In Defense against Pathogens. Both Plant Sentinels and Foot Soldiers Need to Know the Enemy[1[w]]

Paola Veronese, Maria T. Ruiz, Maria A. Coca, Agustin Hernandez-Lopez, Hyeseung Lee, José I. Ibeas, Barbara Damsz, José M. Pardo, Paul M. Hasegawa, Ray A. Bressan*, and Meena L. Narasimhan

Center for Plant Environmental Stress Physiology, 1165 Horticulture Building, Purdue University, West Lafayette, Indiana 47907–1165

Plants are major targets of microbes seeking a source of nutrition. A complex array of interactions between plants and microbes has evolved that reflects both the nutrient acquisition strategies of microbes and defense strategies of plants. Part of plant defense strategy includes an active offense against invading microbes using an array of antimicrobial gene products. Within the context of the overall plant-microbe interaction, we attempt here to emphasize the role of antimicrobial proteins (typically, over 100 amino acid residues) and peptides (typically, 30–60 amino acid residues) in plant defense.

The majority of plant-microbe encounters do not result in disease. Preformed factors including constitutively expressed waxes, cell wall components, antimicrobial peptides, proteins, and non-proteinaceous secondary metabolites that deter invasion have been proposed to contribute significantly to the host range of pathogens (Garcia-Olmedo et al., 1998; Morrisey and Osbourn, 1999; Heath, 2000). The importance of preformed defenses has been inferred from the observation that plants can be rendered susceptible by a deficiency in the production of these secondary metabolites or by the abilities of pathogens to degrade them (Morrisey and Osbourn, 1999; Papadopoulou et al., 1999).

INDUCIBLE DEFENSES REQUIRE DETECTION OF PATHOGENS BY HOST SURVEILLANCE. THE SENTINELS

Plant defense responses are induced by microbial products in non-host (exhibited by an entire plant species to a specific pathogen) and host (exhibited by a particular genotype within a susceptible plant species to a specific pathogen) resistance (Heath, 2000; Kamoun, 2001). Plant defense systems are also induced by microbial products in compatible (resulting in disease) and incompatible (failure to result in disease) plant-microbe interactions.

Specific host-pathogen interaction models describing induced defense responses in plants have been greatly influenced in recent years by the gene-for-gene interactions originally reported by Flor (1956). In these specific host-pathogen interactions, resistance to a particular pathogen is conditional on the presence of a specific *Avr* (avirulence) gene of the pathogen and a specific *R* (resistance) gene (usually a single dominant gene) in the plant host. Widespread interest in gene-for-gene interactions resulted from recognizing that resistance was usually controlled by single dominant genes, making genetic analysis very tractable. The first *Avr* gene was identified from the pathogen *Pseudomonas syringae* pv *glycinae* (Staskawicz et al., 1984). The first *R* gene to be cloned controls resistance to *P. syringae* in tomato (*Lycopersicon esculentum*; Martin et al., 1993). From the cloning of several more *R* genes, much impressive and elegant work has shed a great deal of light on these specific gene-for-gene interactions (for review, see Dangl and Jones, 2001). We now understand that the *Avr* gene system of potential plant pathogens directly or indirectly provides a biochemical target for a plant surveillance system in which the *R* gene plays a central role. In fact, a direct interaction between some *Avr* and *R* gene products has now been demonstrated (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000).

A very important clue to the molecular function of *R* gene-products emerged from the recognition that many of them have sequences resembling, in part, those encoded by *Drosophila melanogaster* and human (*Homo sapiens*) genes that control the innate immune response of insects and animals (Staskawicz et al., 2001). Some of the *R* gene receptors resemble the classic Toll and Toll-like receptors of *D. melanogaster* and vertebrates, respectively. Vertebrate Toll-like receptors directly or indirectly recognize pathogen-associated molecular patterns on microbial cell surface ligands (Hoffman et al., 1999). Individual receptors can recognize specific molecular patterns (Modlin, 2000; Alexopoulou et al., 2001; Garred, 2001; Kirschning and Bauer, 2001) and activate distinct downstream signaling systems (Khush and Lemaitre, 2000; Modlin, 2000; Schnare et al., 2001). It is still uncertain whether plant surveillance systems utilize

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* Corresponding author; email bressan@hort.purdue.edu; fax 765–494–0391.

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molecular pattern recognition to identify pathogen challenges. However, as with Toll-like receptors of animals, plant receptors or other signal components that participate in the recognition of potential pathogen invasion are known to exhibit specificity for the type of pathogen (Ellis and Jones, 1998; Ellis et al., 2000) and can mediate responses through separate downstream components (McDowell and Dangl, 2000). An interesting explanation of plant *R* gene recognition invokes a "guard" role for *R* gene products (van der Biezen and Jones, 1998).

