Journal of Nematology 25(4):507–518. 1993.

Regulation of Defense-related Gene Expression during Plant–Pathogen Interactions[1]

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Abstract: Plants have evolved a broad array of defense mechanisms involved in disease resistance. These include synthesis of phytoalexin antibiotics and proteinase inhibitors, deposition of cell wall materials, and accumulation of hydrolytic enzymes such as chitinases. Resistance appears to depend on the ability of the host to recognize the pathogen rapidly and induce these defense responses in order to limit pathogen spread. Application of molecular technologies has yielded significant new information on mechanisms involved in pathogen recognition, signal transduction, and defense-related gene activation, and is leading to novel strategies for engineering enhanced disease resistance. We are using these approaches to analyze regulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), a key enzyme mediating the production of terpenoid defense compounds. This enzyme is encoded by four genes in tomato; *hmg*2 gene expression is specifically associated with responses to pathogen or defense elicitors. Transgenic plants containing DNA constructs that fuse the *hmg*2 promoter to a reporter gene have been used to analyze both tissue specificity and patterns of defense-related expression. Because this gene is rapidly induced in tissues directly surrounding the site of ingress by a variety of pathogens, it may serve as a valuable tool in engineering new disease-resistance mechanisms.

Key words: disease interaction, gene expression, phytoalexin.

The application of molecular techniques and recombinant DNA methodologies has led to significant advances in our understanding of plant–pathogen interactions and the mechanisms associated with disease resistance versus susceptibility. In addition, recent successes in genetic engineering of plants provide new strategies for directly manipulating these interactions and enhancing disease resistance (51). Our goal here is to briefly review our current understanding of host resistance mechanisms, to describe some of the molecular tools available to the plant pathologist, and where possible, to relate this information to potential experimental strategies to study and manipulate plant–nematode interactions. Where appropriate, examples will be used from our current work addressing the mechanism of defense-related gene regulation controlling the synthesis of terpenoid phytoalexins in tomato and potato.

Received for publication 18 May 1993.

[1] Symposium paper presented at the 31st Annual Meeting of the Society of Nematologists, 2–6 August 1992, Vancouver, British Columbia, Canada. This work was supported in part by NIH award R29-GM39549 to C. L. Cramer.

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Host Resistance Mechanisms

Plants have evolved complex mechanisms including both passive (preformed) and active (inducible) defense responses for protection against pathogenic agents. The tools of molecular biology have been applied primarily to analyses of active defense responses, that is, those responses directly induced by pathogens or by stresses such as wounding or predation. These inducible responses include synthesis of low-molecular-weight antibiotic defense compounds termed phytoalexins, production of hydrolytic enzymes such as chitinase and β-1,3-glucanase, rapid modification of existing cell wall material, and deposition of new cell wall material including lignins, callose, phenolics, and hydroxyproline-rich glycoproteins (10,20,26,30,45,50,98). These responses are localized to the site of infection and, in some interactions, are associated with localized cell death, i.e., a hypersensitive resistance (HR) response. Inducible plant defenses also involve systemic responses that include accumulation of proteinase inhibitors and other defense proteins in tissues distant from the site of

pathogen attack (20,29,30,75,85,94,95). As discussed later in more detail, many of these inducible responses involve activation of defense-related genes that direct the synthesis of these proteins and chemicals. Of particular significance is recent evidence that many of the same genes are similarly activated in response to fungal, bacterial, or viral infections. Thus, it is likely that at least some of these genes would also be triggered during nematode infection. This also suggests that information gained on understanding gene regulation during other disease interactions will be applicable to molecular strategies for enhancing resistance to nematodes.

