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Cellular Coordination of Molecular Responses in Plant Defense

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The attempted infection of plant tissues by a pathogen initiates a complex progression of biological and molecular interactions that culminates in the visual symptoms we associate with disease or disease resistance. The nature, timing, and spatial coordination of the actions taken by either organism are crucial in defining the result of any given interaction and thus the overall outcome of the infection. This finely balanced progression of events is also often subject to marked developmental regulation, environmental influence, and physiological modulation. Thus, if the molecular aspects of host-pathogen interactions are to be understood in perspective with these complex events *in planta,* molecular analyses must be coupled with detailed observations on the spatial and temporal aspects of the infection process and on the developmental, environmental, and physiological status of the specific organisms under investigation.

It has become more and more apparent that the events that determine host resistance or susceptibility often occur in very limited populations of cells at or just in advance of the infection front, the very narrow zone defining the area of immediate contact between host and pathogen. Interactions taking place after infection progresses through a tissue may be largely irrelevant to the outcome of the infection. Thus, it is no longer sufficient to measure responses by gross tissue analysis, even in those cases where they represent discrete lesions, since these lesions may represent a composite of many cellular and molecular events.

The necessity of measuring molecular responses at a cellular level has led to the recent exploration of a number of useful tools in cellular biochemistry that can be applied directly to intact tissues. The new information and perspectives arising from these approaches are the subject of the current review. It is not our intention to review all aspects of cellular research on host-pathogen interactions. Instead, we have chosen to discuss selected research papers to illustrate recently emerging concepts and methodologies relating specifically to the spatial localization and progression of host-pathogen interactions *in planta.*

Three major defense responses will be reviewed separately: the accumulation of host antibiotic and cell wall-bound secondary metabolites; the accumulation of pathogen wall-degrading, hydrolytic enzymes (chitinases and β-1,3-glucanases); and the accumulation of structural host cell wall proteins, the hydroxyproline-rich glycoproteins (HRGPs). As we will see, the questions of central importance to the function of each of these defenses at a cellular level are different. Each thus serves to illustrate a somewhat different aspect of cellular research.

SECONDARY PRODUCT DEFENSE MECHANISMS

Secondary products have been proposed to play many roles in plant defense. These include roles as preformed antibiotics, induced antibiotics (phytoalexins), or covalently linked, cell wall phenolics associated with strengthening of defense barriers.

A full characterization of the deployment of a given secondary product defense response at a cellular level requires the complementary assessment of spatial and temporal events at the transcriptional and translational levels of gene expression as well as at the levels of enzyme activity and product accumulation. Cellular methods for the measurement of certain aspects of these various levels of response, for selected host-pathogen systems, are discussed below. Although products derived from a number of different pathways may play a role in resistance, we have chosen to focus mainly on regulation of the phenylpropanoid pathways because of their universal distribution and the greater amount of information currently available for these metabolites. The molecular biology of these pathways has recently been reviewed (Hahlbrock and Scheel 1989).

Cellular localization of messenger RNAs for enzymes of phenylpropanoid metabolism. Cellular aspects of induction of messenger RNAs for the enzymes of phenylpropanoid metabolism and some of its branch pathways have been examined in a number of hosts in response to wounding, elicitation, or infection. Two major approaches have been taken: analysis of specific messengers by *in situ* hybridizations and *in planta* analysis of promoter-reporter gene fusions in transgenic plants. These approaches have the obvious advantage of directly following the actual activation of defense genes. They are thus very powerful techniques for examination of the specific molecular genetics of host-pathogen interactions. Whereas *in situ* hybridization has the benefit of providing evidence for naturally occurring transcripts directly, the use of reporter genes allows examination of the effects of precisely defined changes in the regulatory sequences of a specific gene on its expression. A limitation of these approaches is that they do not provide a picture of the actual accumulations of the defensive proteins or metabolic products that follow gene activation. Important regulatory events at the levels

of protein translation, protein turnover, protein activity, and metabolism are not seen. These studies are also limited to those processes for which genes have been isolated or gene probes are available.

