PHENOLIC COMPOUNDS AND THEIR ROLE IN DISEASE RESISTANCE

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INTRODUCTION

Antibiotic phenols have been found in all plants investigated to date. Some occur constitutively and are thought to function as preformed inhibitors associated with nonhost resistance (84, 94, 128, 134). Others, which are the subject of this review, are formed in response to the ingress of pathogens, and their appearance is considered as part of an active defense response. Since the first suggestions that phenolic intermediates have a role in the active expression of resistance, an underlying problem in ascertaining that such secondary metabolites are of primary (rather than secondary) importance has been the localization and timing of the host response. In this review we address the problem of response localization and localization of phenolics relative to the sequential development of stages of disease that lead ultimately to resistance expression.

Initial demonstrations that phenols are significant components of the host
response involved the isolation, and sometimes the identification, of phenols from substantially large amounts of tissues following inoculation (76, 125). Although successful within the constraints of the available technology, this approach was flawed by several problems, not the least of which was the demonstration that phenols actually do accumulate within the infection site and not only in a zone surrounding it. The advent of molecular biology and improved technologies, such as high performance liquid chromatography (HPLC) analyses, for the isolation and identification of small amounts of phenols changed our assumptions about the significance of phenolic intermediates relative to the overall host response. In particular, these techniques brought to light the importance of understanding each disease interaction as a unique phenomenon that rarely can be considered, in a generalized sense, as representative of host responses that occur in diverse plant species.

INITIAL EVENTS IN THE HOST RESPONSE

Heath (64) has argued effectively for the differentiation of the responses of plants to pathogens based on host and nonhost interactions. In such relationships it has long been recognized that responses are characterized by the early accumulation of phenolic compounds at the infection site and that limited development of the pathogen occurs as a result of rapid (hypersensitive) cell death (37). Mansfield (82) has proposed that cell death results from irreversible membrane damage that may occur in response to pathogen recognition or as a result of activated host responses. Regardless of the reasons for cell death, it is thought that rapid accumulation of phenols may result in the effective isolation of the pathogen (or nonpathogen) at the original site of ingress (39, 40, 79, 121). These responses include the formation of lignin, the accumulation of cell-wall appositions such as papillae (1, 140), and the early accumulation of phenols within host cell walls (106, 107, 130). Numerous studies suggest that low molecular weight phenols, such as the benzoic acids and the phenylpropanoids, are formed in the initial response to infection (77, 107). Evidence strongly suggests that esterification of phenols to cell-wall materials is a common theme in the expression of resistance (39, 44, 45). Moreover, the accumulation of polymerized phenols occurs as a rapid response to infection (8, 36, 50). A common host response is the esterification of ferulic acid to the host cell wall (88), and it has been suggested that crosslinking of such phenylpropanoid esters leads to the formation of lignin-like polymers (44). Such hydroxycinnamic acids and their derivatives are thought to contribute to the discoloration and autofluorescence of host tissues at the site of infection (2, 8, 36). Unfortunately, the identity of the phenols that accumulate as an initial response to infection is not often addressed, and the assumption for their presence is often based on histochemistry or fluorescence microscopy.
Although it is often suggested that lignin accumulates early in response to infection, little direct chemical evidence supports its formation as an initial host response (see the discussion of lignins below). One response observed consistently to attempted infection, in both compatible and incompatible interactions, is the necrosis of cells. Pertinent questions relative to necrosis are whether phenolic intermediates are involved, whether the response requires phenolic compound synthesis, when the response begins relative to the infection process, and whether specific antibiotic products such as phytoalexins are involved. Matern & Kneusel (88) have proposed, as have others (6, 25, 83), that the defensive strategy of plants exists in two stages. The first is assumed to involve the rapid accumulation of phenols at the infection site, which function to slow (or even halt) the growth of the pathogen and to allow for the activation of “secondary” strategies that would more thoroughly restrict the pathogen. Secondary responses would involve the activation of specific defenses such as the de novo synthesis of phytoalexins or other stress-related substances. They argue that the initial defense response must occur so rapidly that it is unlikely to involve de novo transcription and translation of genes, which would be characteristic of the second level of defense. Thus, the sequence of events in a defense response can be thought to include (in this order) host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers such as appositions or papillae, and, finally, the synthesis of specific antibiotics such as phytoalexins. This hypothesis accords with observations that de novo transcription and translation of genes known to be associated with defense requires more time than is available for the rapid appearance of compounds that would constitute a “primary” response event (31, 55).