Gene-for-gene interactions: A diagram summarizing our current understanding of host responses to an invading pathogen is presented in Figure 1. In a generalized incompatible interaction, signals released from the pathogen are recognized by the host cell, resulting in activation of host defense responses. The precise mechanisms of pathogen recognition and signal transduction remain elusive but are an area of major research emphasis and some recent progress (24,25,29,50,61,76). Significant efforts have focused on understanding the molecular basis of the gene-for-gene interactions and host specificity. The gene-for-gene hypothesis proposes that specific compounds produced directly or indirectly by a dominant avirulence (*avr*) gene in a specific pathogen race interact (again directly or indirectly) with the product of a resistance (R) gene of the host to trigger an incompatible interaction (43). In molecular terms, the simplest conceptualization is that avirulence gene products are signal molecules that directly bind to host cell receptors encoded by R-genes. However, re-

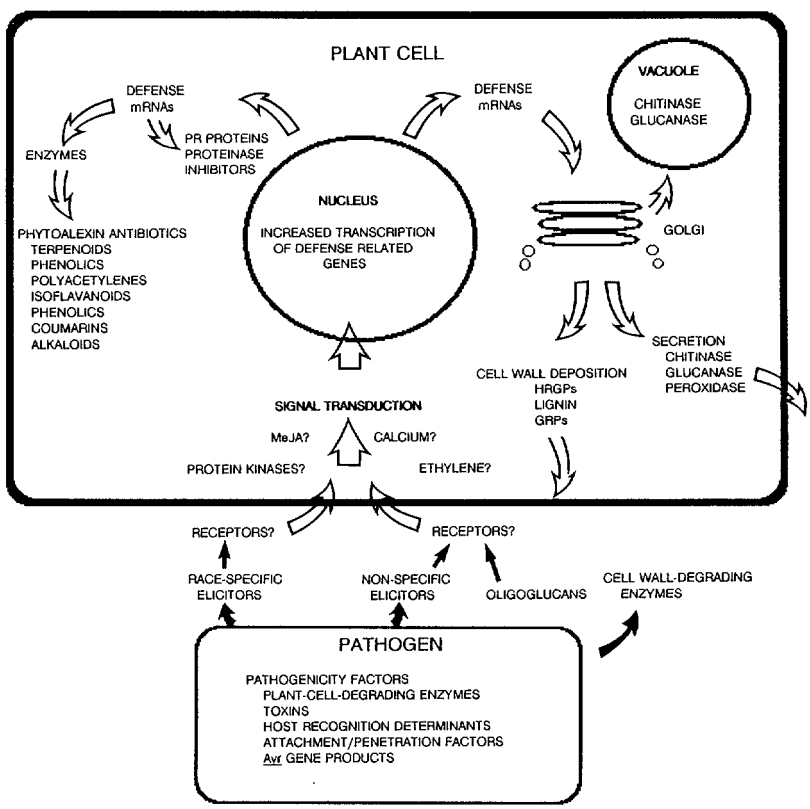

FIG. 1. Simplified diagram of molecular interactions between a plant host cell and a pathogen. This illustration draws primarily on studies of interactions involving fungal or bacterial pathogens but also contains responses known to occur in plants reacting to pathogenic viruses and nematodes. PR = pathogenesis-related; MeJA = methyl jasmonate; HRGP = hydroxyproline-rich glycoprotein.

cent evidence suggests that at least some avirulence genes encode enzymes that function in the modification of cell surface glycoproteins, glycolipids, or elicitor molecules (33,44–46). In several bacterial systems, transfer of a single *avr* gene from one bacterial strain to another can alter host specificity (45,56,57,84,88,96). The first fungal avirulence gene to be characterized, *avr*9 from *Cladosporium fulvum,* encodes a small peptide that activates host defense responses in a cultivar-specific manner associated with the presence of the *cf9* R-gene in the host (91). The cloning of *avr* genes and their use for genetic transformation of host specificity provides definitive evidence of their role as determinants in recognition by host species carrying the appropriate resistance gene. Evidence is mounting that *avr* genes from one bacterial pathogen function in widely different bacterial species on unrelated plant hosts (45,56,96). This suggests that resistance genes may be conserved among some plant species, and more importantly, that R-genes from one plant species, when transferred to distantly related species, may function to trigger new non-host resistance against specific pathogens.

