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Resistance Gene-Dependent Plant Defense Responses

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INTRODUCTION

Plants are constantly being challenged by aspiring pathogens, but disease is rare. Why? Broadly, there are three reasons for pathogen failure. Either (1) the plant is unable to support the niche requirements of a potential pathogen and is thus a nonhost; or (2) the plant possesses preformed structural barriers or toxic compounds that confine successful infections to specialized pathogen species; or (3) upon recognition of the attacking pathogen, defense mechanisms are elaborated and the invasion remains localized. All three types of interaction are said to be incompatible, but only the latter resistance mechanism depends on induced responses. Successful pathogen invasion and disease (compatibility) ensue if the preformed plant defenses are inappropriate, the plant does not detect the pathogen, or the activated defense responses are ineffective. In this review, we examine the essential prerequisites for pathogen recognition and the induction of localized defense responses. Preformed defenses are considered elsewhere in this issue (see Osbourn, 1996, in this issue).

Race-specific pathogen recognition is hypothesized to result from the direct or indirect interaction of the product of a dominant or semidominant plant resistance (*R*) gene with a product derived from the corresponding dominant pathogen avirulence (*Avr*) gene (Keen, 1992; Staskawicz et al., 1995). Subsequent signal transduction events are assumed to coordinate the activation of an array of defense responses.

This "simple" model appears to explain much but begs many questions. For example, *R* gene products are likely to provide key components for recognition, but how do the distinct classes of R proteins characterized to date (see Bent, 1996, in this issue) activate the defense response? Do different *R* gene classes activate distinct responses? The regulation of some components of defense mechanisms has been studied in plant cell cultures in response to non-race-specific elicitors, but to what extent do such studies provide a model for *R* gene function? Plant resistance is often correlated with the activation of specific defense responses, but which (if any) are required to abolish or retard pathogen growth, and how? Which are primary responses and which are secondary? Does the first response involve transcriptional regulation, the activation of preformed enzymes, and/or the opening of ion channels, or are these possibilities nonexclusive? Is the response fine-tuned to the specific pathogen that elicits it? Do the defense responses differ between plant organs, or do they vary according to the attack strategy of the pathogen?

To address these questions, we first review the responses that have been correlated with the activation of defense mechanisms in *R–Avr* gene–dependent resistance and in plant species in which the pathogen never causes disease, that is, nonhost resistance. We then assess the significance of these mechanisms in a few selected examples involving *R* genes. After considering possible signaling mechanisms and the mechanisms that could initially amplify and subsequently attenuate the response, we discuss the difficulties involved in assessing the functional significance of responses that are correlated with resistance. Some interesting related topics, such as the relationship between *R* gene structure and function, the significance of disease lesion mimics, and the phenomenon of systemic acquired resistance (SAR), receive limited attention here and are covered elsewhere in this issue (see the following articles in this issue: Bent, 1996; Dangl et al., 1996; Ryals et al., 1996). In Figure 1, various induced defense responses encountered by invading microbes are depicted.

DEFENSE MECHANISMS

Hypersensitive Response

Incompatible responses are frequently associated with the appearance of necrotic flecks containing dead plant cells at sites of attempted pathogen ingress (Figure 1A). Classically, this hypersensitive response (HR) is defined as the death of host cells within a few hours of pathogen contact (Agrios, 1988), but the HR can be phenotypically diverse, ranging from HR in a single cell to spreading necrotic areas accompanying limited pathogen colonization (Holub et al., 1994). Also, the appearance of the HR can be environmentally contingent and in particular can be attenuated at high humidity (Klement, 1982; Hammond-Kosack et al., 1996).

The HR has been proposed to play a causal role in disease resistance (Heath, 1980). In interactions with obligate biotrophic pathogens that form intimate haustorial associations with host cells, plant cell death would deprive the pathogen of access

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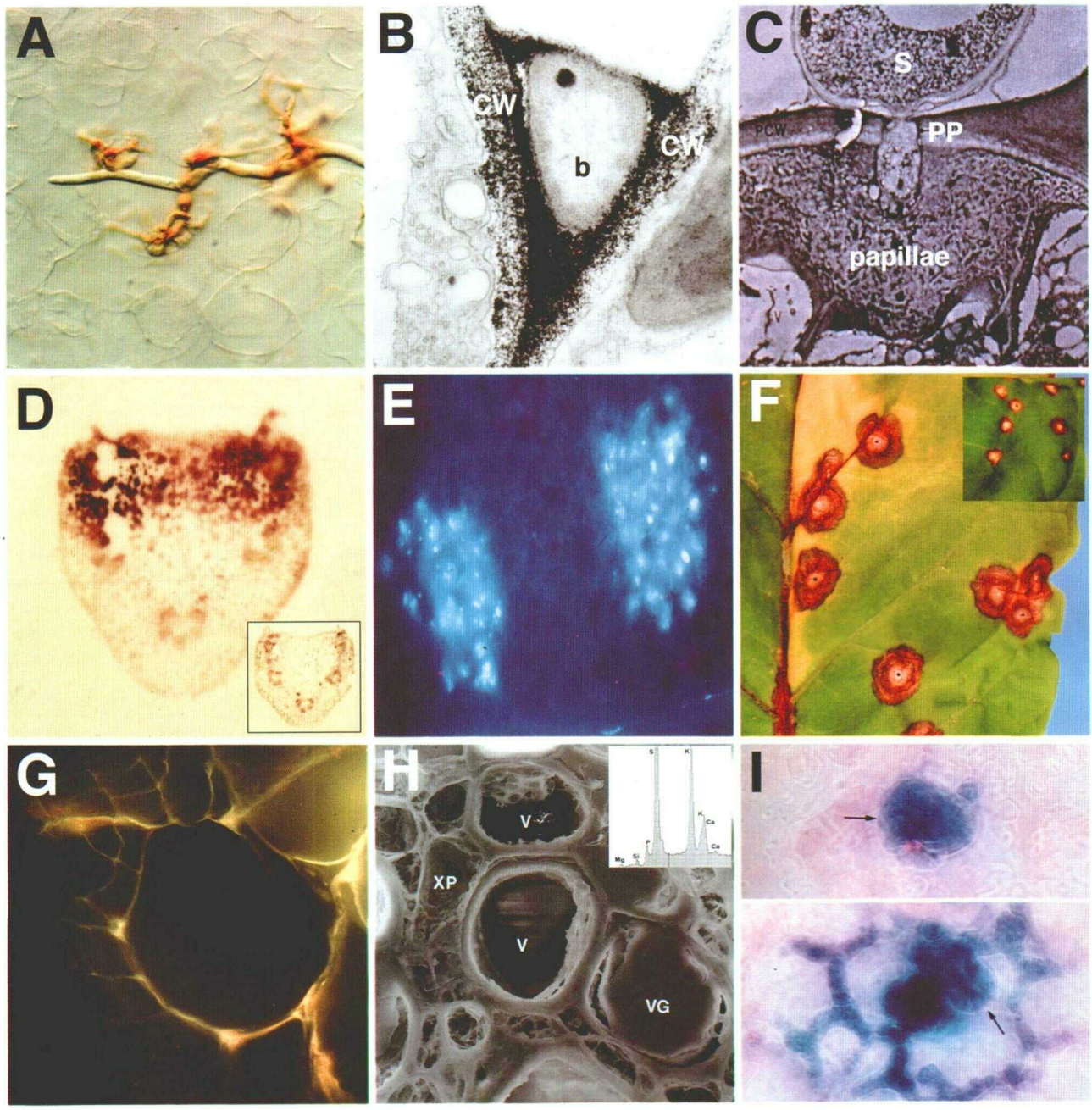


Figure 1. Types of Activated Defense Responses.

(A) Hypersensitive response in single lettuce mesophyll cells penetrated by haustoria of an incompatible isolate of the biotrophic fungus *Bremia lactucae.* The lettuce *R* gene is *Dm7* (Bennett et al., 1996).

(B) H_2O_2 generation in lettuce cell walls, in the vicinity of the incompatible bacterium *P. syringae* pv *phaseolicola.* H_2O_2 was detected in cell walls (CW) 5 hr after inoculation with bacteria (b) as black deposits in the transmission electron image by staining with cesium chloride (J. Mansfield, unpublished data).

(C) Papillae formation. Papillae develop beneath the penetration peg (PP) and germinating spore (S) of an avirulent isolate of the biotrophic fungus *Erysiphe graminis* f sp *hordei* on barley leaves expressing the *Mlg* gene.

(D) Hydroxyproline-rich glycoprotein (HRGP) accumulation in turnip (*Brassicae campestris*) petiole tissue in response to infection by an avirulent isolate of *Xanthomonas campestris.* A compatible interaction is shown in the inset. The HRGP was detected by tissue printing onto nitrocellulose and the use of the monoclonal antibody JIM 11 raised against a pea HRGP and then visualized by alkaline phosphatase enzyme activity (Smallwood et al., 1994; Davies, 1996).

to further nutrients. In interactions involving hemibiotrophic and necrotrophic pathogens, the role of the HR is less clear because these pathogens can obtain nutrients from dead plant cells. However, cellular decompartmentalization may lead to the release of harmful preformed substances that are stored in the vacuole (see Osbourn, 1996, in this issue). Alternatively, the levels of induced phytoalexins, which usually are rapidly turned over in plant cells (see below), may accumulate to inhibitory concentrations because they are no longer metabolized.

