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Phytoalexin Biosynthesis as a Model System for Biochemistry of Plant-Microbe Interaction

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Interaction between plant and microbe, which could be symbiotic or pathogenic, is initiated by mutual recognition of the counter partner. Plant-associated bacteria possess sensory machineries to recognize and process external signals originated from the host plant (Hwang, 1999). For instance, *Rhizobium* recognizes specific flavonoid compounds secreted from the host plant and synthesizes nod factors to initiate nodulation in plant root (Peters and Verma, 1990; Cheon et al., 1999). Plants also have mechanisms to perceive microbial signals to exert proper responses against the microbes (Bent, 1996; Hammond-Kosack and Jones, 1996). The mutual signaling and recognition mechanisms determining plant-microbe specificity have become an important research topic, not only in plant pathology but also in other related areas including biochemistry, molecular biology and genetics.

During the past decades, numerous genes involved in the mutual signaling mechanisms have been identified through genetic and molecular biological approaches. Protein products of plant resistance (*R*) genes may enable plants to recognize specific races of pathogen and transform the signal to inside of the cell to activate defense-related genes, thus acting as receptors (Bent, 1996; Yun, 1999). Bacterial *avr* gene products may act as signal molecules interacting with the corresponding *R* gene product. A number of *R* genes and corresponding bacterial *avr* genes have been cloned through genetic studies, which support the gene-for-gene relationship (Flor, 1956; Keen, 1990; Staskawicz et al., 1995) between plants and pathogens.

Only a small number of signal molecules and receptors, however, have been fully characterized. In many cases, signal molecule is not a simple protein product encoded by an *avr* gene, but rather a complex secondary product synthesized by several enzymes. Thus, signal molecules identified through biochemical studies of plant-pathogen interactions are oligosaccharides, polypeptides, glycopeptides, and glycoproteins (Hahn, 1996). As one of the best examples, nodulation factors secreted from *Rhizobium* have been characterized to be complex lipo-oligosaccharides that are synthesized by a number of *Nod* gene products (Long, 1996). Plant receptor study stands on the same situation. Although a number of *R* genes have been cloned, studies on their function with purified proteins have not been successfully conducted. Moreover, corresponding signal molecules interacting with most of the *R* gene products are still unknown. Therefore, experiments to isolate the complex signal molecules and to identify their mode of action in plant cells should be performed more intensively.

This review focuses on practical approaches to isolate signal molecules and to identify plant receptors, taking the cellular process leading to phytoalexin biosynthesis as a model system. Phytoalexins are absent in healthy plants, but inducibly synthesized when plant cells are exposed to microbes (Darvill and Albersheim, 1984; Ebel, 1986; Dixon, 1986). Availability of homogenous signal molecules (elicitors) has accelerated the progress toward an understanding of the cellular mechanisms regulating phytoalexin biosynthesis at the molecular level. Since information of both ends (signals and final products) has been accumulated, the phytoalexin biosynthesis constitutes a good model system for the study of cellular signaling mechanism. Various efforts in biochemistry to understand the signaling mechanisms in overall plant-microbe interactions have already been well reviewed elsewhere (Dixon et al., 1994; Boller, 1995; Hahn, 1996).

Phytoalexin Biosynthesis

Phytoalexins have been defined as "low molecular weight, antimicrobial compounds that are both synthesized by and

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accumulated in plants after exposure to microorganisms" (Paxton, 1981). These antibiotics are accumulated at the site of infection in sufficient amount to successfully inhibit the growth of both fungi and bacteria (Darvill and Albersheim, 1984). It was recently reported from a study with *Arabidopsis* mutants that deficiency in phytoalexin production caused enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola* (Thomma et al., 1999).

Phytoalexins are a chemically heterogeneous group of compounds distributed among isoflavonoids, sesquiterpenes, diterpenes, stilbenes, and other classes of compounds (Ebel, 1986; Dixon, 1986). A given plant family usually produces a chemically related group of phytoalexins. For example, soybeans (*Glycine max*) accumulate the isoflavonoid phytoalexins such as glyceollin isomers and glycinol (Fig. 1) upon infection by a phytopathogenic oomycete, *Phytophthora sojae*.