Host resistance genes: Molecular cloning of host resistance genes has proceeded more slowly than that of pathogen avirulence genes because of the increased complexity of the plant genome. Additionally, because the products of the R-genes are unknown, the only method for detection involves the generation of an HR response following inoculation with an appropriate pathogen. In well-defined gene-for-gene type interactions, the test pathogen would be one expressing the specific complementary *avr* gene. Strategies being employed to localize and clone R-genes include RFLP mapping coupled with chromosome walking and transposon tagging (7,66). Prime examples of mapping strategies are those focused on cloning the tomato *Mi* gene, which confers resistance to *Meloidogyne incognita* (1), and bacterial resistance genes from *Arabidopsis thaliana* (25). Transposon tagging utilizes an active transposable element to mutagenize an R-gene; the tagged R-gene is then isolated based on known sequences of the transposon. This strategy has been attempted for isolation of the maize *Rp1* locus (7), but the complication of results by a high recombination frequency suggests that resistance loci may be complex and contain multiple linked alleles. Several new strategies are also being applied, e.g., genome subtractive cloning (87) and functional cloning strategies based on pooled DNA clones from resistant plants, which are "shotgunned" into plant tissues using particle bombardment and screened for resistance responses (45). Thus, specific R-genes probably will be cloned and characterized within the next 1–3 years.

Defense elicitors and signal transduction: Many compounds isolated from microbial preparations, fungal cell walls, infected plant material, or plant cells treated with digestive enzymes function as elicitors to trigger host defense responses. With a few exceptions (46,89,91), most elicitors do not show the race–cultivar specificity of the intact plant systems, possibly because of limitations in extraction methods. However, these elicitors have been instrumental in molecular analyses of defense-related gene activation involved in host resistance (18, 26,30,50). Perhaps best characterized of the microbial elicitors is a β-linked heptaglucan isolated from *Phytophthora megasperma* f. sp. *glycinea.* Nanomolar amounts of this glucan trigger defense gene activation and glyceollin phytoalexin accumulation in soybean hypocotyls (81). An elicitor-binding protein in soybean has been identified for this glucan, and efforts are focused on elucidating an elicitor–receptor signal transduction pathway (77,100). Release of endogenous elicitors, for example, oligogalacturonides released from plant cell wall pectins, may also be important regulators of host defenses (27, 47,50).

Molecular mechanisms have not yet been delineated for pathogen-induced signal transduction pathways, i.e., the steps occurring between the cell surface recognition event and the actual defense-related

