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The Hypersensitive Response in Plant Disease Resistance

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5.1 Hypersensitive Response: The Phenomenon

5.1.1 Physical Properties of the Hypersensitive Response

Plants can recognize certain pathogens and activate defenses (called the resistance response) that result in the limitation of pathogen growth at the site of infection. One dramatic hallmark of the resistance response is the induction of rapid and localized cell death, a reaction known as the hypersensitive response (HR), when plants are challenged with an incompatible pathogen. HR cell death is also manifested as a collapse of the infected tissue (see Fig. 5.1) and is considered to be involved in pathogen resistance by creating a physical barrier that may impede proliferation and spread of some pathogens (Goodman and Novacky, 1994; Alfano and Collmer, 1996). Furthermore, the HR is important for limiting the nutrient supply of some pathogens, since the dying tissue rapidly becomes dehydrated. Thus, the antimicrobial defense of plant cells is thought to involve the activation of a suicide pathway in infected cells.

Programmed cell death (PCD) is one of the key mechanisms controlling cell proliferation, generation of developmental patterns, and defense of animals against pathogens and environmental insults (Schwartzman and Cidlowski, 1993). One of the most widely studied forms of PCD is apoptosis, a type of PCD that displays a distinct set of physiological and morphological features (Martin et al., 1994). Morphological hallmarks of apoptosis include the condensation of chromatin at the nuclear periphery, the condensation and vacuolization of the cytoplasm and blebbing of the plasma membrane. Despite these cellular changes, the mitochondria remain relatively stable. These changes are followed by breakdown of the nucleus and fragmentation of the cell to form apoptotic bodies (Schwartzman and Cidlowski, 1993). Among the many biochemical changes commonly found in cells undergoing apoptosis is the systematic fragmentation and degradation of nuclear DNA (Bortner et al., 1995). Large fragments of 300 kb and/or 50 kb are first produced by endonucleolytic degradation of nuclear DNA (Oberhammer et al., 1993). These are further degraded by cleavage at linker DNA sites between nucleosomes resulting in DNA fragments that are multimers of about 180 bp

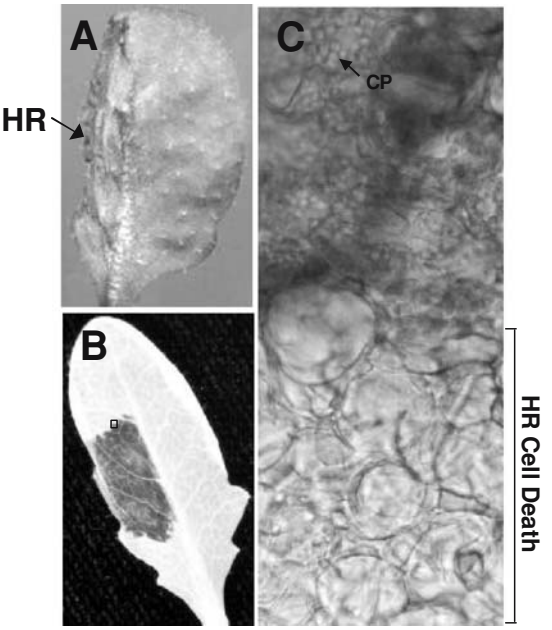

FIGURE 5.1. Morphological observation of collapsed cells of *Arabidopsis* leaf after induction of HR cell death. **(a)** Fully expanded leaf of 4- to 5-week-old *Arabidopsis* plants (ecotype Colombia; Col-0) was infiltrated with *Pseudomonas syringae* pv *maculicola* strain that contains the *avrRpt2* gene (*P.s.m.* ES4326/*avrRpt2*; 10^5 CFU/cm^2) and induced an HR. The leaf was photographed at 20 hour-post-infiltration. **(b)** The leaf was sampled 24 hour-post-infiltration and was fixed in 10% formaldehyde-5% acetic acid-50% ethanol for 3 hours, dehydrated through a graded ethanol series (50, 75, and 100% for 20 min at each step), and incubated in 100% ethanol at 4°C for 3 hours. After rehydrating in water, the collapsed cells were observed by light microscopy. **(c)** shows the higher magnification view of the area which is shown as a red frame in **(b)**. CP, chloroplast.