The HR may cause pathogen arrest but may also occur as a consequence of the activation of other defense responses. Several *R–Avr* gene–mediated resistances appear not to involve an HR. These include barley resistance against all races of *Erysiphe graminis* f sp *hordei*, which is conferred by the *mlo* gene (Freialdenhoven et al., 1996; see also Knogge, 1996, in this issue) and the potato *Rx* gene–mediated resistance to potato virus X (Köhm et al., 1993). Whether an HR is present or absent does not prove that a qualitatively distinct type of resistance mechanism exists. It is possible that all *R* genes initiate responses that could result in HR, but some responses may prevent disease so effectively that cell death is not activated.

What is the mechanism underlying HR formation? Does death arise because of a switch in cell metabolism to biochemical pathways that produce an array of compounds or free radicals that are toxic to both the pathogen and the plant cell, thus causing the latter to necrose rapidly? Or does pathogen recognition switch the attacked plant cell into a genetically programmed cell death or apoptotic response? Recent evidence suggests that both types of cell death process occur, in both incompatible and compatible plant–pathogen interactions (Dangl et al., 1996, in this issue; Mittler and Lam, 1996).

Reactive Oxygen Species

The production of reactive oxygen species (ROS; see Figure 2) probably plays a key role in plant defense. Often the first response activated in many incompatible interactions (Figure 1B), it may be the trigger that initiates the HR. Dolle and colleagues (1983, 1988) were the first to report that superoxide anions ($O_2^{\cdot -}$) were produced in incompatible interactions, initially between potato and *Phytophthora infestans* (late blight fungus) and then between tobacco and tobacco mosaic virus (TMV; see below). From recent studies involving plant cell culture (Levine et al., 1994; Murphy and Auh, 1996), it appears extremely likely that plants contain a mechanism for producing $O_2^{\cdot -}$ that involves an NADPH oxidase analogous to that used in mammalian neutrophils (Segal and Abo, 1993; Groom et al., 1996). The $O_2^{\cdot -}$ generated is usually rapidly dismutated either nonenzymatically or via superoxide dismutase (SOD) catalysis to hydrogen peroxide (H_2O_2; Figures 2A and 2B, equations 2 and 3), and so in most plant systems, H_2O_2 accumulation is detected (Sutherland, 1991; Levine et al., 1994; Mehdy, 1994; Nürnberger et al., 1994). At an acidic pH, such as that in the plant cell wall, the half life of superoxide is <1 sec (Sutherland, 1991). Because H_2O_2 has no unpaired electron, it can cross biological membranes, which the charged $O_2^{\cdot -}$ species can do only very slowly (Halliwell and Gutteridge, 1990).

Both $O_2^{\cdot -}$ and H_2O_2 are only moderately reactive, however, and the cellular damage by ROS appears to be due to their conversion into more reactive species. Protonation of $O_2^{\cdot -}$, which occurs more readily at low pH, yields the hydroperoxyl radical ($HO_2^{\cdot}$; Figure 2B, equation 1). Because $HO_2^{\cdot}$ is less polar than $O_2^{\cdot -}$, it should be able to cross biological membranes about as effectively as does H_2O_2. Unlike $O_2^{\cdot -}$, $HO_2^{\cdot}$ can attack fatty acids directly (Figure 2C, equation 12) and has been shown to convert linolenic, linoleic, and arachidonic acids to lipid peroxides (Halliwell and Gutteridge, 1990). Thus, under appropriate conditions, $O_2^{\cdot -}$ generation, leading to $HO_2^{\cdot}$ formation, could result in membrane damage and the formation of an array of potential lipid peroxide signal molecules (see below).

In the presence of Fe^{2+}, H_2O_2 can undergo the Fenton reaction that gives rise to the extremely destructive hydroxyl free radical ($OH^{\cdot}$; Figure 2B, equations 4 to 6), which can initiate self-perpetuating lipid peroxidation (Figure 2C, equations 12

Figure 1. (continued).

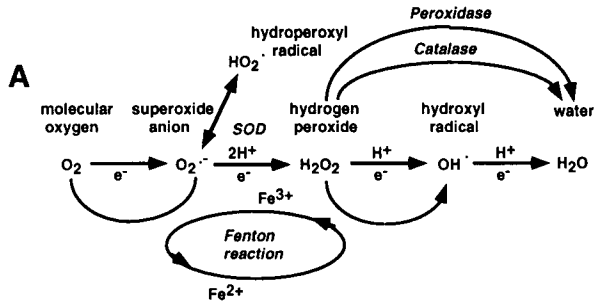
(E) Callose deposition in Arabidopsis leaf mesophyll at the incompatible infection sites of *Peronospora parasitica* isolate Noco2 in the ecotype Ws expressing the *R* gene *Rpp14*. Callose was detected by aniline blue staining and fluorescence under UV light (Parker et al., 1993).

(F) TMV lesion formation in tobacco leaves. A significant increase in TMV multiplication in tobacco leaves is visualized as an enlargement of *N* gene–mediated local lesion formation caused by continuous removal of induced SA. This was achieved through the constitutive expression of the *nahG* gene of *P. putida* encoding the enzyme salicylate hydroxylase (Gaffney et al., 1993; Delaney et al., 1994). Wild-type local lesions at the identical magnification are shown in the inset.

(G) Lignification and the reinitiation of cell division in *Brassica napus* stem tissue in response to a low aggression isolate of the fungus *Leptosphaeria maculans*. Lignin polymers were detected by autofluorescence and confirmed by phloroglucinol-hydrochloride staining (Hammond and Lewis, 1987).

(H) Vascular gel or tylose formation in stem xylem vessels (V) of a resistant genotype of cocoa (*Theobroma cacao*) in response to the vascular colonizing fungus *Verticillium dahlia*. X-ray microanalysis of the scanning electron image within the vascular gels (VG) or adjacent xylem parenchyma cells (XP) reveals the presence of inorganic sulphur, as shown in the inset (Cooper et al., 1996).

(I) Induction of a β-1,3-glucanase promoter: β-glucuronidase (GUS) reporter gene fusion in *Cf-5*–expressing tomato at the site of leaf penetration by an avirulent isolate of the fungus *Cladosporium fulvum*. Upper panel, 3 days after infection; lower panel, 8 days after infection. The arrows highlight the swollen mesophyll cell at the base of the penetrated substomatal cavity (Ashfield et al., 1994).



B

Equilibrium between superoxide and hydroperoxyl

$$O_2^{\cdot -} + H^+ \longleftrightarrow HO_2^{\cdot} \quad (1)$$

Superoxide dismutation

$$HO_2^{\cdot} + O_2^{\cdot -} + H^+ \longleftrightarrow H_2O_2 + O_2 \quad (2)$$

$$2O_2^{\cdot -} + 2H^+ \longleftrightarrow H_2O_2 + O_2 \quad (3)$$

The Fenton or Haber-Weiss Reaction

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2 \quad (4)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^- \quad (5)$$

Overall: $O_2^{\cdot -} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^{\cdot} + O_2$ (6)

Peroxidase

$$\text{Peroxidase} + H_2O_2 \longleftrightarrow \text{State I} + H_2O \quad (7)$$

$$\text{State I} + RH_2 \longleftrightarrow \text{State II} + RH \quad (8)$$

$$\text{State II} + RH_2 \longleftrightarrow \text{Peroxidase} + RH + H_2O \quad (9)$$

Catalase

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \quad (10)$$

C Initiation and propagation of lipid peroxidation

$$-CH_2- + OH^{\cdot} \longrightarrow -\dot{C}H- + H_2O \quad (11)$$

$$-CH_2- + HO_2^{\cdot} \longrightarrow -\dot{C}H- + H_2O_2 \quad (12)$$

$$-\dot{C}H- + O_2 \longrightarrow -\dot{C}HO_2- \quad (13)$$

peroxyl radical

$$-\dot{C}HO_2- + -CH_2- \longrightarrow CHO2H + -\dot{C}H- \quad (14)$$

lipid peroxide

D Proposed route for the generation of the salicylic acid radical leading to lipid peroxidation

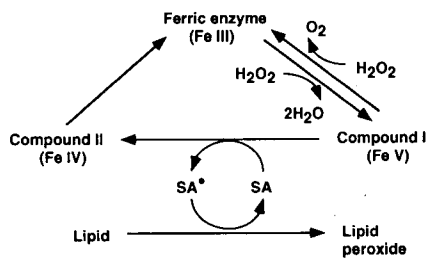


Figure 2. Interconversion of ROS Derived from Molecular Oxygen.

(A) General scheme.

(B) Chemical equations showing key reactions involving ROS.

(C) Chemical equations showing initiation and propagation of lipid peroxidation reactions.

(D) Proposed interaction between catalase and SA (Z. Chen, J. Durner, and D. Klessig, unpublished data). See text for details.

to 14). If H_2O_2 entering the cell cytoplasm survives in sufficient concentrations to reach either the plant or pathogen nucleus, it could react with intracellular metal ions to give $OH^{\cdot}$, which is known to fragment DNA by site-specific attack (Halliwell and Gutteridge, 1990). Thus, ROS production can result in considerable damage to both host and pathogen and requires plant cells to activate an array of protective mechanisms (see below).