TABLE 2. Cloned host defense-response Genes.†

Defense-response gene	Function	Source of clone	Reference‡
Phytoalexin biosynthesis			
Phenylpropanoid phytoalexin			
Phenylalanine ammonia lyase	Enzyme, central pathway	Bean, parsley, potato	(19,23,59)
4-Coumarate CoA ligase	Enzyme, central pathway	Parsley, potato	(4,32)
Chalcone synthase	Enzyme, isoflavanoid branch	Bean, soybean, parsley	(52,97)
Chalcone isomerase	Enzyme, isoflavanoid branch	Bean	(63)
Resveratrol (stilbene) synthase	Enzyme, isoflavanoid branch	Grapevine, peanut	(37)
Isoflavone reductase	Enzyme, isoflavanoid branch	Alfalfa	(71)
Terpenoid phytoalexins			
HMG-CoA reductase	Enzymes, central pathway	Tomato, tobacco, potato	(14,72,73)
Casbene synthetase	Casbene biosynthesis	Castor bean	(58)
Cell wall components			
Lignin			
Phenylalanine ammonia lyase	See above		
Cinnamyl alcohol dehydrogenase	Enzyme, lignin branch	Tobacco	(78)
Caffeic acid *o*-methyltransferase	Enzyme, lignin branch	Alfalfa, tobacco	(41)
Lignin-forming peroxidase	Lignin polymerization	Tobacco	(49)
Hydroxyproline-rich glycoproteins	Structural protein	Bean, tomato	(15,82,98)
Glycine-rich proteins	Structural protein	Bean, potato, pea, rice	(82)
Thionins	Antifungal	Barley	(8)
PR or " pathogenesis-related" proteins			
Chitinases			(39,54,62,74)
Class I chitinase, basic	Vacuolar, antifungal	Tobacco, bean, tomato	
Class I and II chitinase, acidic	Extracellular, antifungal	Bean	
Class II chitinase	Bifunctional lysozyme, chitinase	Cucumber, tobacco, barley, Virginia creeper, petunia	
β-1,3-Glucanase, acidic	Extracellular, antifungal	Bean, tobacco, potato, rice, *Arabidopsis*	(8,62,69)
β-1,3-Glucanase, basic	Vacuolar, chitinase synergist	Bean, pea, tobacco	(8,69)
PR1, PR-1a, PR-1b, PR-1c	Unknown	Tobacco, parsley	(17,64)
Pv PR1, Pv PR2	Unknown, birch pollen allergen-like	Bean (similar in parsley, pea and potato)	
Pv PR3	Unknown	Bean	(80)
PR-5, osmotin	Antifungal, thaumatin-like osmotin-like	Tobacco, maize	(16,86)
Others			
Proteinase inhibitors	Trypsin-, chymotrypsin-inhibitors	Potato, tomato	(43,75)
Superoxide dismutase	Anti-oxidant enzyme	Tobacco, maize, tomato	(9)
Lipoxygenase	Lipid peroxidation, jasmonate biosynthesis	*Arabidopsis*	(65)

† Some of this information was drawn from previous reviews (21,30).

‡ Due to space limitations, not all genes or species are referenced. Researchers interested in accessing specific genes are encouraged to utilize available databanks (GenBank, EMBL). If an institution does not subscribe to these databases, the National Center for Biotechnology Information will facilitate database access through internet [e.g., info@ncbi.nlm.nih.gov or retrieve@ncbi.nlm.nih.gov].

gene activation taking place within the nucleus (Fig. 1). Experimental evidence in some plant–pathogen or plant–elicitor responses suggests that ethylene, calcium, activated oxygen species, and protein phosphorylation may play a role in defense signalling (31,34,35,48,50,76). Recently, salicylic acid and methyl jasmonate (MeJA) have been suggested as key molecules mediating the systemic response (61,65,85,90, 94). Although progress is being made in identifying putative molecular messengers in plant defense signalling, much work remains to develop a comprehensive cause-and-effect relationship in these complex pathways mediating pathogen recognition and defense activation.

Defense-related genes of the host: Pathogen ingress or defense elicitors trigger a rapid change in plant gene expression resulting in increased transcription of defense-related genes (18,21,30). A large number of defense-related genes or cDNA sequences (generated by reverse transcription or PCR-amplification of mRNAs) have now been cloned (Table 1). Many defense-related genes encode biosynthetic enzymes involved in the production of phytoalexins, critical components in many disease interactions (3,11,83). For example, a resistant soybean cultivar, blocked for phytoalexin accumulation by specific inhibitors, showed concomitant loss of resistance against *Phytopthora megasperma* f. sp. *glycinea* (67). Some pathogens utilize either suppression of phytoalexin biosynthesis or detoxification of the host phytoalexin as a key mechanism in successful pathogenicity (93). Phytoalexins have been linked to the localized cell death characteristic of an HR response (83), as have reactive oxygen species and lipoxygenase activities (22,31). The potential to use phytoalexin biosynthetic enzymes to engineer novel disease resistance has recently been demonstrated: a stilbene synthetase gene from grapevine (*Vitis vinifera*) was introduced into tobacco, and the resulting transgenic plants produced a novel phytoalexin, resveratrol, and showed increased resistance to infection by *Botrytis cinerea* (37).