(Wyllie et al., 1984). Degradation of nuclear DNA during apoptosis is coordinated with activation of specific endonucleases that are thought to mediate chromatin cleavage (Peitsch et al., 1993). Cells undergoing HR cell death have some of the features that characterize apoptosis, including condensation and vacuolization of the cytoplasm, blebbing of the plasma membrane, stable mitochondria, and cell shrinkage (Roebuck et al., 1978; Levine et al., 1996; Mittler et al., 1997b; Che et al., 1999; Kawasaki et al., 1999). Moreover, the biochemical events involved in apoptosis, such as activation of specific endonucleases and DNA fragmentation, are also found in plant PCD (Mittler and Lam, 1995; Ryerson and Heath, 1996; Wang et al., 1996b; Mittler and Lam, 1997; Mittler et al., 1997a,b; Sugiyama et al., 2000). These observations indicate that some mechanisms of cell death activation may be conserved between animals and plants.

It is believed that the HR constitutes one of the mechanisms of resistance to plant pathogens. Induction of HR is often associated with elevated levels of salicylic

acid (SA), a key regulator of defense responses and pathogen resistance, synthesis of pathogenesis-related (PR) proteins that exhibit antimicrobial activity such as glucanases and chitinases, thickening and hardening of cell walls, and production of antimicrobial compounds called phytoalexins (Hammond-Kosack and Jones, 1996; Ryals et al., 1996). Furthermore, recognition of an incompatible pathogen triggers the rapid production of reactive oxygen intermediates (ROI) superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in an oxidative burst (Lamb and Dixion, 1997). ROI, in turn, drive crosslinking of the cell wall (Bradley et al., 1992), induce several plant genes involved in cellular protection and defense (Chen et al., 1993; Levine et al., 1994; Jabs et al., 1996), and are necessary for the initiation of host cell death in HR (Lamb and Dixion, 1997). However, these signaling intermediates may not be sufficient to activate cell death on their own. Evidence for involvement of nitric oxide (NO) in the activation of HR cell death has recently been reported (Delledonne et al., 1998; Durner et al., 1998; Clark et al., 2000). It was also suggested that SA, which accumulates during the HR, is involved in the production of ROI (Chen et al., 1993; Chamnongpol et al., 1996; Durner and Klessig, 1996; Takahashi et al., 1997). Thus, multiple secondary signals, such as ROIs, SA, and NO, appear to be essential second messengers for the activation and execution of HR cell death. As a result of the multiple biochemical events during the induction of the HR, growth of the pathogen is restricted.

5.1.2 Genetics of Host-Microbe Signaling

The resistance of some plants to infection by certain pathogens reflects the presence of disease resistance (*R*) genes, which are predicted to encode receptors for pathogen-derived molecules (see in recent review: Shirasu and Schulze-Lefert, 2000; Dangl and Jones, 2001). A single gene in the host (the *R* gene) confers resistance only to those pathogen isolates containing a corresponding *Avr* gene (Flor, 1971). This "gene-for-gene" type of resistance is generally interpreted by an elicitor-receptor model: the plant R proteins recognize directly or indirectly particular Avr proteins produced by different pathogen strains. Most *R* gene-triggered resistance appears to be associated with HR cell death. In other cases, the plant, although infected, may outgrow the pathogen long enough to complete its life cycle. Figure 5.2 shows a typical example of the gene-for-gene system in *Arabidopsis*: the compatible/incompatible interactions between *Pseudomonas syringae* and *Arabidopsis thaliana*. *Arabidopsis* plants of the ecotype Columbia (Col-0) contain the resistance (*R*) gene *Rps2* (Bent et al., 1994; Mindrinos et al., 1994). These plants, but not *rps2* plants, can recognize *Pseudomonas* strains that contain the *Avr* gene *avrRpt2* and mount an HR. However, they are unable to recognize a *Pseudomonas* strain that does not contain the *avrRpt2* gene and are therefore unable to mount a defense response in the form of an HR.