The biosynthetic pathway of phenylpropanoid phytoalexins has been elucidated in several legumes (Dixon, 1986; Hahlbrock and Scheel, 1989). The sequence of reactions converting L-phenylalanine into substituted cinnamic acid CoA esters has been referred to as 'general phenylpropanoid metabolism' (Fig. 2). The enzymes involved in this pathway, such as phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase, and 4-coumarate:coenzyme A ligase (4-CL), have been isolated and characterized from a number of plant sources (Dixon et al., 1983). Several research groups have demonstrated that activities of these enzymes are increased after microbial infection or elicitor application (Hahlbrock and Scheel, 1989). The products of this pathway are common precursors of many classes of natural plant products including flavonoids, isoflavonoids, coumarins, stilbenes, lignin, and other phenolics.

The phytoalexin biosynthesis in plant requires activation of the genes encoding enzymes involved in the synthetic pathway (Lamb et al., 1989). Rapid increases of the gene transcripts have been observed after treatment of plant cells with elicitors (Kuhn et al., 1984). Nuclear transcription run-off experiments has revealed that expression of the mRNAs is regulated at the transcription level (Chappell and Hahlbrock, 1984; Lawton and Lamb, 1987). Corresponding cDNA and genomic clones have been isolated from a number of plants, and regulatory factors involved in the expression of the genes have been identified. For instance, *cis*-acting elements (Douglas et al., 1991; Harrison et al., 1991a) and *trans*-acting regulatory proteins that interact with the elements have been characterized (Staiger et al., 1990; Harrison et al., 1991b).

Biochemical studies of phytoalexin biosynthesis have

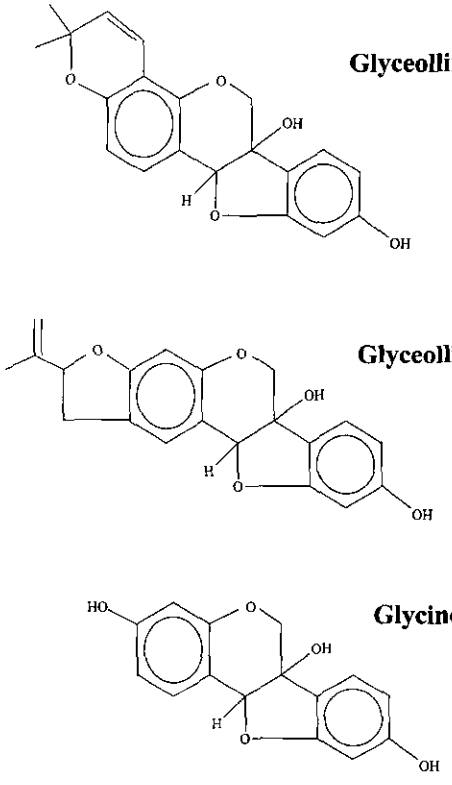

Fig. 1. Structures of isoflavonoid phytoalexins accumulated in soybean tissues infected by pathogens or treated with elicitors.

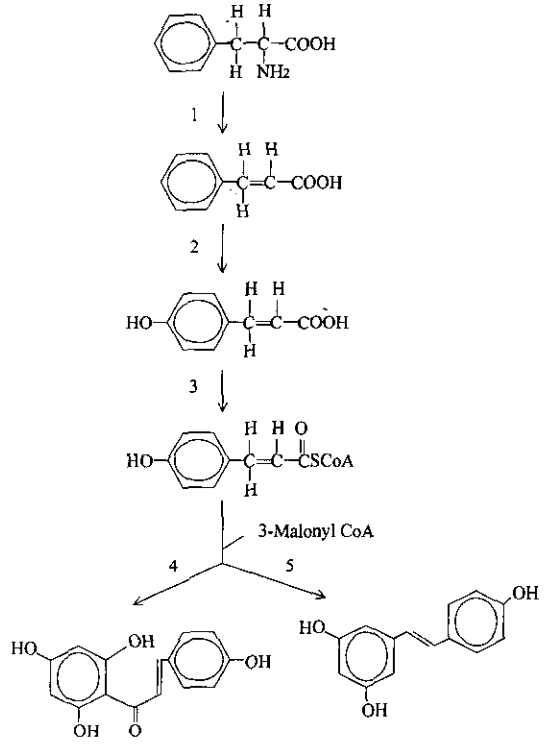

Fig. 2. The general phenylpropanoid pathway leading to the biosynthesis of isoflavonoid and stilbene phytoalexins. Abbreviations are 1, L-phenylalanine ammonia-lyase; 2, cinnamic acid 4-hydroxylase; 3, 4-coumarate:coenzyme A ligase; chalcone synthase; 5, stilbene synthase.