A second group of defense-related genes is involved in the fortification of the plant cell wall, presumably creating additional structural barriers to further pathogen ingress. These include genes encoding extensins or HRGPs, glycine-rich proteins, enzymes involved in lignin biosynthesis, and a novel cell wall protein, thionin, which is toxic to pathogenic fungi (8,15,41, 78,82,98). Additional pathogen-induced changes in cell wall physiology, e.g., localized deposition of callose and increased cross-linking of existing cell wall material, do not appear to function through activation of host gene expression (10,50).

A third group of defense genes encodes hydrolytic enzymes (initially identified as pathogenesis-related proteins, Table 1), some of which are effective in attacking pathogen cell walls. These enzymes, primarily chitinases and glucanases, are localized both to vacuoles and to extracellular compartments and show differential effectiveness against specific pathogens (62,79, 92).

Regulation of defense-related gene expression: Characterization of these defense-activated genes has led to an understanding of temporal and spatial expression patterns during both compatible and incompatible interactions with pathogens (13,18,23,74,97,99). In some cases, regulatory sequences within the gene promoters have been identified; these sequences direct and coordinate transcriptional activation in response to wounding, pathogens, or elicitors (25,38,52,53,59,64,101). Extensive literature has accumulated in the last 10 years on gene expression and regulatory mechanisms of the defense-related genes listed in Table 1 and is more comprehensibly reviewed elsewhere (30,45,50, 94).

Several general points concerning defense-related gene regulation and plant disease resistance can be drawn from this literature. i) The timing of the defense responses seems to be critical in determining the outcome of compatible versus incompatible interactions. Although a susceptible plant appears to have the genetic capacity to generate an effective defense

(e.g., as in response to an incompatible race), either the plant does not recognize the pathogen such that defense responses are not rapidly initiated or else the pathogen somehow suppresses the activation response (5,14,23,30,50,93). ii) Many defense genes are activated in response to a broad variety of pathogens and stress-related stimuli, i.e., viruses, fungi, bacteria, wounding, and in some cases, cold, UV, or drought stress (59,86,97). This broad response may indicate that common or overlapping regulatory circuits are utilized for distinct pathogen or abiotic stresses. However, wounding generates expression patterns distinct from those generated by pathogens or elicitor treatment (14,55,99). iii) Many pathogen-inducible genes show specific patterns of gene expression in the absence of defense-related induction (13, 24,49,59,72,98). These genes may therefore have additional roles in normal growth and development. iv) Many plant defense genes are part of small multigene families, members of which are differentially expressed during development or defense responses (4,13–15,19,55,99). v) Genetic engineering strategies manipulating several of the defense genes listed in Table 1 have generated plants with altered disease interactions to viral, bacterial, and fungal pathogens (11,37,51). These recombinant DNA-based strategies for engineering disease resistance may represent the next generation of integrated disease control.

Compared to studies on viral, bacterial, and fungal diseases, molecular analyses on nematode-induced defense compounds and host defense gene activation are limited (36). It is likely that some of these same defense genes and resulting defense compounds will be involved in determining the outcome of specific host–nematode interactions, especially those interactions characterized by typical HR responses. For example, phytoalexins and oxygen radicals are elevated in nematode-infected roots or in association with nematode-induced HR responses (12,102). The defense-related genes in Table 1 are now available as tools for the nematologist to use in dissecting the molecular basis of these complex interactions.