This hypothesis envisages that *R* gene products "guard" the targets of microbial virulence factors, detect interaction of the microbial virulence factor with its host intracellular target, and subsequently induce defense responses. This hypothesis is supported by the observation that many avirulence gene products constitute a subset of pathogen virulence factors involved in the mediation of disease (White et al., 2000). An intriguing aspect of this hypothesis is its possible explanation of a connection between a more general molecular pattern-type recognition system and the highly pathogen-specific *R* gene system (Dangl and Jones, 2001). It also provides the concept that host protein complexes are responsible for pathogen recognition, leading to the corollary that diversity and specificity of recognition could arise by combinatorial interactions. It also reconciles difficult-to-explain observations that specific *Avr* proteins can be associated with a seemingly inappropriate R protein (Leister and Katagiri, 2000), that a single R protein can recognize two different effectors (Grant et al., 2000), that R proteins can functionally interfere with one another (Ritter and Dangl, 1996), and that direct interaction between R/Avr proteins is not always demonstrable (de Wit et al., 1997; Nimchuk et al., 2001). Genetic analyses have revealed that specificity of *R* gene signaling could also arise from the activation of unique downstream signaling components (proteins) such as *NDR1* and *EDS1* that control separate *R* gene clusters (Dangl and Jones, 2001; Glazebrook, 2001). The reader is referred to several excellent reviews for further discussions on *R* gene diversity/polymorphism, recognition specificities, and mode of action (Ellis et al., 2000; Dangl and Jones, 2001).

Although the *Avr/R* gene interactions control plant disease resistance to very narrow groups of pathogens (specifically, races that contain the appropriate *Avr* locus), broader pathogen-derived elicitors of host defense, or nonspecific elicitors, have been described (Felix et al., 1999; Heath, 2000). These are oligosaccharides (derived from the pathogen or plant), microbial proteins such as flagellin, or nucleic acids (Doares et al., 1995; Ebel, 1998; Felix et al., 1999; van der Luit et al., 2000; Szittya and Burgyan, 2001). Their interactions with plants may more closely resemble molecular pattern recognition, as it is understood in animals. Some nonspecific elicitors, such as cellulolytic enzymes, can cause transmembrane ion fluxes in artificial lipid bilayers (Klüsener and Weiler, 1999).

Other nonspecific proteinaceous elicitors, such as cryptogenin, have been shown to have binding sites on plant membranes, even on membranes of plant species in which they fail to induce a defense response (Bourque et al., 1999). Therefore, it has not been established clearly that interaction with receptors is always a prerequisite for elicitation of a defense response.

SIMILAR DEFENSE RESPONSES ARE INDUCED BY NONSPECIFIC AND RACE-SPECIFIC ELICITORS

Both race-specific (*R* type) and more general (basal) elicitor-mediated defense responses are basically similar in that downstream signal events overlap and the same types of effector molecules are marshaled (Dangl and Jones, 2001). The induced events are ion influx, alkalinization of extracellular spaces, accumulation of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), and transcriptional reprogramming. Because these responses lead to increased production of many antimicrobial substances, they are thought to participate in the protection of the host.

ROIs and RNIs are highly toxic and may directly offer protection against the pathogen, but they are the most non-discriminating defense molecules produced by offended hosts. In animals, they are produced and accumulate in specific self-sacrificing cells only (Nathan and Shiloh, 2000). Perhaps the role of ROI in disease resistance and hypersensitive responses of plants is similar, i.e. cell-autonomous accumulation of ROI in self-sacrificing cells offering protection against pathogens while protecting the host from collateral damage. Despite the overwhelming nonspecific nature of ROIs and RNIs, their efficacy against target organisms can still be rendered specific either because pathogens repress host enzymes catalyzing their synthesis, induce enzymes catalyzing their detoxification, or repair the damage inflicted (Nathan and Shiloh, 2000). ROIs and RNIs also participate in transcriptional reprogramming in and around the affected cell. This transcriptional reprogramming results in other "defense responses," including: (a) synthesis of the signaling intermediates salicylic acid (SA), ethylene (ET), and jasmonic acid (JA); (b) programmed cell death in the form of a hypersensitive response; (c) synthesis of antimicrobial chemicals (e.g. phytoalexins); (d) altered cell walls; and (e) activation of downstream defense genes that encode potent antimicrobial proteins (Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001). Although they do not represent all Arabidopsis expressed sequence tags, recent microarray analyses have shown that there is some sort of spec-

ificity in the transcriptome depending on the signaling intermediate (SA, ET, or JA), the nature of the pathogen, and the type of resistance response (systemic acquired resistance or not). There is also considerable overlap between the transcriptome in response to SA, ET, or JA biotic and abiotic stresses (Maleck et al., 2000; Schenk et al., 2000; Reymond, 2001). Genetic analyses have also confirmed that there is considerable overlap between downstream components involved in the defense response, as exemplified by *NDR1* and *EDS1*, which are required for the function of more than one *R* gene (Dangl and Jones, 2001; Glazebrook, 2001).