The elegant studies by Hahlbrock and co-workers with the potato and parsley systems illustrate the effectiveness of the *in situ* hybridization approach. In potato, the *in situ* localization of phenylalanine ammonia-lyase (PAL) messenger was examined in young leaf tissues of susceptible and resistant cultivars infected with *Phytophthora infestans* (Cuypers *et al.* 1988). The hypersensitive reaction in the incompatible interaction progressed very rapidly, but in a strictly sequential manner from the epidermis through the rest of the leaf. Although hypersensitive cell death was evident within just 3 hr, the accumulation of PAL messenger did not occur in these cells, but in a narrow band of cells immediately surrounding the hypersensitive lesion. PAL messenger in this sharply defined zone was already at high levels by 3 hr and declined to near control levels by 6 hr. Immunohistochemical localization of the fungus demonstrated that this very early activation of PAL involved the apparent transmission of a signal immediately in advance of the fungal hyphae. In contrast, PAL activation was delayed, more diffuse, and nontransient in the susceptible interaction.

Similar experiments were performed in parsley using antisense probes against seven different putative defense-related genes (Schmelzer *et al.* 1989. *Phytophthora megasperma* f. sp. *glycinea* was used to infect parsley leaves, resulting in a nonhost hypersensitive response. Observations were generally similar to those in potato. All seven defense genes were induced transiently in a sharply defined zone of cells immediately outside the necrotic lesion. Within this zone, however, the induction of various transcripts was temporally different. PAL, 4-coumarate:CoA ligase, a pathogenesis-related protein and three other defense-related genes (ELI 3, 5, and 7) were induced rapidly (<4 hr), whereas the gene for a furanocoumarin-specific enzyme, bergaptol *O*-methyltransferase, was induced several hours later. The authors concluded from these studies that hypersensitive cell death *per se* is too rapid to include activation of the defense-related genes studied and that the sharply restricted zone of responding cells may suggest a threshold phenomenon in the signaling process. They also concluded that the furanocoumarin phytoalexins are not among the earliest defense responses. Instead, accumulation of esterified phenolics in the cell walls of responding cells may occur earlier (Jahnen and Hahlbrock 1988; Matern and Kneusel 1988).

In tobacco plants transformed with β-glucuronidase (GUS) gene fusions to two different bean chalcone synthase (CHS) promoters (*CHS8* and *CHS15*), infection of leaves with a nonhost pathogen, *Pseudomonas syringae* pv. *syringae*, or treatment with oxalic acid stimulated activation of only the *CHS8* promoter (Stermer *et al.* 1990). Gene activation was seen both locally and up to 30–40 mm from the primary site of treatment. In contrast, ultraviolet light and $HgCl_2$ stimulated both the *CHS8* and *CHS15* promoters, but only locally. Preliminary work with a fungal elicitor revealed a marked induction of the *CHS8* promoter in leaf tissues immediately adjacent to the elicitor signal and a substantially less intense and more diffuse induction in tissue up to 10–15 mm distant (Schmid *et al.* 1990).

Results from the reporter gene fusions and *in situ* mRNA hybridizations are thus very similar in that they suggest that the major gene activation responses in incompatible reactions are localized in a narrow ring of cells immediately outside the point of hypersensitive pathogen containment. Limited gene activations may also occur in cells more distant from the lesion.

Few studies have examined the *in situ* accumulations of the proteins associated with these gene activations. Approaches similar to those taken with the proteinaceous defense responses discussed below should be applicable and such information should nicely complement the mRNA work.

Measurement of metabolic products in discrete cell populations. If one could conveniently measure the products of the phenylpropanoid pathways in defined cell populations, it should strongly complement both messenger RNA and enzyme localization studies. There are several important advantages of direct product measurements. Perhaps most important is the fact that they reflect the net result of the total regulatory cascade leading to resistance. As such they provide a monitor that is essential to defining the relative importance of various specific events within signal perception, transduction, and response. In addition, defense products are readily quantified and reflect the true stoichiometry of the products presumed to be involved in resistance. When complex mixtures of metabolites can be examined (as in the case of HPLC profiling), there is an added advantage in that the individual metabolites in entire metabolic pathways can often be simultaneously monitored. This provides a particularly powerful tool to define the preliminary nature and regulation of genes of possible future interest. Several approaches to measure defense metabolites directly at a cellular level are reviewed below. Each has its particular advantages, limitations, and uses.