Molecular Analyses of a Defense-Related Gene

In order to demonstrate the application of specific molecular tools for analyses of plant defense genes and their role in disease resistance, we will review some of our recent work on the regulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR). This enzyme catalyzes the rate-limiting step in terpenoid biosynthesis and is thus important not only in disease resistance because of its role in defense compound production (sesquiterpene [e.g., rishitin, lubumin], monoterpene, and diterpene phytoalexins, steroid glycoalkaloids), but also in growth and development (e.g., cytokinins, gibberellins, abscisic acid, chlorophyll, quinones, sterols, carotenoid pigments, isoprenylated proteins). We initially cloned a tomato HMGR gene based on sequence homology with a yeast HMGR gene (72,73). Subsequently, we determined that HMGR isozymes in tomato are encoded by four distinct isogenes that are differentially expressed during development and in response to stress. One of these isogenes, *hmg*2, is the HMGR gene primarily associated with defense responses (72,73). We have monitored changes in HMGR mRNA levels (as an approximation of gene expression) by Northern blot hybridization with isogene-specific probes (72,73,99). Wounding triggers an increase in *hmg*2 mRNA levels in both tomato (leaf, roots, stem) and potato (tuber) with kinetics typical of many defense-related genes (e.g., mRNA maxima at 12–14 hours after wounding). Treatment with elicitors (arachidonic acid or fungal cell wall compounds) or inoculation with the soft-rot bacterium *Erwinia carotovora* spp. *carotovora* triggers a significantly greater induction of *hmg*2 mRNAs than wounding (72,73,99). In contrast, tomato *hmg*1 expression is not induced by defense elicitors but is elevated in tissues undergoing cell

division and thus may be associated with sterol biosynthesis (Cottingham and Cramer, unpubl. data). In potato tubers, *hmg*2 and *hmg*3 are similarly activated by wounding and elicitor treatments; *hmg*1 is wound inducible but, unlike *hmg*2 and *hmg*3, is suppressed by elicitor (14). Thus, HMGR genes appear to show complex defense-related regulation in both tomato and potato. Because *hmg*2 has an expression pattern consistent with an important role in defense, we have utilized it for further analyses on mechanisms of pathogen-induced gene activation.

Expression of hgm2: *reporter gene fusions in transgenic plants:* A powerful tool for precisely delineating patterns of tissue specificity and pathogen induction, and for identifying specific regulatory elements with a gene promoter, involves the fusion of the promoter or regulatory region of a gene of interest to a reporter gene and expressing this construct in transgenic plants (40,42,74,95). In order to further analyze the regulation of the *hmg*2 gene directly in plant–pathogen interactions, we fused about 2.3 kb of the upstream promoter-regulatory region of the tomato *hmg*2 gene to the coding region of the GUS reporter gene (42) encoding β-glucuronidase. This reporter gene is very effective in tobacco and tomato because these plants contain very little endogenous activity and expression of the introduced gene is easily monitored both histochemically and biochemically using a sensitive fluorometric assay (42). The *hmg*2:GUS gene construct was transferred into tobacco and tomato plants using standard *Agrobacterium*-mediated leaf-disk transformation (40). Transgenic tobacco plants expressing the *hmg*2:GUS fusions were used to monitor gene expression directly in plant tissues. Unstressed tobacco leaf or stem tissues show little or no GUS activity, an indication that the *hmg*2 promoter is transcriptionally inactive. Wounding, however, resulted in a rapid and dramatic increase in GUS activity, visualized as intense blue pigmentation (a product of the GUS reaction following incubation with the X-glucuronide substrate [42]) localized to the wound site (Weissenborn, Yu and Cramer, unpubl. data).

In preliminary results, we have monitored *hmg*2:GUS expression in excised leaves inoculated with *Erwinia carotovora* spp. *carotovora* and intact hypocotyls of seedlings inoculated with the fungal pathogen *Rhizoctonia solani.* In both interactions, GUS activity was highly expressed in the host cells directly surrounding the site of inoculation (24 hours after inoculation) and resulting lesion (48 and 72 hours) (Weissenborn and Cramer, unpubl. data). Further analyses monitoring the timing of expression and comparing compatible and incompatible interactions should provide insight into the kinetics of gene activation and signal transduction to adjacent cells.