Two kinds of ROS induction kinetics have been observed in plant cell suspension culture. Pathogen-derived elicitors initiate a very rapid biosynthesis of ROS (within <5 min), which, in at least one example, requires external Ca^{2+} and anion channel activation (Nürnberger et al., 1994). Conversely, avirulent bacteria provoke a small initial oxidative burst (as do near-isogenic virulent bacteria) that is very similar in kinetics to that induced by elicitors, followed by a massive burst 2 to 4 hr after addition of bacteria to plant cells (Levine et al., 1994; Baker and Orlandi, 1995). This delay could simply reflect the time required for the bacterial avirulence signal to be delivered to the plant cells and processed to a form that can elicit recognition mechanisms.

Notably, the production of ROS with either type of kinetics can be prevented by specific inhibitors of the mammalian NADPH oxidase, such as diphenyleniodonium (Segal and Abo, 1993; Jones, 1994). The mammalian superoxide-producing machinery requires a two-component cytochrome consisting of a heme-binding $p22^{phox}$ and a NADPH-binding $p91^{phox}$. The activity of this complex is regulated by the state of phosphorylation of cytoplasmic p47 and p67 proteins and the GTP- or GDP-bound state of the rac2 G-protein. Antibodies to various mammalian NADPH oxidase components cross-react with proteins of similar size found in plant cell cultures (Dwyer et al., 1995; Tenhaken et al., 1995; Desikan et al., 1996). Moreover, a rice gene with high sequence homology to the $gp91^{phox}$ membrane component has been isolated (Groom et al., 1996). Collectively, these data indicate that plants and mammals generate ROS in similar ways during defense responses.

Plant cells have two other ways of generating ROS. First, a germin-like oxalate oxidase protein that can produce H_2O_2 from oxalic acid has been detected in incompatible *Mla1* barley–powdery mildew interactions (Zhang et al., 1995). Second, cell wall peroxidases can produce H_2O_2 (Figure 2B, equations 7 to 9; Bolwell et al., 1995). French bean cell cultures challenged with two different fungal elicitors showed a rapid increase in oxygen uptake, followed shortly afterward by the transient production of H_2O_2 that was accompanied by a rise in peroxidase enzyme activity. A transient alkalinization of the apoplast, to pH 7.0 to 7.2, was absolutely required for H_2O_2 generation in this system. Neither of these alternative routes to generate ROS is inhibitable by diphenyleniodonium, suggesting that plants have evolved at least three mechanisms to produce ROS during defense. It is possible then that the generation of ROS during incompatible interactions (both host and nonhost) will occur via different mechanisms in different plant species. Alternatively, the relative contributions of each

mechanism to the oxidative burst may vary from species to species.

Several roles for ROS in plant defense have been proposed. For example, H_2O_2 could be directly toxic to microbes at levels known to be produced in plants (Peng and Kuć, 1992). H_2O_2 may also contribute to the structural reinforcement of plant cell walls; H_2O_2 is essential for the formation of lignin polymer precursors via peroxidase activity (Bolwell et al., 1995), and Bradley et al. (1992) have demonstrated that hydroxyproline- and proline-rich cell wall glycoproteins were rapidly oxidatively cross-linked in cell walls after fungal elicitor treatment. This protein cross-linking also decreased "protoplastability" (Brisson et al., 1994), thus rapidly making the plant cell wall more refractory to microbial penetration and enzymatic degradation.

A signaling role for some ROS is also likely. H_2O_2 increases benzoic acid-2 hydroxylase (BA2-H) enzyme activity (Léon et al., 1995), which is required for salicylic acid (SA) biosynthesis (see below). Moreover, H_2O_2 activates some protection mechanisms, for example, glutathione *S*-transferase gene expression, in neighboring cells (Levine et al., 1994). However, direct application of H_2O_2 to soybean cell cultures did not induce phenylalanine ammonia-lyase (PAL) or chalcone synthase (CHS) gene expression (Levine et al., 1994). Therefore, H_2O_2 is unlikely to trigger increased synthesis of antimicrobial phytoalexins through the activation of the phenylpropanoid or flavonoid biosynthetic pathways. The lipid peroxides formed as a consequence of ROS generation may also have a direct signaling role in SA accumulation (Léon et al., 1995).

There is a strong likelihood that the generation of ROS will lead to an alteration in the redox balance in the reacting cell. In mammals, many transcription factors, such as the ROS-responsive NF-κB factor and the antioxidant-responsive AP-1 factor, are known to be redox regulated (Schreck et al., 1991; Meyer et al., 1993). Redox balance may also regulate the stability of specific defense-related mRNA transcripts in plants (Mehdy, 1994). In some biological systems, redox changes also induce enzymes with radical scavenging and repair activity, but different ROS trigger different responses (Herouart et al., 1993). For example, in bacteria, two ROS-responsive transcription factor systems, called *oxyR* for the H_2O_2 response (reviewed in Storz et al., 1990) and *oxyRS* for the $O_2^{\cdot -}$ response (reviewed in Demple, 1991) are known. In mammals, agents leading to $O_2^{\cdot -}$ production are not effective in activating NF-κB, whereas those causing H_2O_2 generation are (Schreck et al., 1992). Interestingly, the disease lesion mimic *lsd1*, which constitutively expresses various defense responses, overproduces $O_2^{\cdot -}$ before lesions form (Dangl et al., 1996, in this issue), suggesting that $O_2^{\cdot -}$ may be one of the signals that coordinates plant defense gene induction.

Only one study provides convincing evidence that H_2O_2 is involved in conferring disease resistance. The constitutive expression of an H_2O_2-generating glucose oxidase from *Aspergillus niger* in the apoplast of transgenic potato resulted in good resistance to the bacterial soft rot pathogen *Erwinia carotovora* sp *carotovora* and enhanced resistance to *P. infestans* (Wu et al., 1995). However, the mechanism underlying this general disease protection is not known.

Cell Wall Fortification

Microbes must negotiate the plant cuticle and/or cell wall to reach the cell, although penetration can sometimes occur through a wound or natural opening. Fortifying the plant cell wall can increase resistance in various ways. For extracellular biotrophs, such as *Pseudomonas syringae* or *Cladosporium fulvum*, sealing the wall could impede leakage of cytoplasmic contents, thereby reducing nutrient availability for the pathogens. For necrotrophs, such as *Botrytis cinerea*, that rely on hydrolysis of the plant cell wall in advance of hyphal growth, the diffusion of toxins and enzymes to the sensitive plant cells would be retarded. In addition, the low molecular weight phenolic precursors of lignin and the free radicals produced during polymerization reactions in the cell wall may affect pathogen membrane plasticity or inactivate pathogen enzymes, toxins, or elicitors. Hyphae themselves may also become lignified (Mauch-Mani and Slusarenko, 1996). For sophisticated haustorial biotrophs, preventing entry into the plant cell precludes parasitism.

Microbes produce a number of cutinases and cell wall hydrolyzing enzymes, such as pectinases, cellulases, xylanases, and polygalacturonases (PGs), that attack the various cell wall polymers. Mechanical pressure may also facilitate microbial entry (Agrios, 1988). Although individually none of the above-mentioned enzymes is crucial for particular modes of pathogenesis (see Knogge, 1996, in this issue), these activities produce cell wall fragments, particularly oligomers of galacturonic acid, that might elicit additional defense responses or amplify the original ones (Farmer and Ryan, 1992; Levine et al., 1994). For example, PGs are believed to contribute to cell wall softening by some necrotrophic fungi. Polygalacturonase-inhibiting proteins (PGIPs) inhibit PGs. PGIPs are induced in the bean–*Colletotrichum lindemuthianum* interaction with similar kinetics to pathogenesis-related (PR) proteins (Bell et al., 1986; Nuss et al., 1996). It has been hypothesized that PGIPs may retard PG function, which would lead to an elevated abundance of oligogalacturonides with a chain length of >8 units. These, in turn, may trigger additional defense responses (De Lorenzo et al., 1994). Alternatively, PGIPs may slow the rate of hyphal extension so that other components of the defense response can be more effectively deployed. For example, constitutive expression of the pear fruit PGIP in transgenic tomato plants enhanced resistance to colonization of ripe fruits by *B. cinerea* (Powell et al., 1994). It is intriguing that PGIPs possess a leucine-rich repeat (LRR) domain similar to that predicted for several of the cloned *R* gene products (see Bent, 1996, in this issue), although the significance of this observation is not clear (Jones and Jones, 1996).

One type of cell wall fortification that occurs rapidly in response to fungal invasion is the formation of papillae (Figure 1C). Papillae often form immediately beneath the penetration peg and are heterogeneous in composition (Heath, 1980); they are thought to physically block fungal penetration of host cells (Bayles et al., 1990). However, it is also possible that their formation is required to provide adequate support for subsequent haustorium development, in which case they may be essential for pathogenesis (Heath, 1980).

Rapid callose deposition in cell walls is also frequently associated with sites of pathogen incompatibility (Figure 1E). Callose deposition also occurs when plant cell cultures are challenged with pathogen-derived elicitors or when plant tissue is mechanically wounded (Kauss, 1990). The constitutive plasma membrane–localized callose synthase enzyme catalyses the formation of this β-1,3-glucan polymer and requires both increased levels of the primer β-furfuryl-β-glucoside and Ca^{2+} for activity (Kauss, 1990; Ohana et al., 1993). Blockage of plasmodesmata with callose is an essential component of the defense response required to impede cell-to-cell movement of viruses (Beffa et al., 1996).