Laser microprobe mass analysis and high-performance liquid chromatography for metabolite profiling in defined cell populations. A pioneering report on the direct application of instrumental chemical analyses to the measurement of cellular concentrations of the metabolic products of gene activation was the use of laser microprobe mass spectral analysis (LAMMA) for the measurement of glyceollin in soybean-*P. m.* f. sp. *glycinea* interactions (Moesta *et al.* 1982). LAMMA has remarkable sensitivity, theoretically allowing analyses of individual metabolites in targets as small as 1 μm^3. By making analyses in a grid, precise gradients in concentration can be measured away from any point source. With this approach, Moesta *et al.* (1982) took advantage of the fact that glyceollin shows a characteristic mass fragment at m/e = 321. A dramatic increase in glyceollin concentration was demonstrated as the infection front was approached from uninfected tissues. This was one of the first reports of the use of LAMMA on biological specimens and was the first report of cellular measurement of glyceollin levels. Two major limitations of the method are the need for a characteristic mass fragment of the compound being examined and the fact that accessibility to LAMMA instruments is highly limited.

An alternative method for the direct quantification of metabolites in complex mixtures is the use of high-performance liquid chromatography (HPLC). Although HPLC has been used for many years to analyze small molecules in partially purified fractions, its resolution and sensitivity have not been fully exploited. A high-resolution, HPLC-profiling procedure has been developed that allows the simultaneous analysis of a wide range of soluble aromatic secondary metabolites in unfractionated extracts of very small tissue samples (Graham 1991a). As little as 100 femtomoles of a given metabolite can be detected in samples of less than 1 mg wet weight. Though less sensitive than LAMMA, the simplicity of HPLC allows processing of large numbers of samples. The particular effectiveness that these profiles have in mapping out most, if not all, of the various concurrent metabolic alternatives of a given class of molecules has been demonstrated in a number of plant and microbial systems (Graham 1991a). When used in conjunction with ultrathin sectioning and different sampling times, one can obtain a very dynamic spatial and temporal representation of entire pathways in discrete cell populations, much like combining metabolic snapshots into a moving picture.

The use of HPLC to investigate incompatible infections of soybean cotyledon tissues with *P. m.* f. sp. *glycinea* demonstrated that the isoflavones daidzein and genistein are rapidly released from preexisting conjugates in a narrow zone of cells defining the infection front (Graham *et al.* 1990). Genistein is directly toxic to *P. m.* f. sp. *glycinea* (Graham 1989; L. I. Rivera-Vargas, A. F. Schmitthenner, and T. L. Graham, unpublished). Daidzein is a precursor of the phytoalexin glyceollin, which was shown to accumulate concurrently with the release of daidzein in these same cells. In compatible reactions, hydrolysis of the conjugates occurred later and well behind the infection front. Very low amounts of glyceollin accumulated.

The distribution of daidzein and genistein conjugates differs markedly in different soybean seedling organs and is under intricate developmental and environmental regulation (Graham 1991b). For example, unlike cotyledon tissues, hypocotyl tissues contain comparatively low amounts of the preformed conjugates. Preliminary results with leaf, hypocotyl, and root tissues suggest that, although there is a net hydrolysis of isoflavone conjugates and an accumulation of glyceollin at the incompatible infection front, there is an additional marked buildup of the conjugates in zones immediately beyond the front (T. L. Graham, unpublished). It may be that this distal accumulation of the isoflavone conjugates in tissues ahead of the infection front plays an important role in building up the defense potential of these more isoflavone deficient tissues. Thus a wave of isoflavone synthesis may spatially and temporally precede glyceollin accumulation.

This hypothesis is supported by studies with the *P. m.* f. sp. *glycinea* wall glucan elicitor (Graham and Graham 1991). HPLC profiling of thin sections of elicitor-treated cotyledon tissues led to the finding that this biotic elicitor, which had previously been characterized as a glyceollin elicitor, induces even more massive accumulations of the daidzein and genistein conjugates (Graham and Graham 1991). Intriguingly, while glyceollin accumulated only in cells immediately proximal to (one to four cells away from) elicitor treatment, the isoflavone conjugates accumulated to levels as high as nine times their initial levels in cells five to 20 cells away from the treated surface.