The work of Hahlbrock and colleagues on the response of potato to fungi and biotic elicitors has described the early events in the expression of compatible and incompatible disease responses relative to phenolic compound metabolism. In the interaction with Phytophthora infestans, with certain host cultivars the biochemical and morphological response of foliage to compatible and incompatible races of the pathogen is distinctly different (17, 43, 126). The differential responses of the cultivar Datura provided a tool for the immunohistochemical investigation of compatible and incompatible interactions with races of P. infestans and the incompatible fungus P. megasperma f.sp. glycinea (21). These studies showed that rapid necrosis of mesophyll cells within hours of infection distinguished the incompatible from the compatible response. Histochemical staining of tissues with toluidine blue O together with clearing and fluorescence analysis (13) demonstrated that accumulation of phenols at the infection site occurred as early as 3 hr after inoculation, indicating an association of phenols with the initial stages of the response.

Subsequent studies involved in situ RNA-RNA hybridization in juvenile
leaves and allowed for the demonstration of temporal and spatial differences in the accumulation of phenylalanine ammonia-lyase (PAL) mRNA that occurred as a response to infection (22). As expected, PAL mRNA was rapidly elevated in interactions involving an incompatible race of the fungus, whereas a significantly different profile of mRNA accumulation occurred in interactions involving a compatible race. The expression of a hypersensitive response was established within 3 hr from the time of inoculation and included the death and necrotization of cells at the site of initial penetration. Importantly, the infection site itself was not the site of the maximum PAL response. Rather, the healthy as yet uninfected cells surrounding the infection site exhibited a marked accumulation of PAL mRNA. This was a transient response in that by 6 hr after inoculation, the accumulation of PAL messenger fell markedly and was only marginally greater than that of noninoculated tissue. In contrast, in a compatible interaction with the same host cultivar, the PAL messenger became progressively elevated as the time after inoculation lengthened, and the pattern of mRNA accumulation was diffused throughout the tissue rather than localized within the immediate zone of the infection site.

Because PAL elevation has often been favored as an indicator of resistance (7, 19, 27, 29, 30, 43, 71, 78), these results presented a complex problem relative to activation of phenolic compound synthesis as the primary event in expression of resistance. The likelihood that PAL mRNA accumulation does not always involve increased transcription was negated by experiments involving in vitro runoff transcription with the potato-P. infestans system (43). The PAL gene activation that occurs in a zone of living cells surrounding the area of cell death at the infection site is presumed to require signal transmission in advance of the fungus (22). Thus, the initial event in the response of potato leaves appears to be the highly localized, rapid death and necrotization of a limited number of host cells. In the interaction of potato tubers with Verticillium dahliae, hypersensitive browning and suberization are characteristic of the initial events in resistance rather than the production and accumulation of phytoalexins (143). Similarly, there is good evidence that the resistance of tomato to Verticillium and Fusarium wilt pathogens depends more on the mechanical isolation of the pathogen than on the synthesis of phytoalexins, although phytoalexins cannot be ruled out as contributors to expression of resistance (33).

The kinds of phenolic compounds that accumulate prior to the active defense response as well as their origin has been addressed by Matern and coworkers using parsley cell suspensions as a model system. Inoculation of parsley leaves with P. megasperma f.sp. glycinea (Pmg) or treatment of parsley cell suspensions with a Pmg elicitor results in the accumulation of substantial concentrations of coumarin phytoalexins as well as esterification of phenylpropanoids, in particular ferulic acid, to cell walls (88, 89, 139).
Such esters are thought to be involved in the formation of phenolic polymers by crosslinking (44).

Treatment of parsley cells with the $Pmg$ elicitor causes the synthesis of the coumarin phytoalexins isopimpinellin, psoralen, bergapten, xanthotoxin, and graveolone (139). It was suggested that the rapid induction of coumarin synthesis results from intracellular signaling (136, 137), which may involve polyphosphoinositides in signal transduction (89). Although evidence does not substantiate the involvement of polyphosphoinositides in signal transduction (135), the synthesis of these compounds does occur rapidly, indicating that another mechanism must be involved for the conversion of phenylpropanoids to coumarins following elicitor treatment. In addition to phytoalexin accumulation, lignin-like polymers accumulate as a rapid response to infection (8, 36), and such materials are generally presumed to represent a chemical and/or physical barrier to the pathogen (108).

