuole in older tissues (Pecket and Small, 1980). In the case of the 3-deoxyanthocyanidin phytoalexins of sorghum, colorless vesicles form in the cytoplasm of cells near a fungal infection structure. The color intensity of these vesicles increases as the total 3-deoxyanthocyanidin content increases, and then they coalesce and move toward the site of fungal attachment, finally releasing the phytoalexins near the pathogen (Snyder and Nicholson, 1990). The kinetics and behavior of these vesicles are consistent with their being the site of anthocyanidin synthesis. In contrast to these observations, however, is the cytoplasmic localization of a terminal anthocyanidin methyltransferase activity (Jonsson et al., 1983).

Several general phenylpropanoid pathway enzymes such as PAL and CHS have been described as cytoplasmic (soluble),

but there is some immunolocalization evidence for their loose association with membrane structures (Hrazdina, 1992; Smith et al., 1994), from which they largely dissociate under standard enzyme extraction conditions. Cytochrome P-450 enzymes such as cinnamate 4-hydroxylase, IFS, and IFOH are integral membrane proteins that have been shown to be associated with the endoplasmic reticulum membrane or fragments thereof (Dixon et al., 1992; Smith et al., 1994). It has been proposed that the soluble enzymes form "metabolons," or metabolic clusters of enzymes on a membrane surface, adjacent to the P-450 enzymes (Stafford, 1981; Hrazdina and Wagner, 1985). Intermediates could then rapidly flow from enzyme to enzyme and finally be transported inside the membrane, which may or may not fuse with the vacuole. This model may hold for simple phenylpropanoids; however, the prenyltransferases involved in the synthesis of prenylated pterocarpans and furanocoumarins are associated with plastid membranes, not the endoplasmic reticulum, thus requiring the shuttling of compounds between membranes and/or compartments (Dhillon and Brown, 1976; Biggs et al., 1990).

Metabolic Compartmentation

In many plant species, particularly the legumes, key phenylpropanoid pathway branch point enzymes such as PAL and CHS are encoded by multiple genes. There has been considerable speculation about whether this encoding reflects the organization of different forms of these enzymes in "metabolic compartments" or whether it simply allows for increased enzyme production under stress conditions, during which time expression of the whole gene family is often superimposed upon tissue-specific selective expression of a subset of the family members (Junghans et al., 1993). It is not known whether different isoforms of these enzymes have different subcellular localizations; this can now be addressed by epitope tagging of cloned genes (Mieszczak et al., 1992). There is, however, considerable evidence based on metabolic labeling experiments for the existence of complexes (the metabolons mentioned earlier) that might channel intermediates of phenylpropanoid synthesis from one enzyme to the next without equilibration with cytoplasmic pools (Stafford, 1981; Hrazdina and Wagner, 1985).

PAL is a tetrameric enzyme, and expression of multiple genes in combination with possible post-translational modifications leads to the formation of a range of heterotetrameric forms that may differ depending on the particular stimulus (Liang et al., 1989). Chromatofocusing analysis of native tetrameric forms of PAL from elicitor-induced bean suspension culture cells indicated selective induction of the form(s) with the lowest K_m value for phenylalanine (Bolwell et al., 1985), suggesting that isoforms with specific kinetic properties may be involved in the production of specific metabolic end products related to stress responses. However, the subunit composition of such differentially induced PAL heterotetramers has not been analyzed. There is significant divergence in the amino acid sequences of three bean PAL genes, particularly in the first exons, suggesting the potential for functional differences in the encoded proteins (Cramer et al., 1989). However, cDNAs encoding the four PAL isozymes from parsley, which exhibit greater similarity to one another than do the bean PAL isozymes, were recently cloned and individually expressed in *E. coli*, where they assembled into the corresponding homotetramers (Appert et al., 1994). These exhibited identical kinetic properties, even though the promoter sequences of the four genes are highly divergent, suggesting differential regulation of these PAL isoforms. The coding sequences of most members of the CHS gene families in legumes such as alfalfa are highly similar within the family (Junghans et al., 1993), suggesting that different CHS isoforms may likewise have very similar properties.