An important limitation of HPLC profiling is that it is readily applicable only to soluble defense molecules. However, a combination of HPLC and microanalytical spectrophotometric analyses has been explored for the quantitation of phenolic residues in solubilized wall fractions from *P. m.* f. sp. *glycinea* wall glucan-treated soybean cotyledon tissues (Graham and Graham, in press). Phenolic polymers and esterified phenolic acids accumulated rapidly (10 times over controls within 4 hr) in cell walls immediately proximal to elicitor treatment. Deposition of phenolics in the wall was complete by 24 hr, when it was several times greater than the later glyceollin and isoflavone responses combined. Accompanying the strictly localized deposition of wall-bound phenolics were proximal and distal increases in the activities of a specific group of anionic peroxidase isozymes (Graham and Graham, in press). Equally rapid phenolic polymer deposition also occurs in infected cotyledon, leaf, hypocotyl, and root tissues (T. L. Graham, unpublished), although the precise temporal and spatial aspects are still being examined.

Thus, incompatible infection and *P. m.* f. sp. *glycinea* wall glucan treatment of soybean tissues induce several phenylpropanoid responses that are regulated in different ways in proximal and distal cell populations. The relative flux of metabolites into and out of the isoflavone conjugate, glyceollin, and wall-bound phenolic pools is under intricate spatial, temporal, developmental, and tissue-specific control. These results may reflect, at a product level, gene activations similar to those (PAL, CSH, etc.) discussed above for other hosts. Importantly, the possible involvement of additional enzymes in resistance, such as those involved in isoflavone conjugate turnover, is apparent (Graham and Graham 1991).

Immunologically based assays for small molecules. Small molecules can also be quantified directly in extracts of thin tissue sections by means of immunologically based procedures such as radio-immunoassays (RIAs). The advantages of such assays are their sensitivity (typically picomolar) and the ease of handling large numbers of samples. However, the preparation of antibodies for each class of molecules of interest and the careful characterization of their specificities can be laborious. The latter point cannot be overemphasized because cross reactivity with known or unknown structurally related molecules is very common. These immunological methods usually also do not differentiate between a parent molecule and its conjugates.

An excellent example of the application of these methods is the work of Hahn *et al.* (1985) involving the measurement of glyceollin in relation to the infection front of *P. m.* f. sp. *glycinea*-inoculated soybean roots. To determine the precise spatial and temporal relationship of glyceollin accumulation to infection, an RIA for glyceollin I was combined with an immunofluorescence method for localization of the fungal hyphae. The incompatible response involved penetration of the epidermis and extensive colonization of the cortical cell layers immediately adjacent to the inoculation site. Glyceollin accumulated in both epidermal

and cortical cell layers, where it reached strongly inhibitory levels by 8 hr. In contrast, compatible infections were characterized by unrestricted penetration of both epidermal and cortical cell layers and colonization of the stele. Glyceollin accumulation was predominantly in the epidermis, where it was both delayed and of lower magnitude than in the incompatible response. Although glyceollin accumulation was associated with restricted growth of the incompatible fungus in the cortex, colonization of this tissue nonetheless took place. The inability of the fungus to penetrate into the stele suggests an additional major determinant of resistance.

Histochemical, fluorescence, and cytological approaches as complements to direct molecular analyses. Histochemical methods have been employed to demonstrate localized biochemical responses for many years, but such methods are not highly specific. However, if used in conjunction with other molecular methods, these approaches can provide very valuable complementary information on cellular responses. A particularly important use for histochemical stains has been in the localization of induced changes in cell wall polymers, which are insoluble and thus more difficult to quantitate by conventional means. A large number of stains are available for cell wall polymers (Jensen 1962; High 1984). Most of these react with particular functional groups (e.g., phloroglucinol reacts with free aldehyde functions) and thus are at best semiselective. The results must be complemented by direct chemical confirmation of the presence of the appropriate structural polymer.