Basic hydroxyproline-rich glycoproteins (HRGPs; Figure 1D) are thought to play a key role in the organization of primary cell wall architecture and may act as the foci for the initiation of lignin polymerization (Showalter, 1993; Bolwell et al., 1995). A subset of HRGP genes is also slowly induced in response to incompatible pathogen invasion, indicating that de novo HRGP synthesis is a relatively late defense response (Showalter et al., 1985). In contrast, the rapid oxidative cross-linking of preformed HRGPs and PR proteins via either inter- or intramolecular isodityrosine linkages may constitute one of the earliest defense responses accompanying the oxidative burst (Bradley et al., 1992). Extensins may also act as a kind of cell wall fly paper, capable of immobilizing certain plant pathogens, possibly through electrostatic interactions (Showalter, 1993).

An additional but probably slower mechanism that renders cell walls more impermeable is the local elevation of their lignin content (Figure 1G; Whetten and Sederoff, 1995). The most compelling evidence for a causal role of lignification in resistance has been provided by Moerschbacher et al. (1990) for the *R* gene–mediated incompatible interaction between wheat (*Triticum aestivum*) and the rust *Puccinia graminis* f sp *tritici*. In these experiments, application of OH-phenylsulfinamoyl-tertiobutyl acetate (PAS) and NH_2-PAS, two highly specific irreversible suicide inhibitors of the lignin biosynthetic enzyme cinnamyl alcohol dehydrogenase, significantly decreased the frequency of lignified and necrotic host cells and concomitantly allowed an increase in fungal biomass, to the extent that fungal sporulation sometimes occurred.

Benzoic Acid and Salicylic Acid

Incompatible pathogens, whether fungi, viruses, or bacteria, frequently provoke the accumulation of both free BA and SA and their respective glucoside conjugates, with the highest concentrations forming in the immediate vicinity of the infection site. The induction of these compounds is commonly associated with the HR (Raskin, 1992; see also Ryals et al., 1996, in this issue). The biochemical pathway leading to SA biosynthesis during the defense response is now relatively well established, but its regulation may differ between plant species. SA is derived from the phenylpropanoid pathway, but it appears (at least in tobacco) that SA synthesis is not regulated at the level of PAL transcription. Instead, the release of BA from a preformed BA conjugate induces a soluble cytochrome P450 monoxygenase (BA2-H) that converts BA to SA. BA2-H enzyme activity is strongly induced before the appearance of the HR (Léon et al., 1995). However, in Arabidopsis and other species, the preexisting BA conjugate may be absent. In these cases, increased SA levels require increases in PAL activity (Mauch-Mani and Slusarenko, 1996). Interestingly, oxidative stress caused by ozone or ultraviolet light also triggers SA biosynthesis in tobacco, perhaps by chemical release of BA from its conjugated form (Yalpani et al., 1994).

It is not clear whether SA biosynthesis is a cause or a consequence of the HR. Both hypotheses may be valid. For example, at high concentrations, SA has been reported to inhibit catalase activity. This could exacerbate oxidative stress resulting from the increased synthesis of ROS (Chen et al., 1993; Conrath et al., 1995). Moreover, interactions between SA and catalase and/or ascorbate peroxidase may give rise to the damaging SA free radical (Durner and Klessig, 1995, 1996). SA inhibition of catalase is thought to occur via the shunting of an oxidized catalase intermediate (Compound I) from the very rapid cycling catalase reaction, which restores enzyme function, into the much slower peroxidative reaction cycle, which results in the trapping of catalase in an inactive and partially reduced state (Compound II; Durner and Klessig, 1996; see Figure 2D). As SA donates an electron to Compound I catalase, it is converted into the oxidized form, SA·. This SA free radical could initiate lipid peroxidation (Figure 2D) and may also modify other macromolecules (Savenkova et al., 1994). However, the SA free radical does not inhibit peroxidases involved in lignin biogenesis.

Several other roles for SA and/or BA in plant defense have been proposed. Both compounds may be directly antimicrobial (Raskin, 1992; Klessig and Malamy, 1994). Furthermore, exogenous SA application induces the coordinated expression of a subset of PR genes in numerous plant species (Ryals et al., 1996, in this issue). Elevated SA levels can also inhibit wound-induced gene expression by blocking jasmonic acid (JA) biosynthesis (Pena-Cortes et al., 1993; Farmer, 1994). Thus, at sites of *R–Avr* gene–mediated microbial incompatibility, elevated SA levels should ensure that defense responses required for the arrest of microbial growth are activated, whereas those against chewing insects and migrating nematodes are not induced unnecessarily.

An absolute requirement for SA has been demonstrated in *R* gene–mediated resistance against various viruses, bacteria, and fungi. Transgenic tobacco and Arabidopsis lines have

been made that constitutively express a bacterial *nahG* gene encoding the enzyme salicylate hydroxylase. Salicylate hydroxylase converts SA to catechol, and these transgenic plants have markedly reduced levels of SA (Gaffney et al., 1993; Delaney et al., 1994). The lack of SA accumulation in these *nahG*-expressing plants correlated with weakened local *R* gene–mediated resistance responses (Figure 1F) and also with a block in the induction of various defense genes (Ryals et al., 1996, in this issue). However, in tomato–*C. fulvum* interactions, the presence of the *nahG* gene does not compromise *Cf* gene–mediated resistance (see below). Clearly, the role of SA in defense is complex and may also differ from species to species. In rice, no increases in SA levels occur after pathogen challenge, but constitutive SA levels correlate with general disease resistance (Silverman et al., 1995).

PR Proteins and Other Defense-Related Proteins

The term PR protein was first used to describe numerous extracellular proteins that accumulated in response to TMV infection of susceptible tobacco genotypes. Subsequently, in an array of plant–pathogen interactions, differential PR gene induction was found to be associated with incompatibility (Figure 1I; reviewed in Bol et al., 1990; Linthorst, 1991). More recently, the definition of a PR protein has been broadened to include intra- and extracellularly localized proteins that accumulate in intact plant tissue or cultured cells after pathogen attack or elicitor treatment (Bowles, 1990).

Do PR proteins play a causal role in resistance? Several PR proteins possess either antifungal or antibacterial activity in vitro and are now known to be chitinases, or glucanases, or to bind chitin (Collinge et al., 1993; Melchers et al., 1994). The degradation of fungal cell wall structural polysaccharides, or the alteration of fungal cell wall architecture, could arrest or severely impair fungal growth. Moreover, the constitutive expression of PR proteins of known and unknown function in transgenic plants has led to increased resistance to some fungal pathogens (Broglie et al., 1991; Lui et al., 1994). Overall, two facts have emerged from these overexpression experiments. First, the basic forms of PR proteins, which are targeted to the vacuole, exhibit more effective antifungal activity than their acidic counterparts, which are secreted from the plant cell (Sela-Buurlage et al., 1993); the only exception to date is the tobacco PR-1a protein (Alexander et al., 1993). Second, when two or more PR proteins are constitutively coexpressed, a synergistic increase in the level of disease control can be obtained (Zhu et al., 1994). These findings suggest that the coordinated activity of several PR genes is necessary for resistance. They also indicate that PR proteins targeted to the vacuole or outside the cell are less likely to be components of front line defense action but probably have their major effect, particularly against biotrophic pathogens, after significant cellular decompartmentalization has occurred. However, the genes encoding some of the cytoplasmically localized PRs are activated very rapidly after elicitor treatment, which may indicate that this subset includes components of the front line defense response (Somssich et al., 1989; Hahlbrock et al., 1995).

The basic cysteine-rich thionins, found mainly in cereals, are another group of defense-related proteins with known antimicrobial activity (reviewed in Bohlmann, 1994). Like the PR proteins, these thionins also accumulate differentially during incompatible interactions. Interestingly, a JA-mediated signal transduction pathway, distinct from the typical SA-mediated pathway leading to PR gene activation, controls thionin gene expression in Arabidopsis (Epple et al., 1995). Also, overexpression of a barley α-hordothionin gene in tobacco increased resistance to several bacterial pathogens.

Studies on defense gene induction in parsley cell suspension cultures with an elicitor from *P. sojae*, a nonhost–fungal interaction, have revealed that at least 19 distinct *Eli* (elicitor-activated) genes were transcriptionally induced in the plant cells, some concomitant with the oxidative burst (Somssich et al., 1989). Subsequent studies have shown that in addition to the typically induced PR and phytoalexin biosynthetic genes, some *Eli* gene products are involved in the activated methyl-group cycle that is normally associated with primary metabolism (Kawalleck et al., 1992). The same responses are induced at the sites of attempted fungal penetrations into parsley leaves (reviewed in Hahlbrock et al., 1995). This study highlights the facts that an extreme diversity of plant proteins targeted to various cellular compartments is coordinately synthesized during defense responses and that changes to both primary and secondary plant metabolism are involved. The ligand that triggers these responses in parsley cells has now been defined as a 13–amino acid elicitor, Pep-13 (Nürnberger et al., 1994).