To explain the rapid production of potentially inhibitory phenols that occurs before the synthesis of phytoalexins, the synthesis of components of cell wall-bound polymers in elicitor-treated parsley cell suspensions was investigated. Caffeoyl-CoA is formed from 4-coumaroyl-CoA by an unusual hydroxylase, the activity of which depends on a substantial decrease in cytoplasmic pH (74). Elicitor-treated cells also synthesize a methyl transferase (S-adenosyl-L-methionine:trans-caffeoyl-coenzyme A 3-O-methyltransferase) for the conversion of caffeoyl-CoA to feruoyl-CoA (108–110). This scenario suggests that potentially toxic phenylpropanoids, such as ferulic acid, can form rapidly without the involvement of the traditionally accepted route of phenylpropanoid synthesis and conversion to CoA esters (51, 55). Importantly, the dependence of this synthesis on a drop in cytoplasmic pH explains the rapid accumulation of derivatives of hydroxycinnamic acids during a time span that is generally considered insufficient for transcription and translation of enzymes required for substantial phenylpropanoid synthesis.

As stated above, evidence (47) does not support the suggestion that lignification is an initial (primary) host response (121), although lignin is undoubtedly a component of structural barriers such as papillae that form in response to penetration (14, 122, 140). Rather, it appears that for most plants it is low molecular weight phenols, especially the phenylpropanoids, that are involved in the initial response to stress. In potato, phenols that do not exhibit staining properties for lignins accumulate as an initial response to infection (52, 58). Evidence presented by Friend (39) suggests that these compounds include 4-coumaric and ferulic acid as well as a feruloyl-β-1,4-galactan. Similar rapid changes in phenolic composition have been shown for elicitor-treated bean cells (8) and for chitosan and elicitor-induced changes in phenol composition of soybean cells (36, 72).
An important concept to be drawn from these studies is that the initial response of the tissue occurs prior to the expected stimulation of synthesis of phenolic metabolites. Other plant defense reactions may precede the active synthesis of phenolics such as phenylpropanoids, other phenols, or phytoalexins; and the onset of de novo synthesis of phenols, as evidenced by transcription and translation of enzymes essential to phenol synthesis, may occur as a secondary, albeit rapid, response (21, 22, 71). Thus, in parsley, the synthesis of furanocoumarin phytoalexins does not appear to be the initial defense response, and in potato the earliest responses seem to be associated with the necrotization of cells at the infection site. Regardless of their timing, the initial responses are clearly characterized by the accumulation of phenols in cells that have become necrotized. Thus, in some systems an initial response of the tissue is apparently characterized by phenolic accumulation that does not result from an active synthesis of phenols in response to infection.

As indicated earlier, PAL is often considered to be representative of the initiation of the synthesis of phenols in response to infection. This suggestion may be misleading, as a detectable increase in PAL activity is not always the first response to infection. For example, although increased enzyme activity as well as coordinate regulation of enzymes of phenylpropanoid synthesis are frequently observed following infection or elicitor treatment (54, 87, 90, 99), some reports have demonstrated a lack of correlation between PAL and expression of resistance (9, 18, 26). Such observations led Smith & Rubery (131) to suggest that the transient increases in PAL activity in potato tuber tissue following inoculation with *P. infestans* were related more to wounding than to infection. Similarly, in maize, wounding stimulates PAL expression (112) whereas fungal infection in incompatible interactions does not (26, 111), even though both phenomena increase phenolic compound biosynthesis and oxidation (103). Extractable concentrations of PAL increased in compatible interactions with *Cochliobolus heterostrophus* but only if the tissue was incubated in the dark (111). Moreover, preinoculation with *C. heterostrophus* followed by a challenge with *C. carbonum* reversed the normal expression of resistance to *C. carbonum*. These results suggest that susceptibility of maize to *C. heterostrophus* is due to the fungus interfering with the plant’s normal expression of resistance, which involves activation of phenylpropanoid synthesis, esterification of phenylpropanoids to the cell wall, and the accumulation of phenylpropanoid esters (81). The results further emphasize the danger of using the expression of PAL to indicate either the expression of resistance or the involvement of phenolic metabolism as part of the host response.