Clearly, more studies are required to characterize the catalytic properties of purified native or heterologously expressed phenylpropanoid biosynthetic enzymes. Biochemical approaches cannot, however, easily reveal the physical basis of metabolic compartmentation, in which specific isoforms may exist in complexes with subsequent enzymes. The yeast two-hybrid system, a technique for cloning cDNAs based on the ability of the cloned sequence to encode a protein that associates physically with a target protein (Fields and Song, 1989), has significant potential for studies on the molecular basis of metabolic compartmentation in induced phenylpropanoid synthesis but has yet to be applied to such systems.

One example of protein–protein interactions in the synthesis of stress-induced phenylpropanoids occurs in the formation of 5-deoxy isoflavonoid phytoalexins. This requires the action of CHR, which acts to reduce the polyketide intermediate bound to CHS prior to its cyclization on the synthase (Welle et al., 1991; Welle and Schröder, 1992), thus necessitating physical interactions between the synthase and reductase. Expression studies in *E. coli*, coupled with site-directed mutagenesis, are beginning to reveal the nature of these interactions (J. Schröder, personal communication). The synthase and reductase are co-induced in elicited or infected soybean and alfalfa cells, whereas the synthase alone is expressed in the aerial portions of uninfected plants. It is, however, unlikely that only certain isoforms of CHS are involved in interactions with the reductase, because the reductase is active in combination with the CHS from parsley (Welle and Grisebach, 1989), a plant that does not make 5-deoxy flavonoid derivatives.

MOLECULAR AND GENETIC APPROACHES FOR ANALYSIS OF THE FUNCTIONS OF STRESS-INDUCED PHENYLPROPANOID COMPOUNDS

Flavonoids and Sinapyl Esters as UV Protectants

Flavonoids and other phenylpropanoids have long been thought to play a role in protecting against UV irradiation, because they accumulate primarily in the epidermal and hypodermal layers of leaves and stems (the most illuminated layers) and strongly absorb light in the UV-B wavelengths. More

recent studies have demonstrated that in Arabidopsis leaves, levels of flavonoids (such as kaempferol conjugates) and sinapate esters increase in response to UV irradiation (Li et al., 1993; Lois, 1994), supporting this proposed protective role.

The availability of Arabidopsis mutants lacking wild-type levels of CHS and CHI activity allowed the first direct evaluation of the role of flavonoids in UV protection (Li et al., 1993). Several independent mutants were originally isolated with colorless seed coats (*transparent testa*, or *tt*, mutants). The *tt4* and *tt5* mutants were characterized as CHS and CHI mutants, respectively, and contain no detectable leaf flavonoids. The levels of sinapate esters in the *tt4* line are 30 to 60% higher, and in the *tt5* line are ~42% lower, than in wild-type lines for reasons that are not yet clear. Both *tt* lines grow normally when shielded from UV-B light but are much more sensitive to UV irradiation than the wild type, the *tt5* line being sensitive to very low levels of UV that do not affect the *tt4* line. Taken together, these results suggest that both flavonoids and sinapate esters are important for UV protection (Li et al., 1993).

The importance of flavonoids in UV protection has been confirmed by the results of a direct screen for Arabidopsis mutants with increased sensitivity to UV; one mutant lacked both the constitutive accumulation of a subset of leaf flavonoids and UV-inducible flavonoid synthesis but contained normal levels of anthocyanin leaf pigments and normal seed coat pigments (Lois and Buchanan, 1994). Thus, a specific type of flavonoid may be responsible for UV protection.

A direct thin layer chromatography and HPLC screen for Arabidopsis plants deficient in the fluorescent sinapic acid esters yielded a class of mutant lacking sinapoyl malate biosynthesis, later characterized as F5H mutants and designated *fah1* (Chapple et al., 1992; Chapple, 1994). The isolation of *fah1* mutants thus provided an opportunity to study the role of sinapate esters in UV protection separate from the role of flavonoids. Plants homozygous for the *fah1* mutation are extremely UV sensitive (Chapple, 1994).