Because the function of the pathogen-induced defense gene products (effectors) is to neutralize the invasive nature of the pathogen either by outright killing, inhibiting growth, or somehow blocking its successful colonization, many defense genes encode intrinsically toxic proteins. Here, we specifically review and evaluate the evidence for the contribution of antimicrobial proteins and peptides to plant defense. The contribution of other plant defenses to disease resistance falls outside the scope of this review, and in no way does this imply that those defenses are unimportant. Disease resistance results from the concerted action of the various components that have to be studied and understood in parts to gain a better comprehension of the whole.

ANTIMICROBIAL PROTEINS AND PEPTIDES ARE IMPORTANT COMPONENTS OF INNATE IMMUNITY. THE FOOT SOLDIERS

A common feature of the innate immune system of taxonomically diverse organisms such as mammals, insects, and plants is the ability to marshal the accumulation of antimicrobial proteins and peptides in response to an invasive challenge by foreign organisms (Hoffmann et al., 1999; Maleck et al., 2000; Schenk et al., 2000; Reymond, 2001). More than 500 different antimicrobial proteins and peptides encoded within the genomes of many organisms, including plants, have been described (Andreu and Rivas, 1998; Garcia-Olmedo et al., 1998; Kitajima et al., 1999).

The relevance of antimicrobial proteins to immunity in animals is underscored by the etiology of human cystic fibrosis, a genetic defect carried by one in 3,000 individuals. Impaired ion transport in cystic fibrosis victims results in the inhibition of β-defensin at the surface of lung epithelial cells contributing to chronic respiratory infection caused by *Pseudomonas aeruginosa* (Smith et al., 1996: Goldman et al., 1997). It is difficult or impossible to prove the importance of antimicrobial proteins and peptides to plant defense by mutational or antisense analyses because of their redundancy in the genomes of plant species (Neuhaus et al., 1992; Beffa et al., 1993; Samac and Shah, 1994; Zhu et al., 1996). As elegantly pointed out by Nathan and Shiloh (2000), redundancy of defense components and compensatory induction of a different isoenzyme in antisense transformants upon pathogen infection (Beffa et al., 1993) argues for their utility. Therefore, evidence for the importance of antimicrobial protein and peptide components of plant immunity has been indirect but nonetheless substantial. Expression of genes encoding many antimicrobial proteins and peptides is pathogen induced and is highly correlated with induced disease resistance phenomena such as systemic acquired resistance (Ryals et al., 1996; Maleck et al., 2000). Therefore, they generally are called defense genes and are also often referred to as disease resistance "markers." Many of these genes have been shown to alter the severity of disease symptoms when overexpressed in genetically engineered plants (Logemann et al., 1992; Alexander et al., 1993; Carmona et al., 1993; Liu et al., 1994; Jach et al., 1995; Grison et al., 1996; Molina and Garcia-Olmedo, 1997; Gao et al., 2000). In addition, it has been established that the virulence of a pathogen can be altered by changing its resistance to only one particular defense peptide of the entire repertoire produced by the host (Titarenko et al., 1997; López-Solanilla et al., 1998).

Certainly, genes encoding these proteins/peptides have an important role in host-pathogen interactions. Much less certain is the specific function of each in individual pathogen-plant interactions. In view of this knowledge gap, we emphasize that full susceptibility of any given pathogen to only one host protein toxin should result in immunity. Thus, it becomes clear that resistance mechanisms of pathogens against host defense toxins must be widespread and important to disease development.

DO ANTIMICROBIAL PROTEINS AND PEPTIDES HAVE A SPECIFIC TARGET SPECTRUM OF ANTIMICROBIAL ACTIVITY?

A comprehensive catalog of plant antimicrobial proteins/peptides (classified on the basis of sequence, structure, and/or functional relatedness) and their known microbial targets can be accessed in Supplemental Data Table I (see www.plantphysiol.org). This compilation reveals that many plant antimicrobial proteins/peptides are toxic to some microbes but are ineffective against others in vitro. There are also examples of homologous proteins/peptides from a plant species differing in their toxicity to the same microbe. These data show that the target range of any individual antimicrobial proteins/peptides and comparison of the antimicrobial spectrum of homologous proteins has neither been examined exhaustively nor systematically. Yet, there is at least one member of most antimicrobial protein/peptide families that has already been shown to have specificity of antimicrobial activity (Supplemental Data Tables I and II; www.plantphysiol.org).

HOW IS THE SPECIFICITY IN THE TARGET SPECTRUM OF ANTIMICROBIAL PROTEINS AND PEPTIDES ACHIEVED?

Distinction between Self and Nonself

A hallmark of all successful defense systems is either the nonself recognition of their toxic components or the careful control of their expression at appropriate times and locations so that collateral damage is eliminated or at least minimized. Failure of animal defenses to exhibit proper self-recognition or sequestration leads to various forms of autoimmune dysfunctions and serious collateral damage (Sherman et al., 2000; Medzhitov and Janeway, 2002). Only in a few studies has the specific nonself toxicity of plant-encoded antimicrobial proteins/peptides been examined. Some have been shown to be nonphytotoxic (Broekaert et al., 1995; Garcia-Olmedo et al., 1998), and none have been reported to be toxic to the host plant. The non-discriminate spatial and temporal expression of many others, as in transgenic plants, would suggest very low if any collateral damage results from their production. In addition, toxins either possess selective toxicity or are expressed and sequestered to avoid self-injury. For example, plant ribosome-inactivating proteins (RIPs) do not normally inactivate self-ribosome, but show varying degrees of specificity to nonself ribosomes (Roberts and Selitrennikoff, 1986; Stirpe and Hughes, 1989). Plant RIPs are compartmentalized in vacuoles and intercellular spaces (Yoshinari et al., 1997), which apparently allows the ribosome-inactivating activity to be sequestered from self-ribosomes. Upon release or induction in response to pathogen infection or injury, they penetrate the cell wall of the target microorganism through gaps and natural openings to reach ribosomes.