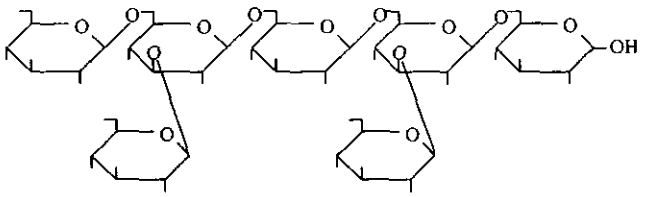

Fig. 3. Structure of the hepta-β-glucoside elicitor. Hydroxyl groups not involved in linkages have been omitted for clarity.

accumulated a great deal of basic information useful for further detailed investigations of this metabolic pathway. Structural characterization of phytoalexin molecules has made it possible to develop rapid and reliable assays for the determination of phytoalexin accumulation in plant tissues, such as spectrophotometric assays (Albersheim and Valent, 1978; Hahn et al., 1992) and a specific radioimmunoassay (Hahn et al., 1985). Moreover, biochemical assays based on enzymology or RNA hybridization have provided valuable tools to quantitate and localize the biosynthetic enzymes in infected plant tissues.

Elicitors of Phytoalexin Biosynthesis

Molecules that trigger plant defense responses including phytoalexin biosynthesis are called elicitors. Several different classes of elicitors have been isolated from culture fluids of pathogenic microbes or directly from microbial cell wall constituents. Elicitors that have been purified to apparent homogeneity include oligosaccharides, glycopeptides, glycoproteins and polypeptides (Hahn, 1996).

Fungal β-glucan fragments are among the best characterized biotic elicitors of phytoalexin biosynthesis. Culture fluids and cell-wall preparations from *P. sojae* were initially observed to elicit phytoalexin biosynthesis in soybean tissue (Ayers et al., 1976a; Ayers et al., 1976b). Elicitor-active components have been released from the fungal mycelial walls either by heat treatment (Ayers et al., 1976b) or partial acid hydrolysis (Sharp et al., 1984c). Chemical composition analyses revealed that these elicitors were neutral polysaccharides consisting of 3-, 6-, and 3,6-linked glucosyl residues having β configuration (Ayers et al., 1976c; Albersheim and Valent, 1978). Using several steps of chromatographic procedure, the smallest elicitor-active glucan fragment consisting of branched seven glucose residues (Fig. 3) was isolated (Sharp et al., 1984b and c). The hepta-β-glucoside was capable of inducing phytoalexin biosynthesis in soybean tissue at concentrations between 10^{-7} and 10^{-9} M. The hepta-β-glucoside was chemically synthesized to confirm the structure and elicitor activity of the mycelial wall-derived oligoglucosides (Sharp et al., 1984a).

A family of oligo-β-glucosides structurally related to the hepta-β-glucoside was chemically synthesized to compare their ability of inducing phytoalexin biosynthesis in soybean cotyledons (Cheong et al., 1991; Cheong et al., 1993). Modifications of the reducing terminal glucosyl residue of the elicitor-active hepta-β-glucoside, including conjugation with tyramine and subsequent iodination, had no significant effect on the elicitor activity. In contrast, the branched trisaccharide at the non-reducing end of the oligoglucosides was essential for the maximum elicitor activity. Substitution of either the non-reducing terminal backbone glucosyl residue or the side-chain glucosyl residue closest to the non-reducing end with glucosamine, *N*-acetylglucosamine, galactose or xylose reduced the elicitor activity of the oligoglucosides by 10- to 10,000-fold. Correct branching pattern of the β-glucan was also important, as the elicitor activity was also reduced 1000-fold if the two side-chain glucosyl residues were attached to adjacent backbone glucosyl residues. These results demonstrated that oligo-β-glucosides should have a specific structure (Fig. 3) in order to trigger the signal transduction pathway which ultimately leads to *de novo* synthesis of phytoalexins in soybean.