Lipoxygenases

Rapid increases in lipoxygenase (LOX) enzyme activity and/or mRNA and protein levels are frequently found to be specifically associated with *R–Avr* gene–mediated incompatibility (reviewed in Slusarenko, 1996). Increased LOX activity may contribute to resistance in a number of ways. For example, LOX may generate signal molecules such as JA, methyl-JA, or lipid peroxides, which coordinately amplify specific responses (see above). LOX activity may also cause irreversible membrane damage, which would lead to the leakage of cellular contents and ultimately result in plant cell death (Keppler and Novacky, 1986). Alternatively, LOX-catalyzed reactions can result in the production of toxic volatile and nonvolatile fatty acid–derived secondary metabolites that could directly attack invading pathogens (Croft et al., 1993). However, at present, only circumstantial evidence exists for any of these roles.

Phytoalexins

Phytoalexins are low molecular weight, lipophilic, antimicrobial compounds that accumulate rapidly around sites of

incompatible pathogen infections and in response to an extensive array of biotic and abiotic elicitors (Smith, 1996). Phytoalexin biosynthesis occurs after a diversion of primary metabolic precursors into novel secondary metabolic pathways. This diversion often arises from the de novo induction of enzymes, such as PAL, that control key branch points in the biosynthetic pathways (Dixon and Paiva, 1995). However, because the synthesis of most phytoalexins requires the activity of numerous enzymes, highly coordinated signaling events must be required in the attacked cells to establish successfully this type of defense response. Interestingly, common sequence elements have been identified in the promoters of several genes encoding enzymes required for different steps in the biosynthesis of flavonoid phytoalexins (Hahlbrock et al., 1995).

Although phytoalexins have an undeniable antimicrobial activity in vitro, the extent of their role in *R* gene–dependent responses in plants remains to be determined (Mauch-Mani and Slusarenko, 1996). Recent genetic experiments have addressed this issue. Wild-type Arabidopsis plants produce the phytoalexin camalexin (Browne et al., 1991), and a number of phytoalexin deficient (*pad*) mutants have been isolated. Interestingly, the *pad1*, *pad2*, and *pad3* mutations do not interfere with *RPS2* gene–mediated resistance to *P. syringae* strains expressing *avrRpt2* (Glazebrook and Ausubel, 1994). One study does, however, provide unequivocal evidence that phytoalexins contribute to disease resistance. The biosynthesis of the phytoalexin resveratol was engineered in tobacco by constitutively expressing the terminal biosynthetic enzyme stilbene synthase (Hain et al., 1993). These transgenic plants exhibited enhanced resistance to the necrotrophic fungus *B. cinerea*. It may well emerge for many plant–pathogen interactions that the purpose of increased phytoalexin synthesis is to reduce the severity of secondary infections or the overall growth rate of virulent pathogens.

Elemental sulphur, produced as cyclo-octasulphur (S_8), was recently revealed to be a novel, highly fungitoxic phytoalexin (Cooper et al., 1996). High levels of S_8 were shown, by energy-dispersive x-ray microanalysis, to accumulate in xylem parenchyma cells and xylem vessels in contact with the fungus *Verticillium dahlia* solely in resistant genotypes of cocoa (*Theobroma cacao*; Figure 1H). The biosynthetic origin of S_8 in plants is uncertain.

SYSTEMS TO STUDY *R–Avr* GENE–DEPENDENT EVENTS

In this section, using four well-characterized plant–pathogen interactions, we explore the temporal order and importance of the individual defense responses described above. In each system, incompatibility is conferred by specific *R–Avr* gene combinations, and for three of these systems, the gene sequences of either or both the *R* and *Avr* genes are known.

Tobacco *N* Gene–Mediated Resistance to TMV

The tobacco *N* gene encodes a nucleotide binding site (NBS)/LRR protein that includes an N-terminal domain with some similarity to the cytoplasmic domain of the Drosophila Toll and the mammalian interleukin 1 receptor-like proteins (Whitham et al., 1994). The corresponding Avr ligand has been identified as the viral replicase (Padgett and Beachy, 1993). At 20°C, *N*-mediated resistance is functional, but it is inactive at 30°C (Weststeijn, 1981). The temperature sensitivity of this interaction provides a molecular switch to study the activation of *N* gene–dependent defense responses in leaves. Thus, it is possible to infect leaves of *N*-expressing tobacco genotypes with TMV, allow the virus to spread systemically at 30°C for 24 to 48 hr, and then to lower the temperature, synchronously imposing incompatibility on a large number of infected cells.

This approach was used in the studies of Doke and Ohashi (1988) on $O_2^{\cdot -}$ generation. When plants carrying *N* that had been infected with TMV were switched from 30°C to 20°C, increased superoxide production, as assayed by nitroblue tetrazolium staining and SOD-abolishable cytochrome *C* reduction, could be detected within 2 min. Infiltration of SOD, or SOD and catalase, attenuated the necrotic lesion formation associated with TMV resistance. The same system has also been used to study SA biosynthesis, which is initiated within 4 to 6 hr of the temperature shift and is associated with a concomitant rise in BA2-H enzyme activity (Léon et al., 1993). Therefore, the production of ROS precedes the induction of SA, which is consistent with the induction of SA biosynthesis by oxidative stress (Leon et al., 1995). Because virus-induced collapse of leaf tissue is first observed at 10.5 hr, SA may be required to amplify the defense response to the point at which cell death occurs.

In the region surrounding TMV–*N* lesions, there is a significant accumulation of an array of PR proteins (White and Antoniw, 1991). However, in *nahG*-transformed *N*-tobacco, both PR gene expression and TMV resistance are severely compromised (Figure 1F; Bi et al., 1995; Ryals et al., 1996, in this issue). Thus, SA can be considered to be an essential signal for the local induction of some PR genes as well as for resistance.

Lignification of host cell walls at the margin of TMV lesions and the formation of various phytoalexins in the surrounding tissue are always associated with *N* gene–mediated resistance (Jaeck et al., 1992). However, when antisense PAL tobacco plants (Maher et al., 1994) were challenged with TMV, the kinetics of local lesion formation, and their final number and dimensions, were unchanged, even though the lesion center now appeared white and not brown due to the absence of oxidized phenolics (Pallas et al., 1996). Thus, it appears unlikely that products of the phenylpropanoid pathway (other than SA) are involved in restricting viral spread. Earlier cytological studies had also revealed that TMV particles could be found

in healthy cells surrounding the necrotic lesion, even when lesion expansion had ceased (Da Graça and Martin, 1976).

Only 3 to 4 hr at the permissive temperature is necessary to establish irreversibly *N*-triggered local lesion formation (Dunigan and Madlener, 1995). Thus, all the temperature-sensitive signaling mechanisms required for resistance are completed at least 5 hr before the visible HR. Dunigan and Madlener (1995) have demonstrated that the inhibition of serine/threonine protein dephosphorylation 1 hr before the temperature shift leads to an 80% reduction in local lesion formation. Thus, reversible protein dephosphorylation appears to be a crucial part of the *N*-mediated signaling cascade.

If the temperature shift from 30° to 20°C is delayed for >4 days after TMV inoculation, the oldest infected areas remain green and a necrotic ring forms around each green island (Da Graça and Martin, 1976). In the central green island, viral synthesis has ceased, whereas at the margins, viral synthesis is still under way. Thus, the *N*-mediated HR appears to require active viral synthesis and not merely the presence of virus in the cell, consistent with the viral replicase being the Avr determinant (Padgett and Beachy, 1993). Alternatively, the infected host cells could be compromised by viral pathogenesis and incapable of responding to the viral signal.

Tomato *Cf* Gene-Mediated Resistance to *C. fulvum*

The tomato *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* genes confer resistance to distinct races of *C. fulvum* that express the corresponding avirulence genes *Avr2*, *Avr4*, *Avr5*, and *Avr9*, respectively (Hammond-Kosack and Jones, 1994). These four *Cf* genes have been cloned, and each DNA sequence predicts a predominantly extracytoplasmic glycoprotein, with numerous LRRs and a short C-terminal membrane anchor (Jones et al., 1994; Dixon et al., 1996; J.D.G. Jones, M. Dixon, and C. Thomas, unpublished data). *C. fulvum Avr* gene products are small proteins of <15 kD that are secreted from fungal cells. *C. fulvum* hyphal growth is exclusively extracellular, so these proteins can be obtained from infected susceptible leaves (De Wit, 1992). Each *Cf–Avr* gene combination results in the arrest of hyphal growth at a distinct stage of colonization, either within the substomatal cavity or in the adjacent mesophyll cell layers (Figure 1l; Hammond-Kosack and Jones, 1994). The availability of Avr elicitors to activate the defense responses synchronously has proven invaluable in the elucidation of the events required for *Cf–Avr* gene–mediated resistance. The activation of plant defense responses has been studied in intact leaf and cotyledon tissue, in cell suspension cultures, and in isolated membrane preparations.