Defense Capabilities and Susceptibility of the Target Organisms

There is evidence that selectivity against target microorganisms may be owed to various defense and susceptibility capabilities of the host and target organism, respectively. Microorganisms may have the capacity to degrade plant toxins (Osbourn, 1996) or synthesize inhibitors of toxic enzyme activities (Simmons, 1994; Ham et al., 1997) just as plants synthesize inhibitors of microbial enzymes for defense (Supplemental Table II; www.plantphysiol.org).

The target specificity of plant antimicrobial proteins/peptides appears to be determined by pathogen-specific cell surface and intracellular determinants (Table II; www.plantphysiol.org). Microbial cell surfaces harbor components that increase or decrease the efficacy of antimicrobials. Osmotin, a tobacco (*Nicotiana tabacum*) PR-5 protein, binds to phosphomannan, the cell wall polyanion of yeast (*Saccharomyces cerevisiae*). Polyanion binding is required for maximal toxicity to walled cells but not spheroplasts, suggesting that it promotes osmotin uptake across the cell wall (Ibeas et al., 2000). Phosphomannans are conjugated to several cell wall proteins and several cell wall mannoproteins could bind to immobilized osmotin in vitro (Ibeas et al., 2000). A correlation between osmotin sensitivity and an anionic cell surface was also observed in other fungi (Ibeas et al., 2000). Uptake of animal/insect antimicrobial proteins across bacterial cell walls is also facilitated by binding to cell wall polyanions, in this case, bacterial lipopolysaccharides or teichoic acids. This interaction is competed by monovalent cations (Hancock and Scott, 2000). However, the interaction in vitro between osmotin and cell wall phosphomannoproteins could not be disrupted by salt alone (Ibeas et al., 2000), suggesting that the carbohydrate moiety is also important for binding. Several other thaumatin-like proteins have been shown to bind glucans in vitro (Trudel et al., 1998), suggesting that carbohydrate binding is a common feature of PR-5 proteins that controls target specificity. Chitinases, glucanases, hevein-like/PR-4 proteins, Ac-AMPs, Pn-AMPs, RIPs, and alfa-thionins also bind to microbial cell wall polysaccharides (Boller and Metraux, 1988; Broekaert et al., 1992; Garcia-Casado et al., 1998; Koo et al., 1998; Muraki et al., 2000; Oita et al., 2000; Peumans et al., 2001; Simmons, 1994). Knottin-like proteins bind to protein, carbohydrate, and lipid (Smith et al., 1998). It has not been proven that this interaction contributes to antimicrobial activity except in the case of chitinases, where it was demonstrated that the chitin-binding domain contributes to the efficacy of antifungal action (Boller and Metraux, 1988; Garcia-Casado et al., 1998). The interaction between β-1,3-glucanses, chitinases and their substrates is multivalent (Hoj and Fincher, 1995; Asensio et al., 2000; Bishop et al., 2000). Interestingly, thaumatin-like proteins also bind only to oligomeric β-1,3-glucosides (Trudel et al., 1998). The interaction between osmotin and yeast cell wall mannans cannot be competed by di- or pentameric mannosides (M.L. Narasimhan, unpublished data). Target cell polymer interactions with antimicrobial proteins is reminiscent of the "pathogen-associated molecular pattern" recognition that has been observed with Toll-like receptors, the sentinels of innate immune response of animals and insects. This presents the intriguing possibility that specificity of plant defense antimicrobial proteins for their target microbes may utilize a form of molecular pattern recognition.

The microbial cell wall also harbors resistance determinants to PR-5 proteins such as osmotin. For example, yeast *ssd1* mutants acquire sensitivity to osmotin because they are deficient in cell wall glycoproteins of the PIR family, alkali-insoluble glucans, and other unidentified cell wall components (Yun et al., 1997; Ibeas et al., 2001). Perhaps these resistance determinants are "barriers" that prevent uptake

across the wall. Susceptibility of *Aspergillus nidulans* to osmotin is negatively correlated with cell wall chitin content (Coca et al., 2000). The activity of antimicrobial peptides is also negatively controlled by some microbial cell wall components (Titarenko et al., 1997). Perhaps the microbial cell wall composition greatly affects susceptibility to all the antimicrobial proteins and peptides, with some components functioning as facilitators (for glucanases and chitinases, this could be their substrates) and others as barriers (this has not been determined, but would explain the specificity of glucanases and chitinases for their fungal targets).