Fluorescence microscopy is also a tool of potential use in the cytological analysis of defense product accumulation. For a number of years, it has been known that deposition of phenolics in the symplast and apoplast leads to autofluorescence of cells responding hypersensitively to infection. Fluorescence microscopy is a very sensitive method and can be applied *in situ* (Cuypers and Hahlbrock 1988). It can be somewhat selective when the specific wavelengths for excitation/emission are known for the molecules of interest. However, because closely related molecules often accumulate in infected tissue, this technique gives only preliminary results and must be complemented with other approaches.

An innovative use of fluorescence microscopy is illustrated by a series of papers by Essenberg and co-workers on the accumulation of sesquiterpene phytoalexins in hypersensitive lesions of cotton responding to *Xanthomonas campestris* pv. *malvacearum* (Essenberg *et al.* 1979; Essenberg *et al.* 1986; Pierce and Essenberg 1987). Importantly, these studies represent an attempt to examine molecular responses to single cell inocula, rather than to massive bacterial infiltrations. Essenberg and co-workers first established (Essenberg *et al.* 1979) that leaves of resistant cotton lines respond to infection by accumulating the phytoalexins 2,7-dihydroxycadalene (DHC), lacinilene C (LC), and the methyl ether of lacinilene (LCME). The mesophyll cells immediately surrounding the site of bacterial colonization had a fluorescence characteristic of LC and LCME (Essenberg *et al.* 1986). To demonstrate that the fluorescing cells were in fact those specifically accumulating the phytoalexins, Pierce and Essenberg (1987) employed fluorescence-activated cell sorting. Brightly fluorescent cells contained 10–25 times as much LC and 40 times as much DHC as less fluorescent cells; thus, more than 90% of the phytoalexins were concentrated at the infection center.

Another excellent cytological investigation relative to the localization of secondary product responses is the recent study of anthocyanin phytoalexin accumulation in sorghum by Snyder and Nicholson (1990). These workers demonstrated that clear vesicles began to accumulate in leaf cells located immediately underneath appressoria of *Colletotrichum* within 2 hr after their formation. By 4 hr, these vesicles had migrated toward the point of appressoria attachment and became red. By 8 hr, the vesicles began to coalesce specifically at the site of appressoria attachment. By 23 hr, the cytoplasm of the responding cell was filled with the phytoalexin, and vesicles from neighboring cells had begun to migrate toward and deposit anthocyanins into the hypersensitive cell. These observations suggest that even hypersensitively dying cells may contribute to active phytoalexin accumulation. It remains to be seen, however, whether this accumulation is accompanied by early gene activation. Intriguingly, phytoalexin synthesis may occur in vesicles as they are actually moving toward their target. This suggests that the host may dynamically package the required enzymes and substrates for phytoalexin accumulation and then deliver the finished product to the appropriate target site. Such packaging and discrete delivery could avoid the immediate toxicity of these metabolites to the responding cell.

Conclusions—secondary product defense responses. Studies on secondary product defenses underscore the complementary nature of different approaches to cellular work. Results to date suggest that containment of the pathogen in the hypersensitive lesion may be accompanied by events occurring both in hypersensitively dying cells and in living cells that immediately surround them. Whether events in these two zones are clearly separate and sharply localized, as suggested by Schmelzer *et al.* (1989), or occur as progressive temporal events in the same cells, as suggested by the work of Synder and Nicholson (1990), may depend on the specific host-pathogen interaction. In any case, depending on the system, these local events may lead first to the activation of genes for early phenylpropanoid and flavonoid metabolism (e.g., PAL, CHS) and somewhat later to activation of the genes for phytoalexin accumulation. At the metabolic level, these events may result first in the deposition of wall-bound phenolics (Jahnen and Hahlbrock 1988; Graham and Graham, in press), followed by the hydrolysis of isoflavone conjugates (Graham *et al.* 1990) and the accumulation of phytoalexins (Schmelzer *et al.* 1989; Graham and Graham 1991).

More distal responses, in cells that are well ahead of the hypersensitive infection front, or point of elicitor treatment, include the induction of transcripts of CHS (Schmid *et al.* 1990; Stermer *et al.* 1990), the accumulation of isoflavone conjugates (Graham and Graham 1991), and increases in the activity of anionic peroxidases (Graham and Graham, in press). Such distal cell responses may build up the defense potential of cells in advance of the infection front (Bell *et al.* 1986; Graham and Graham, in press; Graham and Graham 1991).