Using an in vivo cotyledon assay in which 14-day-old Cf-0, Cf-2, and Cf-9 tomato seedlings were infiltrated with an elicitor preparation containing both *Avr9* and *Avr2* gene products, the chronology of *Cf–Avr* gene–dependent defense responses has been determined (Hammond-Kosack et al., 1996; May et al., 1996). Extracellular superoxide formation (determined by nitroblue tetrazolium staining and SOD inhibition), lipid peroxidation, and increases in the levels of both total and oxidized glutathione (GSH and GSSH) occurred within 2 to 4 hr in both *Cf*-expressing genotypes. However, the supraoptimal opening of stomata that commenced at 3 to 4 hr after Avr injection was specific to the *Cf-9* genotype. Later (6 hr onward), *Cf–Avr* gene–dependent induced events included increased LOX enzyme activity, ethylene and SA biosynthesis, the accumulation of various PR gene transcripts, and eventually host cell death. Overall, the different Cf proteins encoded by genetically unlinked loci activate very similar host responses, and surprisingly, these responses also appear to be very similar to those activated by the N protein in tobacco.

Cf-5–expressing cell cultures challenged with an elicitor preparation that contained Avr5 showed a rapid increase, within 10 mins, in the extracellular production of $O_2^{\cdot -}$ and H_2O_2 (Vera-Estrella et al., 1992). These increases were accompanied by the concurrent acidification of the extracellular medium, which was caused by an increase in plasma membrane H^+-ATPase activity (Vera-Estrella et al., 1994a). Slightly later (1 to 3 hr onward), specifically induced events included increased lipid peroxidation, elevated extracellular peroxidase activity, and the accumulation of extracellular phenolics. Cell death was not induced. Enriched plasma membrane fractions derived from these *Cf-5*–expressing cells also responded specifically to the Avr5 elicitor. Within 20 min, considerable increases in the activity of NADH oxidase and cytochrome *C* reductase, but an inhibition of ascorbate peroxidase activity, were evident. Inhibitor experiments indicated that both protein phosphatases and GTP binding proteins must participate in this defense signal transduction cascade (Vera-Estrella et al., 1994b). However, one serious reservation about the relevance of these data to *Cf-5–Avr5* gene–mediated resistance is the lack of information on the response of a tomato cell line that does not express *Cf-5* to the identical elicitor preparation.

Using ^{125}I-labeled Avr9 peptide, specific, saturable, and reversible binding to plasma membranes isolated from both Cf-0 and Cf-9 leaves has been revealed. Unexpectedly, the same extremely low dissociation constant of 0.07 nM, and a receptor concentration of 0.8 pmol per mg of microsomal protein, was found for both genotypes. Kooman-Gersmann et al. (1996) suggest that the initial plant receptor for the 28–amino acid Avr9 signal is a Cf-9 protein homolog. However, such a conclusion should be considered with some caution until rigorously tested by using additional experimental approaches. Because *Avr* gene products presumably have a function in fungal pathogenesis (see Knogge, 1996, in this issue), the receptor in the binding experiments could be the compatibility target rather than the one specifying incompatibility.

To determine which *Cf–Avr* gene–mediated defense responses are causally involved in *C. fulvum* resistance, several molecular genetic approaches have been taken. For example,

Cf-2– and *Cf-9*–containing tomato plants expressing a *35S:nahG* transgene that almost completely eliminates SA accumulation were found to be as resistant to *C. fulvum* as sibling plants that had not inherited the *nahG* transgene. In contrast, $Tm2^2$-mediated resistance to TMV was compromised in the *35S:nahG*-expressing tomato lines (P. Brading, K.E. Hammond-Kosack, and J.D.G. Jones, unpublished data). These observations suggest that SA is not required for *Cf*-mediated resistance.

An alternative approach was to mutagenize homozygous *Cf*-containing lines before screening with *C. fulvum* to identify mutant plants with reduced resistance. One mutant locus that results in full disease susceptibility and three other loci that cause a partial loss of resistance, all nonallelic to known *Cf* genes, have been identified (Hammond-Kosack et al., 1994; M. Dixon, K.E. Hammond-Kosack, and J.D.G. Jones, unpublished data). These *Rcr* loci (required for *Cladosporium* resistance; Table 1) should help to define whether individual Cf proteins confer resistance via common or distinct mechanisms, and when cloned, to identify proteins that are needed by Cf proteins to arrest the pathogen.

Arabidopsis *R* Gene-Mediated Resistance to *P. syringae*

P. syringae pv *maculicola* strains expressing *avrRpt2* are avirulent on Arabidopsis ecotypes possessing *RPS2*, whereas strains expressing either *avrRpm1* or *avrB* are avirulent on *RPM1*-containing Arabidopsis genotypes. Both genes appear to encode cytoplasmically localized proteins that contain an N-terminal leucine zipper domain, an NBS, and an LRR region (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Bent, 1996, in this issue). The molecular nature of the corresponding bacterial *Avr*-derived ligand is still unclear but could be the *Avr* gene product itself (Gopalan et al., 1996).

The gene combinations *avrRpm1–RPM1* and *avrB–RPM1* condition a visible HR within 5 hr of bacterial inoculation, whereas the HR conferred during the *avrRpt2–RPS2* gene–mediated interaction is not evident for 16 hr. Because formation of the later HR is epistatic over the earlier HR, Ritter and Dangl (1996) suggested that some initial steps in the signal transduction pathway are triggered by both *R* gene products. Similar arrays of defense-related genes are specifically induced in both of the incompatible interactions. Curiously, however, the activation of several genes of unknown function is solely associated with either *RPM1*-mediated resistance (e.g., *Eli3*; Ritter and Dangl, 1996) or *RPS2*-mediated resistance (*PR1*, *AIG1*, and *AIG2*, for avrRpt2-induced gene; Reuber and Ausubel, 1996). Whether these differentially induced genes are causally required for bacterial resistance is not known. However, it is clear that certain aspects of disease resistance mediated by *RPS2* and *RPM1* must rely on a common theme because the Arabidopsis *ndr1* mutation affects resistance conferred by both *R* genes, as well as several *RPP* genes that confer resistance to *Peronospora parasitica* (Century et al., 1995; Table

Table 1. *RDR* Loci—Required for Disease Resistance

Plant Species	*RDR* Locus	Resistance Affected	Pathogen	Function Loss	*RDR* Location	References
Tomato	*Rcr-1, Rcr-2, Rcr-3, Rcr-5*	*Cf-9/Cf-2*	*Cladosporium fulvum*	Partial or complete	Unlinked and unknown	Hammond-Kosack et al. (1994); M. Dixon, K.E. Hammond-Kosack, and J.D.G. Jones (unpublished data)
	Prf	*Pto/Fen*	*Pseudomonas syringae* pv *tomato*	Complete	Tightly linked to *Pto/Fen*	Salmeron et al. (1996)
Barley	*Rar1, Rar2*	*Mla-12*	*Erisyphe graminis* f sp *hordei*	Almost complete	*Rar1* unlinked and *Rar2* linked to *Mla-12*	Freialdenhoven et al. (1994, 1996)
	Ror1, Ror2	*mlo*			*Ror1* and *Ror2* unlinked to *mlo*	
Arabidopsis	*Ndr1*	*RPM1*	*P. syringae (avrB)*	Complete	Unlinked	Century et al. (1995)
		RPS2	*P. syringae (avrRpt2)*	Complete		
		RPPs	*Peronospora parasitica*	Complete		
	nim1	*RPPs*	*P. parasitica*	Complete	Unlinked	Delaney et al. (1995)
	Eds1	*RPPs*	*P. parasitica*	Complete	Unknown	Parker et al. (1996)

1). Also, the *nahG* transgene compromises both resistances (Delaney et al., 1994). Two negative results are also informative: First, Arabidopsis *npr1-1* mutants, which are unable to express PR1, still confer *RPS2*-mediated resistance, even though the SAR response is compromised and the plants are more susceptible to virulent isolates (Cao et al., 1994). Second, Bent et al. (1992) showed that resistance conferred by both *RPS2* and *RPM1* was not compromised by the ethylene-insensitive mutations *ein1* and *etr1*, demonstrating that ethylene signaling is not a prerequisite for incompatibility.

Barley *Mla*, *Mlg*, and *mlo* Gene-Mediated Resistance to the Powdery Mildew Fungus *Erisyphe graminis* f sp *hordei*

E. graminis f sp *hordei* is an obligate biotroph that exclusively attacks epidermal leaf tissue of barley plants. We concentrate here on race-specific resistance conferred by alleles of the *Mla* and *Mlg* loci and compare these with the defense response mediated by the non-race-specific recessive *mlo* gene (reviewed in Jorgensen, 1994; see also Knogge, 1996, in this issue). Nothing is known about the nature of the pathogen avirulence determinants or the *Mla* or *Mlg* gene sequences.

Quantitative cytological investigations indicate that putative *R* gene–mediated host cell defense responses can occur at several distinct stages of fungal ontogeny, depending on the *R* locus involved. *Mla* allele–mediated resistance involves predominantly the HR of cells containing haustoria, and the induction of numerous defense-related genes (Freialdenhoven et al., 1994; Boyd et al., 1995). *Mlg* allele–mediated resistance acts earlier and involves papillae formation (Figure 1C); the HR occurs too late to be considered causal to fungal arrest (Gorg et al., 1993). *mlo* allele–mediated resistance operates as direct fungal penetration of epidermal cells is attempted. Arrest in papillae predominates (>99.5%), and only rarely does the HR occur (Wolters et al., 1993). *mlo* plants also spontaneously form epidermal papillae in axenic culture. This suggests that defense responses in *mlo* plants may be heightened before fungal attack.