Early after infection, low molecular weight phenols accumulate in both incompatible (resistant) and compatible (susceptible) interactions. Whether these compounds are significant in the ultimate host response presents a perplexing problem. It has been suggested (39, 115) that the accumulation of
phenolics as an initial response to infection may reflect a general increase in host metabolism as well as an accumulation of relatively nontoxic secondary metabolites, which could ultimately serve as precursors for compounds essential to expression of resistance. For example, in maize there is a marked accumulation of two caffeic acid esters after inoculation with *Glomerella graminicola* or *C. heterostrophus* in both compatible and incompatible combinations (81). One compound was identified as caffeoyl glucose, whereas the other was a caffeoyl ester of an unknown organic acid moiety. Although neither compound was fungitoxic, a pattern of rapid accumulation followed by a sharp decrease in the amount of both compounds in the tissue suggested that they may serve as a pool of phenols required for diversion to other products. One possibility is that they are associated with cellular browning and tissue necrosis that may result from oxidation and polymerization of *O*-diphenols (118). Such phenomena would be consistent with the relatively nonspecific disruptive effects on cells that result from wounding and lead almost immediately to a variety of physiological changes, including oxidation of secondary metabolites (11, 23). That the accumulation of these esters preceded the onset of visible necrosis of infection sites, and that the concentration of the compounds fell substantially after the onset of necrosis, both strengthen the argument for their involvement in the browning response. This response is somewhat similar to that described for the accumulation of hydroxycinnamoyl esters in response to virus infection, except that ester accumulation occurred after necrosis and was probably not a factor in initiating the hypersensitive response (42, 86, 138).

The maize caffeoyl esters may possibly represent intermediates in the synthesis of other phenols that may occur later in the disease interaction and may be important in the last stages of pathogen restriction. However, this is unlikely since the synthesis of caffeoyl glucose is mediated by UDP-glucosyltransferase rather than 4-hydroxycinnamoyl CoA ligase (144). Also, during the early period of disease development when these compounds are accumulating, there is no apparent turnover of other phenols and the primary change in phenols is reflected by esterification of phenylpropanoids to cell walls beyond the zone of the infection site (P. C. Lyons & R. L. Nicholson, unpublished). The origin and role of these compounds is further complicated by the fact that 4-hydroxycinnamic acid CoA ligase, which is crucial to the diversion of phenylpropanoids, appears to be the enzyme most conspicuously affected by fungal infection (26), and evidence suggests that differences in the synthesis of CoA ligase isozymes are associated with the expression of resistance and susceptibility (145). Isozyme differences in maize CoA ligase may possibly occur in noninfected tissues surrounding the infection site. If so, these differences may be considered as part of a "secondary" host response, similar to that proposed by Matern & Kneusel (88). Evidence for this is that
the final event in lesion restriction in maize involves the formation of a “stress lignin” composed of an exceptionally high level of syringyl units. The formation of such a lignin polymer should require CoA ligase enzyme activity (80), and an isozyme with activity toward 5-hydroxy ferulic acid accumulates late in the disease interaction and before lignin formation (R. L. Nicholson & J. R. Vincent, unpublished). This is important since sinapic acid, the presumed precursor of syringyl units in lignin, does not occur in maize tissue (53), indicating that 5-hydroxy ferulic acid and the CoA ligase specific for its acylation are essential to the final steps of expression of resistance. Note also that phenylpropanoids, found as free compounds, glycosides, and esters, accumulate within diseased maize tissues at the termination of lesion development (103). This phenomenon may reflect the source of phenylpropanoids necessary for the flavonoid anthocyanins to accumulate in healthy maize tissues surrounding lesions of incompatible interactions (62, 65).

In potato tubers chlorogenic acid accumulates slower following inoculation with *P. infestans* than in noninoculated controls, regardless of cultivar resistance (39, 46). In contrast, in some susceptible cultivars lacking major genes for resistance, chlorogenic acid accumulates at an accelerated rate after inoculation (66). Friend (39) suggested that chlorogenic acid may act as a reservoir for the caffeoyl moiety that, as an activated phenylpropanoid, could be shunted to the synthesis of other phenolics possibly involved in containment of the pathogen. The lack of accumulation in resistant interactions could also represent the direct conversion of CoA esters of phenylpropanoids to other phenols more essential to the defense response. Thus, the accumulation of chlorogenic acid (not a particularly toxic compound) may represent a general rise in phenolic biosynthesis. Such synthesis can be envisioned to ultimately result in the accumulation of compounds with sufficient toxicity to be involved in resistance. When conversion occurs, relative to the time of expression of resistance, remains in question.