A serpentine receptor class protein on the plasma membrane of yeast is required for full sensitivity to osmotin. This protein binds to osmotin in vitro but the mechanism by which it controls osmotin susceptibility remains unknown (M.A. Coca, unpublished data). Specific binding to plasma membrane sphingolipid has been shown to be required for binding, permeabilization, and toxicity of dahlia defensin to yeast (Thevissen et al., 1996, 1997, 1999, 2000a, 2000b). Experiments suggest that there are also specific unidentified binding sites for thionins and nonspecific lipid transfer proteins on target cell surfaces (Florack and Stiekema, 1994).

Just as perception of the microbe modulates plant intracellular signaling pathways to determine susceptibility or resistance, the antimicrobial plant defense protein, osmotin, induces intracellular signaling in the target fungus to promote apoptosis and increase cell wall permeability (Yun et al., 1998; Narasimhan et al., 2001). The likelihood that antimicrobial peptides also induce intracellular signaling in the target (Thevissen et al., 1996) show that selectivity may also result from intracellular determinants of microbial susceptibility.

A long history of specific selection pressure and counter-selection pressure between defense protein genes and their microbial targets has also been inferred from an analysis of gene sequences of glucanases and chitinases (Hoj and Fincher, 1995; Bishop et al., 2000; Stahl and Bishop, 2000). Some proteinaceous inhibitors of insect α-amylases and trypsin, which could function in plant defense against insects, have domains resembling thaumatin-like proteins, lectins, thionins, defensins, knottins, 2S albumins, or lipid transfer proteins, again suggesting that these domains have "recognition" functions (Moreno and Chrispeels, 1989; Bloch and Richardson, 1991; Broekaert et al., 1995; Franco et al., 2002). All of these observations clearly point to the existence of a second tier (Fig. 1) of recognition specificity between the foot soldier defense proteins and the target microbes. In addition to the R gene sentinels, this second tier of recognition specificity may represent an important underestimated component of disease resistance that is especially effective in delimiting host range.

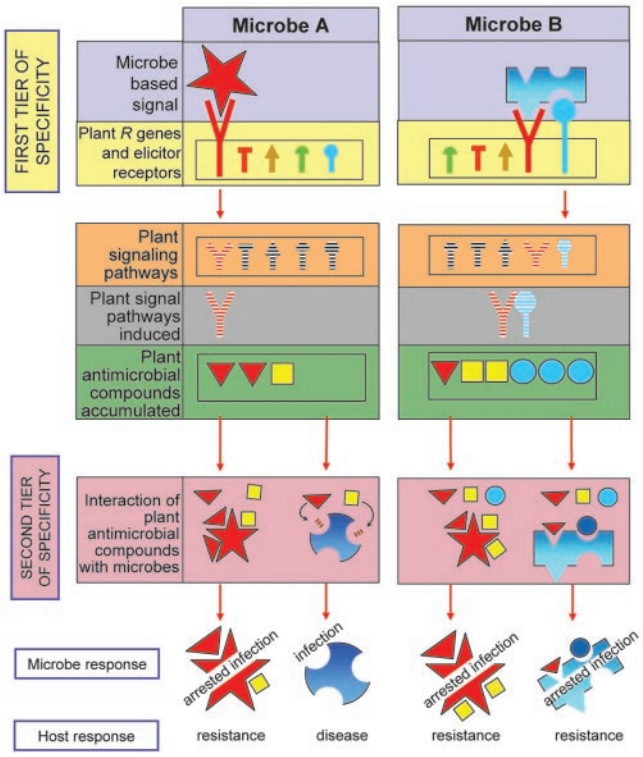

Figure 1. Model for the generation of plant disease resistance specificities. Pathogens (A or B) interact directly or indirectly with specific subsets of receptors/*R* gene products (sentinels). The combinatorial interaction of these receptors/*R* gene products with downstream signaling components results in the activation of a unique web of signaling pathways resulting in the production of a subset of the host arsenal of antimicrobials (foot soldiers). There is limited evidence that a unique assortment of antimicrobial proteins and peptides is induced by each pathogen (see Supplemental Table III; www.plantphysiol.org; Maleck et al., 2000; Schenk et al., 2000). Each of these antimicrobial proteins and peptides then has the capacity to interact with specific microbe-derived macromolecules (Supplemental Table II; www.plantphysiol.org). Additional synergistic interactions between the antimicrobial proteins and peptides (and, possibly, unknown interactions with other plant antimicrobials) further contribute to generating specificity against the microbial target. The two tiers of specific interaction of plant defense molecules with microbes are indicated by the yellow- and pink-shaded boxes.

Host Signaling Capability of Antimicrobial Proteins and Peptides

Several cationic peptides of non-plant origin possess specific host signaling capabilities. For example, some antimicrobial peptides suppress host genes that are induced by bacterial lipopolysaccharide, a virulence factor, and induce host genes involved in cell cycle regulation and apoptosis (Hancock and Scott, 2000; Zasloff, 2002). This may limit pathogen spread by killing host cells in the vicinity of the primary site of infection, resulting in host resistance. Co-option of these signals may hyperinduce host defenses around the apoptotic host cells, resulting in host resistance associated with a hypersensitive response.