Induced and Constitutive Phenylpropanoids in Plant Defense

Proposed roles for phenylpropanoid compounds in plant defense have traditionally been based on biological activities in vitro and on correlations between rates of accumulation and expression of resistance in vivo. Because plant defense responses are invariably multicomponent in nature, it is not easy to define which components are both necessary and sufficient to confer protection. Both the isolation of mutants selectively impaired in the production of specific phenylpropanoid end products and the production of phenocopies of such mutants by reverse genetics offer useful approaches to address the functionality of stress-induced phenylpropanoids. Unfortunately, the plants that have been most studied at the biochemical level as model systems for the phytoalexin response, such as soybean, alfalfa, green bean, chickpea, and parsley, are less than ideal subjects for classical mutational and genetic mapping approaches, whereas Arabidopsis, with its advantages for genetic studies, does not produce the isoflavonoids or furanocoumarins characteristic of the aforementioned species.

The first direct demonstration of the potential significance of phytoalexins in plant defense was provided by the introduction of a grapevine SS gene into tobacco plants (Hain et al., 1993). The foreign gene product was able to divert a portion of the substrates of CHS to the synthesis of the stilbene phytoalexin resveratrol, resulting in plants with increased resistance to the fungal pathogen *Botrytis cinerea.*

There is also evidence that individual phytoalexins may not be essential for defense. For example, analysis of phytoalexin-deficient Arabidopsis mutants has recently indicated that the indole phytoalexin camalexin is not required for resistance to avirulent *Pseudomonas syringae* pathogens (Glazebrook and Ausubel, 1994). However, camalexin may help retard the growth of virulent pathovars in the plant. Whether this situation will also hold for other host–pathogen interactions is not yet known, although the role of the isoflavonoid phytoalexin pisatin in resistance of pea to the fungal pathogen *Nectria hematococca* has also recently been questioned. Genetic studies had shown that virulence segregated with the ability of this fungus to detoxify pisatin by the action of an inducible cytochrome P-450 enzyme, pisatin demethylase (PDA) (Kistler and VanEtten, 1984). PDA genes were recently cloned (Maloney and VanEtten, 1994), and gene disruption experiments were performed by homologous recombination into the *pda* locus. Loss of PDA function only marginally impaired the virulence of the fungal pathogen (H.D. VanEtten, personal communication), suggesting, contrary to the results of the classical genetic studies, that pisatin is not in itself sufficient to account for resistance of pea to *N. hematococca.*

The availability of cloned genes encoding terminal enzymes for the biosynthesis of isoflavonoid or stilbene phytoalexins (Hain et al., 1993; Oommen et al., 1994) provides the means to assess phytoalexin function by reverse genetic (i.e., antisense or sense suppression) approaches in species that are amenable to genetic transformation. The antimicrobial activities of the isoflavonoid precursors of the alfalfa phytoalexin medicarpin against a range of fungal pathogens have been determined (Blount et al., 1993), facilitating interpretation of results should such intermediates accumulate in transgenic plants whose production of isoflavonoid or stilbene phytoalexins is blocked.

Analysis of transgenic tobacco plants with reduced levels of constitutive phenolic compounds as a result of sense suppression of PAL expression has revealed an important role for preformed phenolic compounds in limiting the extent of infection by the virulent fungal pathogen *Cercospora nicotianae* (Maher et al., 1994). Infection by *C. nicotianae* does not induce phenylpropanoid synthesis in wild-type or sense-suppressed plants, although induced defense responses such as chitinase production appear normal in sense-suppressed plants. The rate of development of fungal lesions is considerably greater in plants containing reduced levels of constitutive phenolic compounds, of which chlorogenic acid may be the most important

antimicrobial component, than in wild-type plants. Similar results have been observed in transgenic potato tubers expressing the *Catharanthus roseus* tryptophan decarboxylase gene (N. Brisson, personal communication). In these plants, redirection of tryptophan into tryptamine resulted in decreased phenylalanine pools, corresponding decreases in wound- and elicitor-induced chlorogenic acid and wall-bound phenolics, and increased susceptibility to the potato blight fungus *Phytophthora infestans.*