**LIGNIN AND POLYMERIC PHENOLS: IDENTIFICATION AND IMPORTANCE**

The deposition of lignin and other polymeric phenols has been implicated as a defense response (140). Because of the polymeric nature of these compounds and their covalent attachment to cell walls, quantification of lignins and lignin-like polymers is more difficult than the soluble phenols discussed above. However, the insolubility of the compounds and their characteristic reactions with histochemical reagents does allow their direct observation in tissues following pathogen ingress.

A major difficulty in the study of cell-wall phenolic polymers is identifying their chemical nature. Histochemical reagents such as phloroglucinol-HCl,
chlorine-sulfite, and toluidine blue provide information (80, 100, 140), but no one reagent can be used to determine if the observed change in wall composition is due to lignin. Although precaution is advised because of the possibility of false positive and negative assays (80), histochemistry is nevertheless a valuable tool. It can establish whether the phenolic polymer is associated with the host cell wall, can assist in ascertaining the location of lignin in relation to the pathogen, and can provide preliminary data for further chemical analysis. Ultraviolet fluorescence microscopy has also been used to localize lignins in host tissue (100). However, precaution is also needed with this method since other materials associated with cell walls may fluoresce (e.g. wall-bound ferulic acid).

If a lignin is suspected by histochemistry, the identity should be confirmed by one or more chemical tests. For the analyses, cell walls should be prepared that are free of potentially contaminating substances such as soluble phenols and wall-bound esters of phenolic acids (100). If possible, preparations should not be contaminated with xylem and schlerenchyma tissues. The extracted and deesterified cell-wall materials can then be analyzed by alkaline oxidation with nitrobenzene or cupric oxide or by acidolysis in dioxane-HCl (80, 100, 140). These methods provide recognizable aldehydes (the oxidation procedures) or ketones (acidolysis) that can be analyzed by HPLC or GC/MS to confirm the presence of the expected lignin-degradation products. Further details on these and other chemical methods, and their advantages and disadvantages are given in recent reviews (80, 100).

Quantification of pathogen-induced lignins also presents problems not associated with the soluble phenols. Several methods (100, 140), e.g. the thioglycolic acid procedure (100), have been used to quantify lignins in infected tissues. Although Freudenberg indicated that the formation of a thioglycolate derivative is proof for the presence of lignin (38), additional chemical analyses should be performed for lignin confirmation. Recently, Barber & Ride (5) demonstrated a direct spectrophotometric method for lignin determination in leaves. In this method, the tissue is treated with p-nitrobenzenediazonium tetrafluoroborate. Since this reagent will couple with any phenolic hydroxyl, these authors indicated the need to extract the tissue with a solvent (e.g. 70% ethanol) to remove soluble phenols and then to saponify wall-esterified phenolic acids by alkaline hydrolysis. The stained tissue is then scanned with a densitometer and the results corroborated with the thioglycolic acid method. The advantage of the procedure is that the location of the lignin in relation to pathogen growth or elicitor treatment and quantitative analysis can be performed on the same tissue. Its usefulness was recently demonstrated by Reimers & Leach (117) in a study of the interaction of rice and *Xanthomonas oryzae*.

Despite problems associated with lignin analyses, the role of pathogen-
induced lignins and related polymers has been closely correlated with the defence responses of several plants (140). Lignification also has the potential for acting in several ways in the defense strategy. As Ride pointed out (120), lignin or the lignification process can act in plant defense against infection by establishing mechanical barriers to pathogen growth, chemically modifying cell walls to be more resistant to cell-wall degrading enzymes, increasing the resistance of walls to the diffusion of toxins from the pathogen to the host and of nutrients from the host to the pathogen, production of toxic precursors and free radicals, and the actual lignification and entrapment of the pathogen. Each of these possible events provides a testable hypothesis, and some of these mechanisms have been demonstrated.