Although nothing is known about the ability of plant-encoded antimicrobial peptides to affect host gene expression directly, a number of observations suggest that this may occur. A defensin-like pollen coat protein interacts with the *S*-locus glycoprotein that controls self-incompatibility in *Brassica* spp. (Doughty et al., 1998), and a lipid transfer protein has been reported to share binding sites with an elicitor of defense responses on tobacco membranes (Buhot et al., 2001), suggesting that plant-encoded antimicrobial proteins can directly affect host gene expression. PR-5 proteins have been ascribed properties other than antifungal activity on the basis of in vitro experiments, such as: (a) cryoprotection (Hon et al., 1995), (b) β-glucanase activity (Grenier et al., 1999), (c) chitinase activity (Pan et al., 1999), (d) actin binding (Takemoto et al., 1997), and (e) carbohydrate binding (Trudel et al., 1998; Ibeas et al., 2000). In fact, biological roles other than defense have been proposed for most plant antifungal proteins and peptides (Apel et al., 1990; Collinge et al., 1993; Florack and Stiekema, 1994; Simmons, 1994; Broekaert et al., 1995; Garcia-Olmedo et al., 1995), and many of them are known to interact with carbohydrates, proteins, and lipids (Boller and Metraux, 1988; Broekaert et al., 1992; Garcia-Casado et al., 1998; Koo et al., 1998; Smith et al., 1998; Ibeas et al., 2000; Muraki et al., 2000; Oita et al., 2000). Genes encoding putative receptor kinases with PR-5-like or lectin-like domains have been identified in Arabidopsis (Wang et al., 1996; Shiu and Bleecker, 2001), suggesting that domains of plant antimicrobial proteins can recognize self signal molecules. The products of glucanase, chitinase activity, and RIP are elicitors of the plant defense reactions and, thus, can indirectly potentiate antifungal/antimicrobial activity (Simmons, 1994; Doares et al., 1995; Ebel, 1998; Peumans et al., 2001). Taken together with the apoplastic location of some family members of each class of pathogenesis-related proteins (Linthorst, 1991; Kitajima and Sato, 1999), these observations suggest that defense proteins and peptides may have a role in initiating the spread of secondary waves of defense response in the host, perhaps by themselves acting as secondary sentinels.

Synergistic Interactions

Non-plant antimicrobial peptides function synergistically with one another (Hancock and Scott, 2000). Plant glucanases and chitinases exhibit synergism in their antimicrobial activity in vitro and in vivo (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995). Plant nonspecific lipid transfer proteins, 2S albumins, proteinase inhibitors, and puroindolines act synergistically with thionins (Molina et al., 1993; Terras et al., 1993; Dubreil et al., 1998). RIPs and PR-4s act synergistically with chitinase or β-1,3-glucanase (Leah et al., 1991; Hejgaard et al., 1992; Ponstein et al., 1994; Jach et al., 1995). Snakin acts synergistically with defensin (Segura et al., 1999). Osmotin antifungal activity is synergistic with chitinase (Lorito et al., 1996; L.R. Abad, unpublished data). Thus, synergism of antimicrobial activity is a feature shared by the antimicrobial end products of nonadaptive immunity in all species. Synergism probably results from the multihit mechanism of action of antimicrobial proteins and peptides. Synergistic interaction between antimicrobial proteins and peptides has the potential of amplifying their effectiveness, achieving a wide range of target specificities by combinatorial interactions, and modifying target specificity by small changes in one or few components. These are probably the reasons for conservation of this feature of innate or nonadaptive immunity.

Why Do Plants Make Antimicrobial Proteins in Addition to a Combinatorial System of Antimicrobial Peptides?

Synthesis of large number of antimicrobial proteins in addition to antimicrobial peptides appears to be a unique feature of plant immunity. Recognition of specific molecular structures is a characteristic of proteins, as exemplified by enzymes, receptors, and antibodies. In animals, the adaptive immune system interacts with the innate immune system, and protein components of the adaptive immune system (antibodies) provide extensive target recognition capability to the entire immune system (Schnare et al., 2001; Zasloff, 2002). Also, because they have a circulation system for cells, animals can utilize clonal expansion of cells to provide an efficient gene-based nonself detection and elimination system. For plants to have survived without this feature, either their cell-autonomous innate immune systems must have some features that compensate for the lack of an adaptive immune response or the unlikely alternative that plants just do not require the capabilities of the animal immune system must follow. Therefore, it is quite likely that the ability of many plant antimicrobial proteins and peptides to interact with carbohydrates, proteins, and lipids provides some capacity for target recognition that compensates for the lack of an adaptive immune system.