A Hypothetical Model of the Signaling Pathway Leading to Phytoalexin Biosynthesis

A hypothetical model for cellular signaling mechanisms leading to phytoalexin biosynthesis in plants has been established (Fig. 4). In the initial stage, signal molecules would be generated in the contact area between the invading microbe and the host plant. By analogy to animal cells, the elicitors would then be recognized and bound by specific receptors. Because oligosaccharide and glycoprotein elicitors are hydrophilic molecules, the receptors for these membrane-impermeable signal molecules are presumably located on the plasma membrane. Upon binding of elicitors, the receptor would then initiate a signaling pathway by transmitting the external signal to inside of the cell to activate a cascade of intracellular biochemical events. This cascade eventually results in the expression of genes encoding the enzymes required for phytoalexin biosynthesis. Experimental results from a number of studies based on this model are described in detail in the following sections.

Generation of Elicitors during Plant-Pathogen Interactions

The fungal oligosaccharide elicitor preparations were obtained by treatment of the cell walls with acid or heat. Therefore, it has not been fully understood how these elicitors are generated during *in vivo* interactions between plants and microbes.

It has been hypothesized that either constitutive or inducible cell-wall degrading enzymes of host plants are involv-

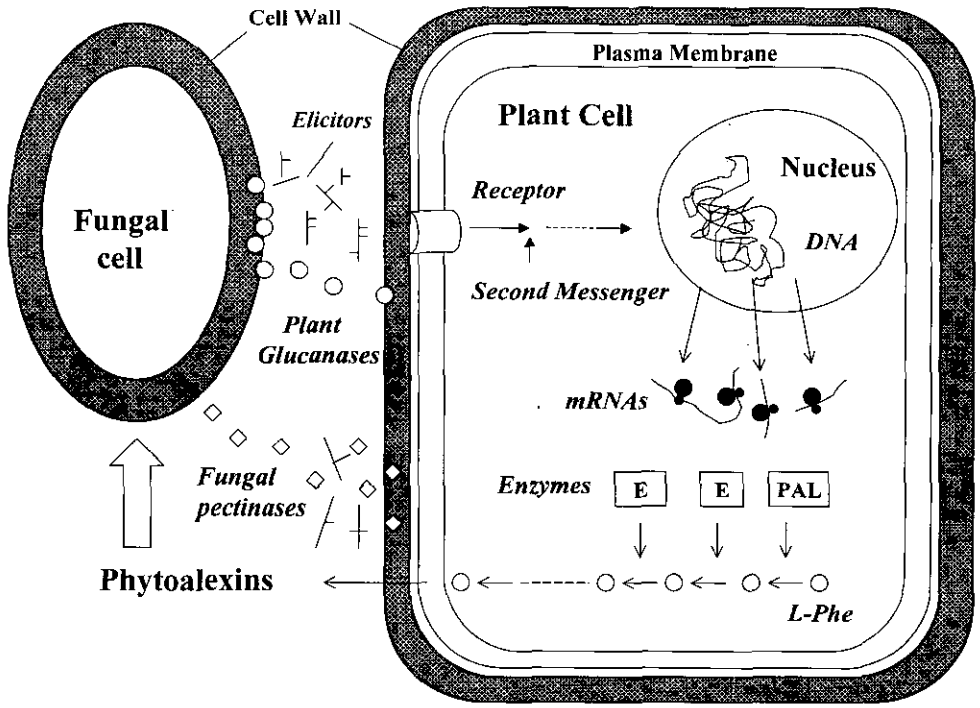

Fig. 4. A hypothetical model for the cellular signaling pathway leading to phytoalexin biosynthesis. Abbreviations are E, enzyme; L-Phe, L-phenylalanine; PAL, L-phenylalanine ammonia-lyase.

ed in the process of oligosaccharide elicitor generation (Côté et al., 1998). A number of hydrolytic enzymes are localized in cell wall, extracellular space, and vacuole (Boller, 1987; Mauch and Staehelin, 1989). Thus, the presence of a family of β-glucanase isoforms expressed either constitutively or inducibly in plants has implicated that these enzymes, individually or in combination, may be involved in the production of β-glucan elicitors from pathogens at the plant-pathogen interface. Some isoforms of the glucanases were purified and used in experiments to release elicitor-active fragments from fungal cell walls (Yoshikawa et al., 1981; 1990; Ham et al., 1991). The fragments obtained from the enzyme digest consisted of β-1,6-linked glucan frequently branched by β-1,3-linked glucose residue (Okinaka et al., 1995). However, structural characterization for the enzyme-generated fragments has not been completed.