The proposal by Hijwegen (67), that lignification could be a general resistance response, seemed a likely hypothesis since terrestrial vascular plants have the ability to synthesize this polymer (80). In his studies, Hijwegen showed that cucumber plants resistant to *Cladosporium cucumerinum* deposited lignin more rapidly than susceptible plants. Hammerschmidt et al (61) later confirmed these observations and demonstrated the relationship between lignification and resistance in additional cucumber cultivars. Studies by Robertson (123, 124) expanded on this by demonstrating induction of lignin in cucumber either in response to infection by *Cladosporium* or treatment with elicitor. The role of lignification as a general defense response in cucurbits was also shown by work demonstrating that induced systemic resistance of cucumber was associated with enhanced deposition of lignin at the site of attempted penetration (24, 60). Hammerschmidt & Kuć (60) further verified the deposition of lignin on fungal hyphae in vitro, providing support for one of Ride’s hypotheses (120). Their work indicating that nonhost resistance in cucurbits is associated with lignification and that the lignin deposited is primarily p-coumaryl in nature (59) corroborated the hypothesis that to deposit lignin as a component of resistance expression is a general response of cucurbits.

In early work on the role of lignification, Friend and associates found that potato tuber tissue infected with incompatible races of *P. infestans* deposited a phenolic polymer (41). Although the chemistry of the polymer has not been elucidated, a recent report by Ampomah & Friend (3) suggests that it may be composed of polymerized chlorogenic acid, a fact that would explain why concentrations of chlorogenic acid in tubers drop after infection (39). Nevertheless, the ability to prepare a ligninthioglycolate derivative from arachidonic acid-elicited tissue suggests that lignin is a normal component of the tuber defense response (10). Note that lignin, as determined by thioglycolic acid and cupric oxide oxidation analyses, is also deposited in tuber tissue in response to pathogens that do not induce a hypersensitive resistance response (58).
Some of the most extensive work has dealt with lignification in graminaceous species. Vance & Sherwood (129, 140, 141) as well as Ride and colleagues (5, 113, 119, 120, 122) demonstrated that nonhost resistance to fungal infection in reed canary grass and wheat, respectively, was associated with lignin deposition. It was further shown that lignified tissues were more resistant to degradation by cell-wall degrading enzymes (119), substantiating the hypothesis initially put forth for the function of lignification (120). Various cell-wall polysaccharides derived from fungi also induce lignification in wheat (113), an observation that expands the role of elicitors of phytoalexins to include the induction of other plant defense processes.

The role of lignification as part of the hypersensitive response of wheat to *Puccinia graminis* has been presented by Moersbacher and coworkers (95–98). In these studies, the interaction of incompatible races of *Puccinia* resulted in the deposition of lignin and the induction of enzymes associated with the lignification process (PAL, 4-coumarate CoA ligase, cinnamyl alcohol dehydrogenase, and peroxidase) (98). Although initial studies indicated the presence of a race-specific elicitor of lignification (96), subsequent work (98) has negated this suggestion. Of particular significance is the recent report that the inhibition of lignification by inhibitors of PAL and the lignin-specific enzyme cinnamyl alcohol dehydrogenase also resulted in the inhibition of the hypersensitive response (97). These studies suggest that the accumulation of lignin precursors is causally related to the development of resistance to *Puccinia*. The failure of host cells to undergo hypersensitive cell death may reflect the absence of either lignin precursors or peroxidase-generated free radicals that, under normal conditions, are toxic to the plant cell itself.

Lignin precursors, or their free radicals, were also proposed to potentially act in defense as toxic compounds (120). This was demonstrated by Keen & Littlefield (73) who identified coniferyl aldehyde and coniferyl alcohol as phytoalexins in the flax-*Melampsora lini* interaction. They not only demonstrated the toxicity of these compounds, but also showed that the degree of resistance was directly correlated with the amount of coniferyl aldehyde produced. Phloroglucinol-HCl staining of the flax tissue indicated that some lignin was also being deposited. The deposition of lignin in the flax rust interaction, based on histochemistry, was confirmed by Coffey & Cassidy (16).