To provide an explanation for the efficiency of nonself detection by the sentinel *R* genes of plants, Fluhr (2001) has used a probability model developed by Lancet et al. (1993). The calculations show that a repertoire of 300 to 1,000 small receptors with low affinity (10^{-5} M range) for their ligand, aided by further integration of information by combinatorial interactions with other receptors, would suffice to serve the olfactory detection needs of an animal. Based on this model, Fluhr (2001) has predicted that the similar number of *R* genes found in a plant genome is sufficient to detect pathogens by combinatorial interactions.

A similar calculation can probably be applied to explain discrimination by antimicrobial proteins/

peptides, the foot soldiers of plant defense. (a) The numbers of these foot soldiers appear to be similar to the number of sentinels, because it has been documented that there are a large number of antimicrobial protein/peptide genes in the genome of every plant. Although the exact number is unknown even for one plant species, in Arabidopsis, researchers have compiled at least 15 members in the lipid transfer protein gene family, several members in each of the two defensin gene families, and 60 members in the β-glucanase family (Epple et al., 1997; Arondell et al., 2000; Stahl and Bishop, 2000), and several members in the PR-1, chitinase, PR-4/hevein-like, and PR-5 gene families (Maleck et al., 2000; Schenk et al., 2000). (b) Antimicrobial proteins and peptides are involved in combinatorial interactions with one another, because there is synergism in their antifungal activity. (c) As documented below, there is some specificity in the induction of particular isoforms by a given pathogen or signaling intermediate, indicating "integration" of information equivalent to that observed for the R-genes. (d) Many plant antifungal proteins/peptides are effective at about 10^{-5} M in in vitro assays, and this has often raised questions about their relevance to plant defense. (e) Although not considered here, non-proteinaceous antimicrobials such as phytoalexins are also synthesized for plant defense, and their potential contribution to interactions with the antimicrobial proteins/peptides toward plant defense would increase the number of possible combinatorial interactions. In view of the calculations of Fluhr (2001), these observations suggest that this strategy (i.e. using a limited number of antimicrobial compounds of moderate, but specific, toxicity that interact with one another) is employed to meet the specific pathogen extermination needs of a plant by helping to provide a sufficient range of target specificities. Combinatorial interactions at both ends of the defense system (the receptor sentinels and antimicrobial foot soldiers) would greatly increase the effectiveness/range of plant defense and could be the mechanism whereby plants compensate for the lack of an adaptive immune system (Fig. 1).

IS THERE SPECIFICITY IN THE COMPLEMENT OF ANTIMICROBIAL PROTEINS AND PEPTIDES THAT ARE INDUCED BY INDIVIDUAL PATHOGENS OR SIGNALING INTERMEDIATES?

A common strategy proposed to achieve broad-range host resistance is to modify the narrow pathogen specificity of *R* gene-mediated resistance. Therefore, delineation of R protein domains that control recognition of specific pathogens and subsequent activation of the downstream defense response has been the subject of intense research. The question of whether or not the triggering of the host defense system always unleashes the full repertoire of defense responses also has important implications for this strategy. Many studies have assumed that once the presence of a pathogen is recognized and multiple defense gene expression is triggered, at least some induced defense proteins will be active against the inducing pathogen. This view is supported by the finding that compatible (unrecognized) and incompatible (recognized) plant-pathogen interactions result in similar patterns of defense gene induction but differ in the rate of induction (Zhou et al., 1997; Maleck et al., 2000), implying that the ability to recognize a pathogen quickly is more important than the particular genes that are subsequently activated. Some caution is due here because no studies have examined the relative activity against the inducing pathogen of all of the induced gene products.

The most extensive antimicrobial gene expression data is available for Arabidopsis, and a comprehensive compilation of northern-blot data may be accessed from Supplemental Data Table III (www.plantphysiol.org). Different researchers have often used the same antimicrobial gene probes, therefore allowing comparison between various sets of data. It is evident from Supplemental Data Table III that the expression pattern of the complete set of host-encoded antimicrobial proteins has not been monitored after exposure to even one pathogen species or signaling intermediate by northern blotting. Yet, there is some evidence that individual microbes do induce different antimicrobial proteins. For example, *Alternaria brassicicola* induced PR-1 (the probe is known to detect two isoforms but cannot discriminate between them), a PR-4 isoform, and a defensin (the probe is known to detect four gene products but cannot discriminate between them; Penninckx et al., 1996, 1998; Thomma et al., 1998, 1999). On the other hand, turnip crinkle virus induced PR-1 and the same PR-4 isoform but not defensin (Potter et al., 1993; Kachroo et al., 2000). *Erwinia carotovora* did not induce PR-1 but induced the PR-4 isoform and defensin (Norman-Setterblad et al., 2000). The microarray data available presumably distinguishes between isoforms (Maleck et al., 2000; Schenk et al., 2000). It is clear from the microarray data that within families of antimicrobial proteins such as PR-1, PR-2, PR-3, or PR-5, the pathogen *A. brassicicola* or the signaling intermediates SA, ET, and JA can have differential effects on the induction/repression of each isoform and that this pattern is unique for each inducer. Because it is probably more energetically economical to express defense genes only when needed, it likewise should be more economical to express only the specific subset of genes needed for each microbial challenge. Also, if induction is preferred over constitutive expression to avoid toxicity to potentially helpful microbial interactions, specificity of induction would further decrease the likelihood of inadvertent toxicity.