A variety of glycosylhydrolases ultimately degrade the plant and fungal polysaccharides to short, biologically inactive oligomers. Therefore, it has been postulated that regulatory mechanisms exist *in planta* to control the activities of polysaccharide-degrading enzymes for the production of longer, biologically active oligosaccharides. Proving this postulation, a glucanase inhibitor protein (GIP-1) was purified from fungal culture filtrate (Ham et al., 1997). The inhibitor protein may have a role in the regulation of β-glucan elicitor generation at the plant-fungal interface.

Alternatively, β-glucan elicitors could be released from the pathogen in the absence of host-produced enzymes. Accumulation of β-glucan elicitors was observed in the culture medium of germinating cysts of *P. sojae* (Waldmüler et al., 1992). This observation suggests that plant cells could interact directly with β-glucan elicitors released by the growing pathogens themselves. The cyst elicitors have not been characterized in detail. Thus, relationship of these β-glucan elicitors to those isolated from older mycelial walls by chemical or enzymatic means remains unknown.

Pectic-degrading enzymes secreted by fungal and bacterial pathogens are capable of degrading pectic polysaccharides of host plant cell walls and releasing oligogalacturonide elicitors (Fig. 4). This endogenous plant-origin elicitor will not be described further in this review, since other good reviews on this topic have been published elsewhere (Collmer and Keen, 1986; Hahn et al., 1989)

Receptor(s) for Elicitors of Phytoalexins

It has been speculated that receptors capable of recognizing elicitors of phytoalexin biosynthesis are present in plants (Darvill and Albersheim, 1984; Dixon, 1986). Several lines of experiments using crude elicitor preparations have suggested that elicitors have significant effects on plant plasma membranes. Fungal glycopeptide elicitors induced electrolyte leakage by changing the membrane permeability (Dow and Callow, 1979; Young et al., 1982). In addition, rapid

depolarization of transmembrane potential has been observed after treatment of plant cells with fungal wall-derived elicitor preparations (Katou et al., 1982; Pelissier et al., 1986). These results suggested that plasma membrane is the primary site of interaction of elicitors with plant cells.

Presence of sites that could bind elicitor molecules has also been examined in a number of studies. For instance, it was shown that a crude glucan elicitor preparation isolated from *P. infestans* caused rapid agglutination of potato protoplasts, indicating that glucan binding sites exist in plant plasma membrane (Peters et al., 1978). Experiments to demonstrate the presence of binding sites for fungal glucan elicitors in plant membranes have been performed using ligand-binding assays. Through extensive efforts in several laboratories, a number of binding sites have been identified for oligosaccharide, glycopeptide and oligopeptide elicitors (Hahn, 1996). An example is a high affinity binding site recognizing a 13-amino acid peptide (Pep-13) identified within a fungal glycoprotein elicitor (Nürnberger et al., 1994b).

Ligand-binding assays to identify binding sites for fungal glucan elicitors in plant membranes were performed with heterogeneous glucan fragment mixtures that contained both active and inactive fragments (Yoshikawa et al., 1983; Schmidt and Ebel, 1987; Cosio et al., 1988; Cosio et al., 1990b). Later, experiments with homogeneous elicitor-active compounds were performed using radioiodinated hepta-β-glucoside in ligand-binding assays (Cheong and Hahn, 1991). Binding of the radiolabeled hepta-β-glucoside elicitor to soybean root membranes was saturable, reversible, and of high affinity (apparent Kd=0.75 nM). Specificity of the ligand binding was demonstrated in competitive inhibition assays using a number of synthetic oligosaccharides that are structurally related to the hepta-β-glucoside elicitor. A strict correlation was observed between the binding affinities and elicitor activities of these oligoglucosides. Thus, the elicitor-binding site fulfills criteria expected of a physiological receptor.