It will be interesting to determine if the two zones of

local response noted above and the distal cell responses each involve unique signal-response events or simply reflect the progressive expressions of a wave of gene activations stimulated by a central elicitation event.

HYDROLYTIC ENZYMES

Of the various defense-related proteins in plants, the accumulations of the hydrolytic enzymes, chitinase and β-1,3-glucanase, have been the most extensively investigated. Both of these hydrolytic enzymes have been identified as true pathogenesis-related (PR) proteins and share certain attributes with other PR proteins, such as low molecular weight, resistance to proteolytic enzymes, extraction at low pH, and presence in the apoplast of infected tissues (Van Loon 1985). Chitinases and β-1,3-glucanases are thought to play multiple roles in resistance. First, they may release pathogen cell wall fragments that act as elicitors of active host defense responses and, secondly, they may degrade the pathogen's cell wall or disrupt its deposition, contributing to pathogen death. On this latter point, chitinases and β-1,3-glucanases have been shown to act synergistically in the lysis of hyphal tips, which is the site of new wall deposition (Mauch *et al.* 1988). In either of these roles, the apoplastic localization of these enzymes would seem important. Thus, for this group of enzymes, efforts have focused on the subcellular localization of these responses as well as on their spatial and temporal coordination.

The apoplastic localization of both chitinases and β-1,3-glucanases in infected tissues was first suggested from several studies in which intercellular washing fluids were examined (DeWit *et al.* 1986; Kauffman *et al.* 1987; Legrand *et al.* 1987; Kombrink *et al.* 1988). The accumulation of these enzymes was found to be more rapid in resistant than in susceptible tissues, supporting their involvement in a defensive role (Joosten and Dewit 1989).

To circumvent problems inherent to infected tissues, Mauch and Staehelin (1989) used ethylene to induce chitinases and glucanases in bean leaf tissue and undertook a more detailed examination of the subcellular distribution of the enzymes by a combination of immunocytochemistry and biochemical fractionation. Ethylene-induced enzymes in this system accumulated nearly exclusively in protein aggregates within the vacuole (100% of the chitinase and >95% of the β-glucanase). The small amounts of β-glucanase that were found to be apoplastic were primarily associated with the middle lamella. These results led Mauch and Staehelin (1989) to propose a general model in which these hydrolytic enzymes enter the apoplast only after cell death accompanying the hypersensitive response.

Although this is an interesting model, and may explain some host pathogen interactions, observations from several other studies strongly suggest that regulation of the distribution of these enzymes is complex and not simply the result of cellular disruption. In tobacco, for instance, both acidic and basic isoforms of the β-1,3-glucanases exist, which are encoded by different genes (Van den Bulcke *et al.* 1989). The two basic isoforms, similar to the previously characterized cytokinin- and auxin-regulated enzymes, were located in the central vacuole. Salicylic acid treatment or infection with *Pseudomonas syringae* led to the specific, apoplastic accumulation of three additional, acidic isoforms (Van den Bulcke *et al.* 1989). The vacuolar isoforms were not secreted into the extracellular spaces following infection. Thus, at least in this system, the genes regulated by hormones (basic, intracellular isoforms) are distinct from those regulated by infection (acidic, extracellular isoforms).

The active accumulation of β-glucanase in the apoplast of infected tissues is further supported by the elegant studies of Benhamou *et al.* (1989) on resistant and susceptible interactions of tomato with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. The precise locations of the infecting hyphae and the accumulations of β-glucanase were compared. In compatible interactions, the fungus spread throughout several tissues, finally entering the vascular stele by 120 hr. The accumulation of β-glucanase, as measured by immunogold techniques, occurred in the cell walls of all cells encountering the advancing fungus. Significantly, this enzyme was localized only in that area of the host cell wall immediately in contact with the fungus. In incompatible infections, which were strictly limited to the outermost tissues of the root, massive depositions of β-glucanase were detected in the cell walls of healthy cells well ahead of the infection front. Thus, the deposition of this enzyme in the wall is a specific phenomenon and is not the result of tissue disruption.