With the caveats that the gene expression data from Arabidopsis is incomplete (for example, thionins were not represented in either microarray studies) and that in vitro antimicrobial activities have not been demonstrated for all these gene products, the overall picture of defense gene induction nevertheless suggests that only a specific subset of antimicrobial proteins are induced in a specific plant-pathogen interaction. Distinct expression profiles of antimicrobial protein/peptide genes or other defense genes in response to different pathogen infections can account for three scenarios. (a) Different sets of defense genes are induced for specific resistance to different pathogens. (b) Differential induction of plant genes (including defense genes) by distinct pathogens may actually reflect different virulence mechanisms employed by pathogens. (c) It is also possible that some of the induced genes reflect a general stress response. The incomplete information about the microbe target spectrum of different defense gene products and some indifference to the importance of this information has resulted in a serious gap in the knowledge needed to answer the question of the degree to which the array of induced defense genes is specifically tailored to protect against each particular pathogen. As more gene expression data are collected, the compilation of data relating to the target spectrum of individual antimicrobial proteins and peptides (Supplemental Table I; www.plantphysiol.org) should help to examine possible correlations between expression and efficacy of defense gene product arrays against specific microbes.

ARE THE CONSPICUOUS SENTINELS OVERSHADOWING AN IMPORTANT ROLE OF THE LOWLY FOOT SOLDIERS?

Given the impressive collection of antimicrobial proteins/peptides encoded within a plant genome and other metabolically derived defensive agents, we may ask the following questions.

Why are microbes still able to successfully colonize and nutritionally exploit plants? It is very tempting to ascribe this ability solely to the pathogens avoidance of recognition by the plant surveillance system (sentinels) so that the toxic arsenal of antimicrobial agents (foot soldiers) will not be unleashed and the pathogen can escape inhibition. This avoidance may result from a less than robust surveillance interaction or a defective signal transduction between surveillance and response components. However, even if a microbe triggers activation of the defense gene system but is sufficiently able to avoid recognition and attack by all of the induced host defense toxins, it would allow successful colonization of the plant. In fact, some animal pathogens have evolved very complex and subtle mechanisms to evade host defense even after their presence has been detected by the host (Klein, 2000; Rhen et al., 2000; Knodler et al., 2001). Thus, the pathogen can be successful by avoiding recognition (by the sentries or the foot soldiers) or by neutralizing the action of foot soldiers.

It follows that plant pathogens would subsequently face selection pressure to avoid recognition at both of these interaction levels. In such situations, resistance to any specific antimicrobial protein/peptide that could provide even a partial advantage to the invader would be selected. Analysis of conserved and variant amino acid residues at the active site of a large number of plant chitinases reveals a history of plant-microbe interactions leading to conservation of certain amino acid residues important for catalysis (Bishop et al., 2000). In addition, specificity of several host-encoded toxins could possibly be altered simultaneously by the use of defensive barriers, which shield the pathogen against several toxins at once. This has not been tested experimentally.

In contrast to the challenging microbe, the host plant can avoid invasion only by success at both levels of interaction. It must succeed at detecting the invader because its defense arsenal needs to be activated. Also, the subsequently activated defense genes must encode proteins that can actually recognize and attack the invader or they will not be effective, just as they do not harm the host that makes them. As we have just outlined (Supplemental Table I; www.plantphysiol.org), isoforms of particular plant defense proteins have been described that display considerable differences in activity against a specific microorganism, indicating that counter-evolution against resistance to specific antimicrobial proteins has occurred in the plant.

A model for the generation of plant disease resistance specificities is presented in Figure 1. Although achieving broad-range defense by altering the recognition specificity of the *R* gene product (sentinels) is a major goal of much ongoing research, this may be a formidable objective (Nimchuk et al., 2001; Stuiver and Custers, 2001). An important clue that this may be difficult or impossible may be taken from the observation that *R* gene mutation and even mutations in signal components downstream of *R* genes do not lead to a very broad-range susceptibility indicating the existence of a complex signal system (Rogers and Ausubel, 1997). It is predicted from the model that increasing either the range of target microbes, or the level of activity against a specific microbe, of antimicrobial proteins and peptides (the foot soldiers) is a viable alternative, albeit underexplored, approach toward improved disease resistance. Further systematic research on the foot soldiers of plant defense should increase our understanding of the plant immune system and aid in the development of better strategies of disease control, eventually including molecular evolution to increase their range and degree of effectiveness.

Note Added in Proof

The Arabidopsis gene *DIR1* that is required for the production or transmission of a mobile signal for systemic acquired resistance encodes a "foot soldier-class" putative apoplastic lipid transfer protein (**Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK** (2002) A putative lipid transfer protein involved in systemic resistance signaling in Arabidopsis. Nature **419:** 399–403).

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