Mutagenesis of *Mla* and *mlo* homozygotes has led to the identification of several other barley loci required for resistance gene function. Mutations at the *Rar1* and *Rar2* loci (originally named *Nar1* and *Nar2*) significantly compromised *Mla*-mediated resistance and caused reduced HR formation and defense-gene induction (Freialdenhoven et al., 1994). However, mutant *rar* alleles do not affect *Mlg*-mediated resistance (J. Jorgensen and P. Schulze-Lefert, unpublished data). Two other barley loci, *Ror1* and *Ror2* (Freialdenhoven et al., 1996), abolish resistance and spontaneous papillae formation mediated by several *mlo* alleles. Interestingly, mutations at neither *ror* locus affect resistance mediated by several alleles of the *Mla* and *Mlg* loci (P. Schulze-Lefert, unpublished data). Thus, in barley it appears that resistance conferred by different *R* genes that map to distinct genetic locations operates by dissimilar mechanisms. In some but not all cases, the barley loci required for disease resistance (*rdr*) are linked to the corresponding *R* gene (Table 1).

SIGNALING MECHANISMS

An overview of the complexity of signal transduction pathways required to activate and coordinate plant defense responses is given in Figure 3. It is envisaged that R proteins act as receptors to detect the microbial Avr-dependent signal and thus initiate downstream signaling. Alternatively, Avr signal recognition may involve another protein(s), with R protein function residing either at an early rate-limiting step in the signal transduction cascade or at a point of potential cross-talk between distinct signaling pathways. Identification of the exact cellular locations of the different classes of R proteins in both healthy and challenged plants is eagerly awaited. For non-host-induced resistance, similar roles for key proteins are likely (Boller, 1995; Nürnberger et al., 1995; Mithöfer et al., 1996), but the initial triggering events may be different.

Immediately downstream of pathogen perception, the activation of preexisting protein kinases, phosphatases, and G proteins are the most likely next steps (Figure 3; Staskawicz et al., 1995; see also Bent, 1996, in this issue). A second protein kinase, Pti, has been shown to interact with the tomato Pto protein, thus implicating a protein kinase cascade in R protein action (Zhou et al., 1995). Other rapidly induced events that have been detected include protein phosphorylation/dephosphorylation, changes in Ca^{2+} concentration, ion fluxes, increased inositol triphosphate and diacylglycerol levels, and alterations to the ratio of proteins with bound GTP or GDP (Dixon et al., 1994; Low and Merida, 1995; Ward et al., 1995).

Is the purpose of these initial signaling events to activate de novo gene expression? Or do they upregulate preexisting defense-related biosynthetic enzymes? Or lead to the release of stored precursors of defense compounds? These questions are unresolved, and the answers probably vary from interaction to interaction. The extremely rapid induction of the oxidative burst and/or ethylene biosynthesis (Baker and Orlandi, 1995; Boller, 1995) suggests that gene induction is not required for these responses. The cross-linking of cell wall proteins and callose deposition also do not appear to involve gene activation. In contrast, rapid increases in PAL and CHS activities correlate well with gene activation (Logemann et al., 1995a). Elevated SA levels probably occur by increasing preexisting BA2-H activity to convert stored BA to SA and/or by de novo PAL and BA2-H protein synthesis (Léon et al., 1995; Mauch-Mani and Slusarenko, 1996).

Once the earliest defense responses have been activated, the complexity of the biochemical pathways within the responding cell is likely to increase enormously as new signal molecules are generated (Figure 3). This hierarchy of signaling events probably provides the overall framework to induce coordinately

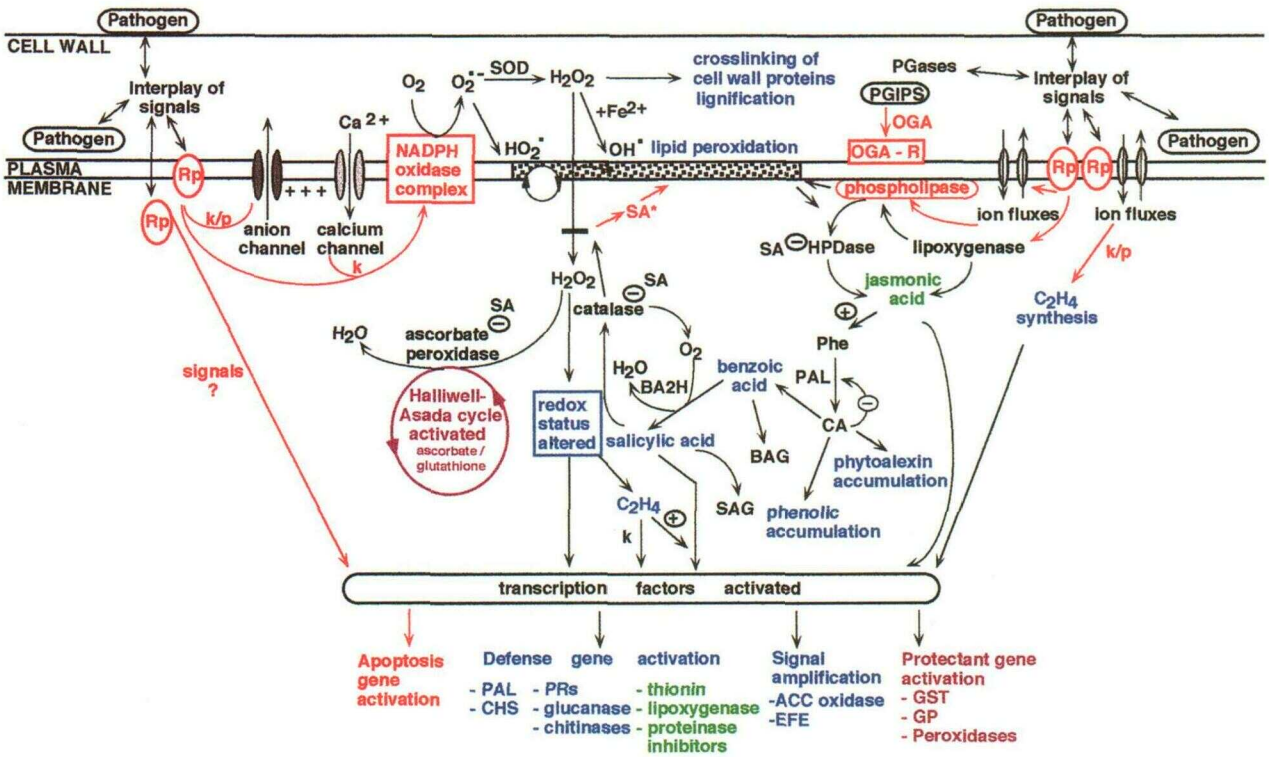


Figure 3. Complexity of Signaling Events Controlling Activation of Defense Responses.

Plant receptor proteins (Rp) intercept pathogen-derived or interaction-dependent signals. These signals include the direct or indirect products of *Avr* genes, physical contact, and general components of each organism, such as chitin, enzymes, and plant cell wall fragments. Plant receptor proteins may or may not be the products of *R* genes. The immediate downstream signaling events are not known but involve kinases, phosphatases, G proteins, and ion fluxes. Several distinct and rapidly activated outcomes are recognized, including the production of ROS, direct induction of defense gene transcription, or possibly apoptosis genes, JA biosynthesis, and/or ethylene biosynthesis. Amplification of the initial defense response occurs through the generation of additional signal molecules, that is, other ROS, lipid peroxides, BA, and SA. These, in turn, induce other defense-related genes and modify defense proteins and enzymes. Concomitant alterations to cellular redox status and/or cellular damage will activate preformed cell protection mechanisms (that is, the Halliwell–Asada cycle, plastid-localized SODs, and catalases) and induce genes encoding various cell protectants. Defense-related stress may also induce cell death. Cross-talk between the various induced pathways will coordinate the responses. (+) indicates positive and (−) indicates negative interactions. Components and arrows indicated in red are only postulated to be present in plant cells, whereas those in blue indicate known plant defense responses; green indicates plant defense responses also activated by JA, and purple indicates plant protection mechanisms. ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; BAG, benzoic acid glucoside; BA2H, benzoic acid-2 hydroxylase; CA, cinnamic acid; CHS, chalcone synthase; EFE, ethylene-forming enzyme; $HO_2^{\cdot}$, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; GP, glutathione peroxidase; GST, glutathione *S*-transferase; k, kinase; $O_2^{\cdot -}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; OGA and OGA-R, oligalacturonide fragments and receptor; p, phosphatase; PAL, phenylalanine ammonia-lyase; PGases, polygalacturonases; PGIPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA and SAG, salicylic acid and salicylic acid glucoside; SA•, SA radical; and SOD, superoxide dismutase.

the diverse array of defense responses in the various cellular compartments. Considerable amplification of specific defense responses then occurs, via either positive feedback or signal cross-talk. The induction of various housekeeping genes is also likely to accompany the defense response to ensure that adequate pools of precursor compounds are maintained (Kawalleck et al., 1992). Furthermore, in young plant tissues, histone and cell-cycle-regulated gene expression may be repressed either to redirect all available cellular resources to defense-related metabolism (Logemann et al., 1995b) or to preclude cell death.