Parallel studies with chitinase showed that in both compatible and incompatible interactions, induction is confined to those cells in immediate contact with the fungus (Benhamou *et al.* 1990). Induced chitinase was not found in vacuoles, but was predominantly associated with the fungal cell wall in the intercellular spaces. Often it was clearly associated with areas of fungal cell wall degradation. These results led the authors to hypothesize that induction of glucanases spatially ahead of the chitinases, in incompatible responses, may be critically important to 1) earlier dissolution of pathogen wall glucans in which the chitin polymers may be imbedded, and 2) release of the pathogen glucan elicitors that may function to induce the chitinases.

Although protein localization studies have predominated in examinations of hydrolytic enzymes, transcriptional induction at a cellular level has also been investigated. In transgenic tobacco, a GUS reporter gene driven by a β-glucanase promoter from the *gn1* gene of *Nicotiana plumbaginifolia* was shown to be constitutively expressed in root and lower stem and leaf tissues (Castresana *et al.* 1990). This gene encodes a protein that is different from the previously characterized intra- and extracellular isoforms in tobacco (Van den Bulcke *et al.* 1989). In incompatible bacterial infections of these same transgenic plants, GUS activity was not expressed in the developing hypersensitive lesion, but was strictly localized to living nonhypersensitive cells just outside of the necrotic lesion.

Roby *et al.* (1990) examined the activation of the bean chitinase 5B promoter fused to a GUS reporter gene in transgenic tobacco plants infected by *Botrytis cinerea*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. Although there were subtle differences in the responses to the different pathogens, marked induction was seen predominantly in the lesion itself or in a narrow zone of cells immediately surrounding the lesion. Although of lower magnitude, GUS activity between lesions and in the uninoculated half of

an inoculated leaf was often severalfold that of controls. In *Botrytis* infections, for example, GUS activity was nearly 200 times that of controls in the lesion *per se*. Apparently healthy tissue up to 3 mm away from the lesion showed a 12-fold reduction in GUS activity, while tissues 3–6 mm away showed a further sevenfold reduction, being only double that of controls.

Analyses of transcriptional gene activations in potato leaf tissues infected with *Phytophthora infestans* indicated that, in contrast with the local activation of genes for enzymes of phenylpropanoid metabolism, the expression of both chitinase and β-1,3-glucanase is stimulated more slowly and systemically throughout the leaf (Kombrink *et al.* 1990). No differences were seen in total activities of these hydrolytic enzymes in resistant or susceptible interactions.

Summary—hydrolytic enzymes. In general, the accumulation of the hydrolytic enzymes appears to be a relatively late event (see e.g., Joosten and DeWit, 1989; Kimbrink *et al.* 1990). Although this is consistent with the debilitation and ultimate containment of the pathogen in resistant reactions, it would seem to be too late to account for release of the first elicitor signals from the pathogen. Possibly, low levels of specific isozymes may be constitutively present in the apoplast (see, e.g., Fink *et al.* 1988). Elicitor release may depend initially on these constitutive enzymes. Released elicitor signals may then trigger multiple defense strategies, including the extracellular β-glucanases and chitinases needed to generate greater levels of the elicitors themselves. Such a signal amplification mechanism is reminiscent of the induction of cutinase genes in pathogenic fungi (Woloshuk and Kolattukudy 1986; Podilla *et al.* 1988).

Like secondary metabolite responses, the accumulations of chitinases and β-1,3-glucanases have been shown to include both local and distal cell responses. Current research suggests that the glucanases may be induced spatially in advance of the chitinases (Benhamou *et al.* 1989; Benhamou *et al.* 1990). How distal induction of glucanases relates specifically to distal induction of CHS (Stermer *et al.* 1990), isoflavones (Graham and Graham 1991) and peroxidase (Graham and Graham, in press) is an intriguing question.

HYDROXYPROLINE-RICH GLYCOPROTEINS

Several classes of hydroxyproline-rich glycoproteins (HRGPs), which differ slightly in amino acid sequence and patterns of glycosylation, exist in plants (Cooper *et al.* 1987; Showalter and Varner 1989). Those proposed to be involved in host defense are: 1) the insoluble extensins, which are thought to play primarily a structural role in strengthening the cell wall, and, 2) both the soluble extensins and lectin-active HRGPs, which display agglutination activity and have been proposed to be involved in pathogen recognition and immobilization (Sequeira and Graham 1977; Leach *et al.* 1982; Mellon and Helgeson 1982; Bolwell 1987).