Although most research on the deposition of lignin as a defense response deals with fungal infection, evidence indicates that resistance to bacterial infections may also be related to enhanced lignin deposition. Reimers & Leach (117) have recently shown that the race-specific resistance of rice carrying the Xa-10 gene to *X. oryzae* is correlated with the deposition of lignin at the site of infection. Lignin was identified with a variety of histochemical stains at infection sites of both compatible and incompatible in-
teractions. However, the deposition of lignin in the incompatible interaction occurred within 18 to 24 hr after inoculation, whereas no increased accumulation of lignin was seen in the compatible interaction until 96 hr after inoculation. Monitoring the populations of the bacteria in host tissue further demonstrated a correlation between the initiation of the lignification process and lignin deposition with the cessation of bacterial multiplication in the incompatible interaction. These results indicate that lignin and/or toxic lignin precursors play a role in the active defense of rice to *Xanthomonas*.

**PHYTOALEXINS: DO THEY ACCUMULATE AT THE RIGHT TIME AND PLACE?**

The conceptualization of phytoalexins (101) and the subsequent identification of the pterocarpan pisatin (20, 114) from pea established a different basis for attempting to decipher the elements of plant resistance. In spite of substantial evidence for the role of phytoalexins in resistance, it is often difficult to conclusively demonstrate their direct involvement in the in vivo containment of a pathogen (83). Clearly, not all plants produce phytoalexins, and dicotyledonous species represent the majority of plants from which such compounds have been identified. Their synthesis does not appear to be a characteristic of monocotyledonous species as, to date, they have only been shown to occur in oat (92, 93), rice (15, 75), sugarcane (12), and sorghum (68, 105). The compounds from oat, sugarcane, and sorghum are phenols. Furthermore, although many phytoalexins are phenols, these compounds are clearly chemically diverse and grouped together as a “family” based on their antimicrobial properties. Phytoalexin synthesis is but one of a vast array of phenomena that, taken together, contribute to the expression of resistance. Recent reviews of phytoalexins and the regulation of their synthesis are available (28, 31, 32, 55, 57, 142). In spite of the demonstration that many plants produce low molecular weight antibiotic compounds as a result of de novo synthesis in response to infection (4), substantial questions remain concerning the validity of the phytoalexin concept and its application to our understanding of disease resistance mechanisms.

In this section we address issues that are important to understanding the role of phytoalexins and their efficacy in disease interactions. One question is whether phytoalexin synthesis actually represents a determinative event in expression of resistance (25). Do phytoalexins accumulate before cell death or as a consequence of cell death? Are accumulations of phytoalexins within the infection site sufficient to be effective antimicrobial agents?

Phytoalexins are synthesized in living cells (56, 71, 85, 102) affected by the ingress of pathogens (28, 32), and as disease progresses these compounds accumulate in dead or dying cells (28, 63, 69, 102). For most interactions the
compounds are thought to be found in tissues that surround the original infection site but not necessarily in the cells that are the first to come into contact with the pathogen (28, 32, 57, 71, 85, 91, 127). Because most phytoalexins are not visible, it is difficult to assess their cellular localization. Mayama and colleagues (92) demonstrated that oat produces nitrogen-containing phenolic phytoalexins, the avenalumins, and that these compounds accumulated only in incompatible host-pathogen interactions. To address where the compounds appear at the cellular level, Mayama & Tani (91) took advantage of the UV-absorbance and autofluorescence spectra of the avenalumins and used microspectrophotometry to reveal the presence of intense fluorescence only in cells immediately associated with the infection site. In similar studies with the broad bean-Botrytis cinerea interaction, the furanoacetylene phytoalexins wyerone and wyerone acid accumulated in necrotic cells at the infection site as well as within vacuoles of adjacent healthy cells (85).

Recent work with sorghum has also addressed the issue of phytoalexin localization and whether it occurs in cells undergoing a hypersensitive response. Sorghum phytoalexins identified to date are unusual flavonoids of the 3-deoxyanthocyanidin class and include luteolinidin, apigeninidin, and a caffeic acid ester of arabinosyl-5-O-apigeninidin (68, 105). Fungitoxicity assays with the anthracnose pathogen G. graminicola showed that each compound was highly inhibitory at concentrations less than 9 μM (68). Because these phytoalexins are pigmented their accumulation could be localized to within 1 to 3 cells in living sorghum leaves, an area encompassing about 2300 μm² (104). It was then found that the compounds accumulate in a site-specific manner within individual cells associated with the site of attempted fungal penetration (133). With G. graminicola as the pathogen, appressorium formation was complete at approximately 20 hr after inoculation. Shortly after appressorium formation, colorless vesicle-like inclusions appeared in the cytoplasm of the host cell directly underlying an appressorium. These inclusions, which were initially less than 1 μm in diameter, enlarged by coalescence (often attaining diameters of 15 to 20 μm) and moved within the cell toward the site of appressorium attachment. During this process the inclusions became intensely pigmented and appeared red, a color that corresponds to the pigmentation of the mixture of phytoalexins. The inclusions then burst, depositing their contents within the cytoplasm of the host cell itself, a phenomenon that results in the hypersensitive death of the cell. The water-soluble phytoalexins leached out of the cell and entered the overlying appressorium, killing the fungus. These events occur synchronously and require but 5 to 8 hr after the formation of a mature appressorium on the tissue.