Salicylic acid has been implicated as an important regulatory molecule in plant defense. A considerable body of evidence links production of salicylic acid to the phenomenon of SAR, in which plants inoculated with an avirulent pathogen on a lower leaf develop resistance against a secondary inoculation on other leaves of the plant (Ryals et al., 1994). SAR is also induced by environmental stress factors such as UV irradiation and ozone (Yalpani et al., 1994). Salicylic acid levels increase in leaves responding hypersensitively to inoculation with an avirulent pathogen; the compound appears to travel in the phloem and is somehow involved in the transcriptional activation of defense response genes.

A metabolic engineering approach has now provided direct evidence for the role of salicylic acid in SAR. Transgenic tobacco plants were produced expressing the *nahG* gene from *Pseudomonas putida*, which encodes a salicylate hydroxylase that converts salicylic acid to catechol (Delaney et al., 1994). These plants had greatly reduced salicylic acid levels and were unable to establish SAR. Moreover, not only did they fail to exhibit resistance to virulent challenges following inoculation with avirulent pathogens, but also they were no longer able to express hypersensitive resistance against the primary avirulent challenge (Delaney et al., 1994), confirming an important role for salicylic acid in the expression of local resistance. However, if plants expressing the *nahG* gene were used as the stock in grafting experiments with wild-type plants as scion, primary inoculation of the stock still led to resistance in the scion, indicating that salicylic acid alone is not the systemic signal (Vernooij et al., 1994).

A range of defense response genes, including those encoding the so-called pathogenesis-related proteins, are activated in systemically protected leaves and in response to exogenously applied salicylic acid (Ryals et al., 1994). However, functional promoter analyses have not led to the identification, in plant genes, of a common salicylate response element that could be involved in coordinating the transcriptional response. It has recently been shown that the cauliflower mosaic virus (CaMV) 35S promoter contains a salicylate response element identical to the previously characterized activation sequence-1 (*as-1*) (Lam et al., 1989), suggesting that the virus uses a plant defense signal to regulate its transcription (Qin et al., 1994).

Transcriptional activation of the CaMV 35S promoter by salicylate is very rapid and does not require protein synthesis (Qin et al., 1994). In contrast, transcription of the tobacco PR-1a defense response gene is slower and is inhibited by cycloheximide. These results suggest a model in which salicylate may "sensitize" cells for rapid defense gene activation by acting as a transcriptional activator for the production of signal transduction components necessary for the activation of the downstream defense response genes. This model predicts that functional homologs of the CaMV *as-1* element will be present in plant genes associated with the establishment of SAR. Whether salicylate can act directly as a transcriptional regulator is not clear; the identification of a salicylate binding protein as a peroxidase specifically inhibited by salicylate and SAR-inducing derivatives of salicylate implicates hydrogen peroxide as a key component in SAR signaling upstream of salicylate (Chen et al., 1993). The development of screens for Arabidopsis mutants that lack SAR (Uknes et al., 1993; Bowling et al., 1994) opens up the possibility of a systematic dissection of the processes underlying signal generation, transmission, and reception during SAR, including the factors involved in salicylate formation/release and site of action.

PAL sense-suppressed tobacco plants are unable to establish SAR, which is consistent with the involvement of PAL in the synthesis of salicylic acid and possibly other signal molecules. However, primary inoculation of PAL sense-suppressed plants with tobacco mosaic virus results in the formation of normal-sized hypersensitive lesions that appear white due to the reduction in phenolics (J. Pallas, C.J. Lamb, and R.A. Dixon, unpublished results). These results indicate that phenylpropanoid compounds are unlikely to be involved in limiting the size of tobacco mosaic virus lesions.

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