The elicitor-binding sites appeared to be (glyco)proteins integrated in the plant plasma membrane (Cheong et al., 1993). The elicitor-binding proteins were solubilized from soybean root membranes using biological detergents (Cosio et al., 1990a; Cheong et al., 1993). The detergent-solubilized elicitor-binding proteins retained the ligand-specificity and binding affinity determined for the membrane-localized form (Cheong et al., 1993). The elicitor-binding protein(s) were partially purified by ligand-affinity chromatography using a column containing immobilized β-glucan (Cosio et al., 1992; Mithöfer et al., 1996; Umemoto et al., 1997) or synthetic hepta-β-glucoside (Côté et al., 2000).

The protein fraction eluted from β-glucan affinity column contained a prominent polypeptide with apparent molecular mass of 70 to 75 kDa (Cosio et al., 1992; Mithöfer et al., 1996; Umemoto et al., 1997). In addition, a 78 kDa protein homologous to the polypeptide was isolated from French bean (Mithöfer et al., 1999). In a recent study, the detergent-solubilized β-glucan binding proteins were incorporated into lipid vesicles to demonstrate that the reconstituted binding proteins were functional (Mithöfer and Ebel, 1999). Binding of the hepta-β-glucoside ligand was saturable, reversible and of high affinity (Kd=6-7 nM). However, it is not clear whether the proteins present in the affinity-purified fractions are essential for specific binding of the hepta-β-glucoside elicitor. The binding protein preparation eluted from a column containing immobilized hepta-β-glucoside elicitor contains two major polypeptides of 55 and 70 kDa (Côté et al., 2000). However, the polypeptides cross-react with antisera raised against callose synthase-associated proteins. Furthermore, these antisera are unable to immunoprecipitate elicitor-binding activity from detergent-solubilized membrane preparations. These results raise a significant question as to the involvement of the polypeptides in the specific recognition of the hepta-β-glucoside elicitor.

Upon the purification completed, proof that the hepta-β-glucoside elicitor-binding protein(s) are, in fact, the elicitor receptor(s) will require reconstitution of the purified binding protein(s) or transgenic expression of the cDNA in an elicitor responsive system. Sequence analysis of cDNA encoding the elicitor-binding protein(s) would make it possible to postulate function(s) of the binding proteins by examining sequence homology to those encoding proteins that are known to be involved in signal transduction process in animal cells. In addition, transformation of soybean cells with antisense nucleotide sequences could also be performed to demonstrate that the hepta-β-glucoside elicitor-binding protein(s) function as part of the signal pathway. Thus, further purification and characterization of the hepta-β-glucoside elicitor-binding proteins will open the way toward elucidating, at the molecular level, how the β-glucan elicitor triggers the signal transduction pathway that ultimately leads to phytoalexin biosynthesis in soybean.

Signal Transduction Mechanisms Leading to Phytoalexin Biosynthesis

By analogy to interactions between cells and hormones in mammalian systems, the elicitor signals perceived by specific receptors might be transmitted by second messengers to activate related genes. Examples of such second messengers include cyclic adenosine monophosphate (cAMP), phosphoinositides and calcium. Involvement of these putative second messenger molecules in the signaling process leading to phytoalexin biosynthesis has been examined in a

number of studies.

The possible role of cAMP in phytoalexin biosynthesis has been reported with contradictory results. Exogenous addition of cyclic AMP to carrot cells induced phytoalexin accumulation (Kurosaki et al., 1987a). Moreover, addition of crude elicitors prepared from carrot cells caused a rapid increase in intracellular level of cAMP (Kurosaki et al., 1987a). In contrast, in a study using a sensitive radioimmunoassay, no significant change in endogenous cAMP level was detected in soybean hypocotyls infected by *P. sojae* or in suspension-cultured soybean cells treated with a crude elicitor preparation (Hahn and Grisebach, 1983).