Activation of the HR triggers a systemic resistance response known as systemic acquired resistance (SAR). This response includes the accumulation of the signal molecule salicylic acid (SA) throughout the plant and the expression of a characteristic set of defense gene, including PR genes (Malamy et al., 1990; Métraux

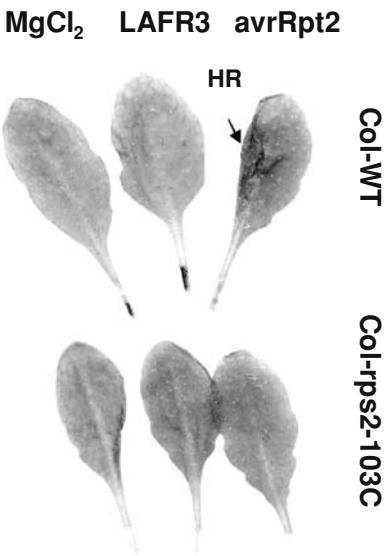

FIGURE 5.2. Illustration of "gene-for-gene" interaction: activation of HR cell death via the *Rps2*/*AvrRpt2* pathway. Fully expanded leaves of 4- to 5-week-old *Arabidopsis* plants (Col-0) were infiltrated with *Pseudomonas syringae* pv *maculicola* strain ES4326 (*P.s.m.*; 10^5 CFU/cm^2) that does not induce an HR, *P.s.m.* ES4326/*avrRpt2* (10^5 CFU/cm^2) which induces an HR, or mock infected with 10 mM $MgCl_2$ as described by Greenburg et al. (1994) or Mittler et al. (1997a). Leaves of 4- to 5-week-old *Arabidopsis rps2-103C* plants were infiltrated with *P.s.m.* and *P.s.m.* ES4326/*avrRpt2*, or mock infected with 10 mM $MgCl_2$. These leaves were sampled and photographed at 20 hour-post-infiltration.

et al., 1990; Gaffney et al., 1993) [salicylate-mediated induced systemic resistance is also called ISR in some literature]. Plants expressing SAR are more resistant to subsequent attacks by a variety of otherwise unrelated virulent pathogens (Ryals et al., 1996). Many defense responses that are characteristic of SAR also contribute to local resistance that is mediated by *R* genes, and to the local growth limitation of moderately virulent pathogens. In addition to SA, the *NPR1/NIM1* gene product is a key mediator of SAR as well as gene-for-gene disease resistance (Dong, 2001). The SA signal is transduced through NPR1/NIM1, a nuclear-localized protein that interacts with TGA transcription factors, which may be involved in SA-mediated gene expression (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000b).

The past decade has seen the isolation of various *R* genes from different plant species that can specify resistance to viruses, bacteria, fungi, nematodes and insects. An important observation from sequence alignment of the encoded proteins is a modular R protein structure. Despite a wide range of pathogen taxa and their presumed pathogenecity effector molecules, *R* genes encode only several classes of structurally related proteins (see reviews: Shirasu and Schulze-Lefert, 2000; Dangl and Jones, 2001). These include the *Cf-X* class of tomato R proteins (Cf-2, Cf-4, Cf-5, Cf-9), containing extracellular leucine-rich repeats (LRRs), a