Because of their well-established localization in the cell wall, research on HRGPs has focused mainly on their physical association with induced paramural cell wall appositions. For example, incompatible infections of bean hypocotyl with *Colletotrichum lindemuthianum* were characterized by an early (<4-day) and massive accumulation of HRGP transcripts in the epidermal, cortical, and perivascular tissues immediately adjacent to the inoculation site (Templeton *et al.* 1990). Because the fungus did not penetrate beyond the epidermal layer, these results demonstrate both proximal and distal cell responses. The compatible reaction was markedly different. By 7 days, colonization had progressed past the epidermis and throughout the cortical cell layers, but at no time was message accumulation seen in epidermal or cortical cells. Only a late accumulation (7 days after inoculation) of transcripts was seen in the perivascular tissue.

Immunocytochemical localization of extensin in melon and bean plants after inoculation with several pathogens has been examined (O'Connell *et al.* 1990). The saprophyte *Pseudomonas fluorescens* caused no visual symptoms in leaves of either host, but within 6 hr of inoculation, extensin antibodies were bound to the paramural region associated with membrane invagination and with amorphous material encapsulating the bacteria. The authors conclude that the early appearance of HRGPs in the encapsulating material is consistent with the involvement of these glycoproteins in agglutination and immobilization of bacteria on plant cell walls. *Pseudomonas syringae* pv. *phaseolicola* race 3 induced typical hypersensitive reactions in leaves of both hosts. Within 3 hr of inoculation, extensin was localized in the paramural area at sites of membrane convolution. By 12 hr, the developing electron translucent papillae were labeled and after 48 hr, the cell walls and paramural papillae in living cells adjoining hypersensitive cells were intensely labeled. The extracellular polysaccharide surrounding bacterial cells was not labeled.

Incompatible infection of bean hypocotyl with the fungal pathogen, *C. lindemuthianum*, led to extensin localization results quite similar to those described above for bacterial pathogens (O'Connell *et al.* 1990). Thus, antibody labeling was mainly associated with the cell walls of living cells immediately adjacent to hypersensitive cells and with the development of paramural papillae in these cells. In contrast to the *in situ* mRNA hybridization results of Templeton *et al.* (1990) with this same host-pathogen combination, however, distal cellular responses were not detected.

CONCLUSIONS

Exciting progress has been made in the emerging area of the cellular biochemistry of host-pathogen interactions. It is becoming more and more evident that plants are remarkably sophisticated in the coordination of their resistance responses. Although similar classes of defense-related genes are induced in different plant species, the specific genes involved and the products of their induction are often species-specific or even organ-specific. The induction of the various defense-related genes within a given species, moreover, is under intricate temporal and spatial control, which may be specifically tailored for the particular pathogen invading the plant.

It is also apparent that different discrete cell populations in the host carry out different activities relating to the overall defense of the plant. Although some responses

appear to be truly localized in relation to the advancing infection front (e.g., hypersensitivity *per se*, the accumulations of phytoalexins and phenolic polymers and, in some cases, the accumulation of chitinases), others seem to be generated in a progressive wave outward from the initial site of infection (e.g., CHS gene activation and the accumulations of isoflavones, β-1,3-glucanases and peroxidases).

Related to these aspects of cell response are the processes involved in the generation and perception of elicitors for the various molecular events (Lamb *et al.* 1989; Dixon and Lamb 1990). Although both proximal and distal cell reactions are generated by elicitor treatments, research on the nature of the actual signals that elicit these responses and on signal perception and signal transduction in differently responding cells is needed. Purified elicitors, with a defined relationship to proximal and distal events in infected tissues, would greatly facilitate cellular studies.

The involvement of putative molecular defense mechanisms in plant resistance has always been largely correlative. As our understanding and our methodologies become more refined, and attuned to events *in planta*, these correlations should become more meaningful. The application of complementary technologies, the refinement of existing tools, and the development of new *in situ* methods will undoubtedly facilitate future studies and the comparison of common schemes and subtle differences in parallel systems.

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