We have generated a series of promoter deletions (ranging from 58 to 2,300 base pairs from the transcription initiation site) from the 5′-upstream region of the tomato *hmg*2 gene. These truncated promoters have been fused to GUS and transformed into tobacco. Analysis of the pathogen induction patterns in these transgenic plants will aid in delineating the regions within the *hmg*2 promoter responsible for the pathogen-specific responses. Analogous approaches with other defense-related genes have identified within these promoters critical regions that bind specific proteins and thereby mediate the rate of gene transcription and tissue specificity (38,52, 90,101). This information has recently led to the isolation of specific DNA-binding regulatory proteins and may lead to alternative strategies for genetically manipulating an entire battery of defense genes and thus enhancing resistance.

Nematode-induced expression of hmg2 *gene activity:* Terpenoid phytoalexins are toxic to nematodes, and increases in at least some of the biological activities mentioned in Table 1 have been noted in various plant–nematode interactions (12,60,102). Because of HMGR's role in mediating sesquiterpene phytoalexin production and *hmg*2's activation by both fungi and bacteria, we were interested in determining if

*hmg*2 expression was activated by nematode infection. To date, we have only very preliminary results obtained with transgenic tomato seedings (germinated on agar) containing the *hmg*2:GUS gene and inoculated with second-stage juveniles of *Meloidogyne incognita* and *M. hapla*. No GUS activity was seen in root tips of uninoculated seedlings or in infected roots within the first 48 hours after inoculation. However, once feeding and galling was initiated, high levels of GUS activity were observed localized to the galling tissue (Weissenborn, Eisenback, Radin and Cramer, unpubl. data). This result suggests that *hmg*2 may be a nematode-response gene. Obviously, these experiments require confirmation with appropriate controls and defined susceptible and resistant cultivars.

Genetic engineering strategies for disease resistance: The ability to genetically engineer new or altered genes into plants is a valuable tool not only for assessing changes in gene expression but also potentially for manipulating the plant–pathogen interaction directly (28,51). As described above, the difference between disease susceptibility and resistance often appears to be determined by how quickly the pathogen is detected and a defense response is activated. Thus, one can speculate that, short of isolation of specific recognition factors (e.g., R-genes), mechanisms accelerating the rate or magnitude of the response or constitutively expressing specific defense genes may result in enhanced disease resistance.

There are examples of enhanced disease resistance in transgenic plants based on altered expression of a specific defense-related transgene. Constitutive expression driven by the cauliflower mosaic virus 35S promoter (6) of a bean (or tobacco) chitinase in transgenic tobacco yielded plants with enhanced resistance to *Rhizoctonia solani* (11). Chitinase overexpression, however, did not yield resistance to *Cercospora nicotianae* in *Nicotiana sylvestris* (68) and would not be expected to function against pathogens (e.g., *Pythium* or *Phytopthora*) without a chitinous cell wall. Perhaps chitinase overexpression would affect nematode propagation by attacking the chitinaceous egg shell. Enhanced resistance against a variety of bacterial pathogens was demonstrated in transgenic tobacco plants expressing lysozymes or insect-derived bacterial toxins (2,28,70). Overexpression of proteinase inhibitors has successfully enhanced host resistance to specific insects (43). In addition, introduction of a grapevine stilbene synthase gene into tobacco resulted in the production of the phytoalexin resveratrol in the transgenic plants (37), demonstrating the potential of circumventing specific pathogenicity mechanisms by expressing new phytoalexins.

The interaction of host plants with nematode pathogens, especially endoparasitic nematodes, is a highly complex interaction that, in many ways, is distinct from interactions with pathogenic viruses, bacteria, or fungi. The application of molecular techniques to studies of these plant–microbial pathogen interactions has clearly led to enhanced understanding of the molecular basis of disease resistance and to novel strategies for disease control. Hopefully, an increased research focus on the molecular basis of plant–nematode interactions will generate new genetic engineering-based strategies for nematode control. Identification of plant gene regulatory sequences expressed constitutively in root target tissues or induced during susceptible interactions could be used to direct localized accumulation of specific nematicidal or nematostatic compounds. Enhanced plant-based nematode resistance would positively impact crop productivity and hopefully result in long-term benefits to the environment derived from reduced utilization of chemical pesticides.

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