Experiments examining the possible involvement of another second messenger, phosphoinositides, have also yielded ambiguous results. An isotope-labeling experiment revealed that elicitor treatment did not cause any significant change on the incorporation of ^{3}H or ^{32}P into phosphoinositides in either soybean or parsley cells (Strasser et al., 1986). In contrast, breakdown of phosphatidylinositol occurred in carrot cells upon elicitor treatment (Kurosaki et al., 1987b). In a study with lucerne suspension culture, fungal elicitor rapidly induced generation of inositol 1,4,5-trisphosphate and concomitant hydrolysis of phosphatidylinositol 4,5-bisphosphate (Walton et al., 1993), suggesting that inositol trisphosphate, a second messenger providing a calcium-mobilizing signal, involve in the phytoalexin elicitation.

Evidence supporting a role of calcium in the elicitor-induced signal transduction is more consistent. Phytoalexin accumulation in cell suspension cultures was induced by calcium ionophores, but inhibited at low extracellular calcium concentrations (Kurosaki et al., 1987b; Stäb and Ebel, 1987). Moreover, tobacco tissues expressing transgenic aequorin, a calcium-sensitive luminescent protein, showed an increased level of cytoplasmic calcium in response to fungal elicitors (Knight et al., 1991). It was reported that the oligopeptide elicitor Pep-13 induced rapid and transient influxes of Ca^{2+}/H^{+} as well as effluxes of K^{+}/Cl^{-} (Nürnberger et al., 1994b). Certain channel blockers inhibited the phytoalexin production, while amphotericin B stimulated the ion fluxes to induce the phytoalexin production in the absence of elicitor (Nürnberger et al., 1994a). By chemical crosslinking, a 91 kDa parsley plasma membrane protein was identified to be a receptor of the peptide elicitor (Nürnberger et al, 1997).

The possible role of protein phosphorylation in elicitor-induced signal transduction has also been examined. The pattern of phosphorylated proteins was rapidly (within 5 min) and specifically changed after treatment with various elicitor preparations (Dietrich et al., 1990; Farmer et al., 1991; Felix et al., 1991). In addition, inhibitors of protein kinases (Felix et al., 1991) or protein phosphatases (Kauss and Jeblick, 1991) inhibited elicitor-induced responses of the cells. However, identities of the phosphorylated proteins and their possible role(s) in signal transduction remain to be determined.

Recently, a role of salicylic acid (SA) mediating the process of systemic acquired resistance was suggested in relation to phytoalexin biosynthesis. In *Arabidopsis* plants that express NahG proteins to inactivate SA, accumulation of the phytoalexin (camalexin) were down-regulated (Zhao and Last, 1996). Application of SA to the SA-deficient *Arabidopsis* mutant, *pad4*, restored camalexin production (Zhou et al., 1998). However, the *cpr1* mutant exhibiting constitutive expression of SA-inducible pathogenesis-related (PR) proteins showed no alterations in the pathway leading to the formation of camalexin, despite having high levels of SA (Zao and Last, 1996). In addition, the SA induction-deficient mutants of *Arabidopsis*, *sid*, accumulated high levels of camalexin (Nawrath and Métraux, 1999).

In summary, signal transduction mechanisms involved in phytoalexin biosynthesis are still unclear. In addition, it should be noted that most of the studies described above have been carried out with heterogeneous preparations of elicitors. Therefore, it is difficult to relate the observed effects unambiguously to a single elicitor-stimulated signal pathway. More detailed studies using homogeneous elicitor preparations are required to elucidate the transduction mechanisms involved in the phytoalexin biosynthesis.

Conclusion

Together with another related research area, biochemistry is one of the essential tools for molecular studies of plant-microbe interactions. This review has directed to describe the efforts in biochemistry to elucidate how plant cells perceive and respond to extracellular signals from microbes. The model system taken is the induction of phytoalexin biosynthesis in soybean by a pathogenic oomycete, *P. sojae*. Purification of the receptor(s) for fungal cell wall-derived β-glucan elicitor will initiate more detailed molecular studies aimed at furthering our understanding of the signal transduction pathway in plants interacting with microbes. In addition, it would provide much available information about plant receptor structure and function, as no plant receptor with a proven physiological function has been purified and characterized. Thus; contribution of biochemistry to this research area has not been limited to the phytoalexin biosynthesis, but extended to another plant defense mechanisms such as hypersensitive response and systemic acquired resistance.

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