At some stage in the incompatible interaction, damage is inflicted on both the responding host cell and the pathogen. As a result, the formation of additional signal molecules occurs at the host–pathogen interface, probably in a less controlled manner. The particular microbial species and its mode of pathogenesis are likely to influence the diversity of second-generation elicitors that are produced. These new signals could include, for example, chitin fragments, lipid peroxides, arachidonic acid, cell wall oligosaccharide fragments, and a localized change in cellular redox state (Figure 3; Farmer and Ryan, 1992; Baker and Orlandi, 1995; Boller,

1995). As a consequence, a second wave of signal perception and transduction events could occur that could activate additional defense responses, amplify/repress the original response, or induce cell death.

The activation of specific cellular protection mechanisms is likely to accompany the defense response. These mechanisms include upregulation of the cytoplasmic Halliwell–Asada cycle that minimizes the consequences of oxidative stress (Figure 3). Furthermore, increased transcription of specific SOD and catalase genes may occur to ensure that maximal enzymatic activity is maintained within the appropriate cellular compartments (Bowler et al., 1994). For example, the expression of glutathione peroxidase, glutathione *S*-transferase, and polyubiquitin genes has been detected in incompatible interactions (Mauch and Dudler, 1993; Hahn and Strittmatter, 1994; Levine et al., 1994). Glutathione peroxidase activity can block cell death in mammalian systems (Hockenbery et al., 1993), whereas glutathione *S*-transferase detoxifies the products of lipid membrane peroxidation and other products of cellular oxidative stress (Berhane et al., 1994). Polyubiquitin is required for the recycling of damaged proteins; tobacco plants that overexpressed a mutant form spontaneously developed necrotic lesions (Becker et al., 1993). BA, SA, and other phenolics may act as free radical scavengers that protect cells from oxidative toxicity (Léon et al., 1995). Thus, mutations in genes conditioning the signal pathways for the activation of cellular protection genes could account for the phenotype of uncontrolled spreading of lesions in response to avirulent pathogens that is typical of some disease lesion mimics (see Dangl et al., 1996, in this issue).

Overall, precise temporal and spatial coordination of induced defense responses is required to successfully kill or contain the invading microbe while simultaneously minimizing the damage to host tissue. In the initially attacked cell(s), rapid responses may ultimately lead to cell death, whereas in the surrounding cells, induced defense may be more transcription dependent. The magnitude and type of signals perceived by neighboring cells depend on the relative rates of signal production, diffusion, and reactivity toward macromolecules. Also, as plasmodesmata become plugged with callose, as cellular protection mechanisms become less overloaded, and as cell wall architecture becomes modified by the cross-linking of cell wall proteins and lignification events, both symplastic and apoplastic routes for signal molecules become blocked. This could result in the progressive shutting down of defense signaling pathways after the invading microbe has been successfully contained.

FUNCTIONAL ASSESSMENT OF DEFENSE AND SIGNALING MECHANISMS

What are the responses correlated with *R–Avr* gene–mediated recognition that actually stop the pathogen from growing? To ascribe a function to each induced defense response, two complementary molecular genetic approaches can be undertaken. First, targeted loss of function can be achieved by constitutive antisense suppression, cosuppression, or transposon tagging to eliminate one or all members of a particular defense gene family. Alternatively, the targeted loss of individual signal molecules, through the same experimental approaches, may reduce fluxes through specific signaling cascades and simultaneously eliminate a subset of defense responses. However, because of the inherent redundancy of many defense responses, probably only partially impaired resistance will be obtained by either route.

Nontargeted mutagenesis of *R* gene–expressing lines, followed by screening for disease-sensitive mutants, is already proving useful in the identification of other genes required for disease resistance (Table 1). Such mutants provide excellent tools to dissect genetically the contribution of various defense responses and signaling pathways to resistance. In this context, mutations found to affect potentially related biological processes, such as cold stress and sensitivity to pathogen toxins, should also be tested for their ability to compromise resistance. Re-mutagenesis of mutants to look for restoration of resistance, that is, gain of function, may identify negative regulators in defense response pathways. Alternatively, defense gene promoters could be fused to various reporter genes, such as those encoding β-glucuronidase (GUS), green fluorescent protein (GFP), or luciferase (LUX). Homozygous transgenic plants could be mutagenized and screened to identify mutations conditioning nonresponsiveness (i.e., no reporter gene expression) after microbial or elicitor challenge. Subsequent testing will ascertain whether such mutants are also compromised in disease resistance.

To identify the signaling networks required for defense activation, either interaction cloning (Phizicky and Fields, 1995) or the molecular tagging of *R* and/or *Avr* gene products should identify the immediate downstream interacting proteins. The more tortuous approach is to work backward in the defense response by identifying transcription factors that bind to specific promoter domains or enzymes that activate particular protein complexes. The increasing complexity and decreasing specificity of the responses activated as incompatibility proceeds (Figure 3) suggest that selecting even relatively rapidly induced responses as a starting point for the second strategy is unlikely to yield results directly relevant to *R* protein action.

Simplification of the experimental system should in theory help to identify key induced responses. Using defined elicitors or synchronizing the microbial infection has already yielded good temporal resolution of the more rapidly induced events as well as potential targets for gene knockout experiments. Marking the microbe with GUS, GFP, and LUX allows both spatial localization and pathogen biomass quantification. This permits the chronology of host-induced responses to be superimposed on microbial ontogeny, which will indicate whether a specific defense response is activated in an appropriate location and before the divergence in growth rates of virulent and avirulent isolates. Plant cell cultures are frequently used to simplify the experimental system still further. However, due to the distinct morphology of cultured cells, which could cause

differences in oxygen concentration and nutrient status, and the potential for a much greater rate of signal diffusion than in an intact system, wherever possible the results obtained should be verified using intact plant tissue.

CONCLUSIONS

Exciting times lie ahead now that several *R* genes have been cloned and other *rdr* loci have been identified (Table 1). Obviously, there is considerable conservation of defense signaling mechanisms between plant species because several different classes of *R* genes also confer resistance when expressed in heterologous plant species. Examples include the tobacco *N* gene in tomato (Whitham et al., 1996), tomato *Pto* gene in tobacco (Rommens et al., 1995; Thilmony et al., 1995), and tomato *Cf* genes in tobacco and potato (K.E. Hammond-Kosack and J.D.G. Jones, unpublished data). But do different R proteins activate distinct resistance mechanisms? On the whole, the evidence provided by the case studies discussed above suggests the contrary. These data demonstrate that there may be a rapid convergence of the initially activated *Avr–R* gene–dependent signaling events into one or a few common pathways that coordinate the overall defense response. Whether these same pathways are also activated by nonspecific elicitors is not known. Either way, the identification and characterization of additional mutants affecting different classes of *R* gene– mediated and nonhost resistance would help to answer this question.

Pathogen growth is restricted even in genetically compatible interactions. A proportion of infections always abort (Ashfield et al., 1994), and the colonized/sporulating area is usually delimited within a lesion, pustule, or canker (Agrios, 1988). However, some microbes such as *Phytophthora* species, bacterial fire blight, and citrus tristeza virus are plant destroyers. Why? By screening for Arabidopsis mutants with increased disease symptoms to virulent *P. syringae* bacteria, Glazebrook et al. (1996) have identified 10 *eds* loci that cause enhanced disease susceptibility. Three of the mutant loci are identical to those that compromise particular aspects of *R* gene–mediated defense responses (i.e., two *pad* loci involved in phytoalexin biosynthesis, and *npr1*, which is possibly allelic to *nim1*, which compromises PR1 gene expression and the SAR response [Cao et al., 1994; Delaney et al., 1995]). Thus, some defense mechanisms appear to restrict bacterial growth in both incompatible and compatible interactions. In plant–symbiotic interactions, for example, with *Rhizobium* or mycorrhizal species, many of the known defense responses are activated (Gardner et al., 1996; see also Gianinazzi-Pearson, 1996, and Pawlowski and Bisseling, 1996, in this issue). Is defense-gene activation in these compatible interactions of functional significance for pathogenesis or symbiosis or does it stop the entry of unwanted competitors? Probably both functions are important.

R–Avr gene–mediated resistance is a cell-autonomous trait (Bennetzen et al., 1988) in which the hallmark of successful pathogen containment is rapid pathogen perception leading to the coordinate induction of a diverse array of defense mechanisms both within the initially attacked cell as well as in the surrounding cells. But even this resistance is really a combination of induced responses superimposed on preformed defenses. Most individual defense responses appear to be additive in their effect on resistance, and the most effective engineered resistance is often observed when heterologous gene expression results in the pathogen encountering a novel response (Hain et al., 1993). But the importance of several induced responses associated with incompatibility remains enigmatic. For example, why does the plant nucleus rapidly move toward the site of pathogen entry into a cell (Gross et al., 1993; Freytag et al., 1994)? Why is there induction of plant retrotransposon mRNAs (Pouteau et al., 1994; Moreau-Mhiri et al., 1996)? Why is cell division or leaf abscission, to discard the infection, only sometimes provoked (Samuel, 1927)? As we strive to increase our understanding of the mechanisms underlying incompatible plant–pathogen interactions, we will also learn much about normal plant growth and development.

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