Microspectrophotometric procedures indicate that the apparent concentra-
tion of phytoalexin, based on luteolinidin as a standard, within individual vesicle-like inclusions can attain levels as high as 0.15 M (132). In addition, the accumulations of phytoalexins reached ng concentrations in individual cells. Thus, the concentration of phytoalexins within single host cells that are undergoing attempted penetration greatly exceeds that which is necessary for the in vitro inhibition of the fungus. These observations demonstrate that phytoalexin synthesis in sorghum does occur before host cell death, that it occurs first at the site of attempted penetration, and that concentrations of the compounds far exceed those required for fungal inhibition.

Based on the observations of vesicle development and change in pigmentation, the synthesis of the phytoalexins probably occurs within the vesicles themselves as they move toward the site of appressorium attachment. If true, the enzymes and substrates necessary for synthesis are probably packaged within the vesicle-like inclusions. This mode of synthesis and accumulation within subcellular organelles may indicate the means by which the host cell avoids contact with the toxic phytoalexins that it produces.

Studies by Essenberg and colleagues have demonstrated that in the interaction of Xanthomonas campestris pv. malvacearum with resistant cotton there is an accumulation of aromatic sesquiterpenoid phytoalexins (34). Through the use of fluorescence microscopy and cell sorting, fluorescent cells within infection sites were isolated from healthy, nonfluorescent cells surrounding infection sites (35, 116). The results demonstrated, as with sorghum, that phytoalexins occurred in a concentrated fashion in cells immediately at the infection site such that greater than 90% of the compounds were highly localized. Eventually these compounds accumulated to concentrations sufficient to account for bacteriostasis. In studies somewhat analogous to the sorghum and cotton systems, Huang & Barker (70) investigated the site accumulation of glyceollin I in soybeans resistant to the cyst nematode Heterodera glycines. The spatial and temporal distribution and accumulation of the phytoalexin in root tissues was assessed using a radioimmunoassay specific for glyceollin I, a method that proved successful for the localization of the phytoalexin in roots responding to P. megasperma f.sp. glycinea (56). The results demonstrated that the phytoalexin accumulated specifically within cells adjacent to the head region of the nematode. Moreover, phytoalexin accumulation was detected within 8 hr from the time of nematode penetration into the tissue.

Why phytoalexins appear in tissues as an immediate response to pathogen ingress is of concern since the de novo synthesis of these compounds typically involves a presumably time-consuming route of biosynthesis. Recent investigations with soybean have shown that precursors of the glyceollin pterocarpan phytoalexins are present in tissues as glycosylated conjugates (49). This work, which was done with cotyledons, showed that in a resistant
cultivar conjugate hydrolysis results in the release of an excess of the isoflavone daidzein, which serves as an immediate precursor of glyceollin. Thus, if these results can be confirmed for other organs, phytoalexin synthesis in soybean may not depend solely on the induction of phenylpropanoid and flavonoid metabolism. Rather, it is likely to involve the hydrolysis of high concentrations of precursor compounds present as natural constituents of the tissue. The importance of pre-existing pools to such a mechanism has now been confirmed by studies indicating a wall glucan elicitor from P. megasperma f. sp. glycinea specifically elicits extraordinarily high accumulations of the glycosylated daidzein conjugate (48).

CONCLUDING REMARKS

Phenols have long been associated with passive and active defense responses of plants. Because of their universal presence in vascular plants and their accumulation in both compatible and incompatible interactions, the relative contribution of any group or class of phenols to expression of resistance or the ultimate restriction of pathogen development in compatible interactions remains in question. Future research should address the time, location, and concentration of phenols, as well as their relationship to other putative defense responses. With current technology, the ability to achieve these critical experiments is at hand.

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