single-span transmembrane domain, and a short cytoplasmic region with no known homologies; Xa21 and FLS2, transmembrane proteins containing extracellular LRRs and a cytoplasmic serine-threonine kinase domain; and Pto, a cytoplasmic soluble serine-threonine kinase. More recently, two genes containing novel structures for a disease resistance gene were cloned. One gene is tomato *Ve*, which encodes a putative cell surface-like receptor that has N-terminal LRR domain containing 28 or 35 potential glycosylation sites, a hydrophobic membrane-spanning domain, and a C-terminal endocytosis-like signal sequence (Kuwchuk et al., 2001). The other gene is barley *Rpg1*, which encodes a receptor-kinase that contains an N-terminal domain that does not resemble any previously described receptor and two tandem protein kinase domains (Brueggeman et al., 2002). Aside from these noted exceptions, the majority of cloned R genes contain nucleotide-binding site (NBS) and LRR motifs. The *Arabidopsis* genome sequence annotation predicted that ~150 genes with homology to the NBS-LRR class of *R* genes exist in this species alone (The *Arabidopsis* Genome Initiative, 2000). Proteins containing LRR motif are thought to be involved in protein–protein interactions, and the specificity of these interactions is likely to be determined by the composition of the variable amino acids in the consensus core of the LRRs (Kobe and Deisenhofer, 1995). In addition, the NBS motif is thought to be critical for ATP or GTP binding, although to date there is no direct biochemical evidence for the postulated nucleotide binding activity via this domain of R proteins. The NBS-LRR class of R proteins can be divided into two subclasses based on the conserved N-terminal motif. One subclass has a coiled-coil domain (CC) that consists of a putative leucine zipper motif: this CC-NBS-LRR subclass includes *Arabidopsis RPM1, RPS2, RPP8*, *RPS5*, tomato *Prf* and *Mi*, and potato *Rx1*. The other subclass contains an N-terminal domain that has significant homology with the Toll/interleukin receptor domain (TIR): this TIR-NBS-LRR subclass includes the tobacco *N*, flax L_6 and *M*, and *Arabidopsis RPS4, RPP5*, and *Rpp1*. Surprisingly, the NBS region of the *R* genes shares sequence homology with the NBS region of cell death genes such as *CED4* from *Caenorhabditis elegans* and *Apaf-1*, *FLASH*, *CARD4*, and *Nod1* from human (Aravind et al., 1999). The presence of conserved TIR, NBS, and LRR structural motifs in different R proteins may imply their involvement in protein complexes that recognize pathogen-derived ligands (Avr products) and trigger signal transduction leading to defense response. Moreover, identification of the TIR domains in the N, L_6, M, RPP5, and RPS4 proteins suggests that plants and animals might use proteins with similar domains to resist infection.

5.1.3 Relationship to Disease Resistance

In most studied cases, HR appears to correlate with activation of resistance to a broad range of pathogens. However, HR cell death does not protect plants against infection by necrosis-causing pathogens (necrotrophic pathogens) such as the fungi *Botrytis cinerea* and *Sclerotina sclerotiorum*, although HR is thought to deprive the pathogens of the supply for food and confine them to initial infection site. The disease is manifested by appearance of necrotic lesions. Necrotrophic pathogens

usually kill the host cells before deriving food from them, often through secretion of toxin (Weymann et al., 1995; Hunt et al., 1997). Recent study has provided interesting evidence showing that infection of plants by necrotrophic pathogens can induce an oxidative burst and HR cell death with a marker of apoptosis, such as nuclear condensation, and with induction of HR-specific gene *HSR203J* (Govrin and Levine, 2000). The degree of *B. cinerea* and *S. sclerotiorum* pathogenicity was directly dependent on the level of generation and accumulation of superoxide or hydrogen peroxide. Interestingly, growth of *B. cinerea* can be suppressed in the HR-deficient mutant *dnd1*, and enhanced by HR caused by simultaneous infection with an avirulent strain of *P. syringae* (Govrin and Levine, 2000). Thus, HR induced by incompatible strain of bacterial pathogen (biotrophic pathogen) or elicited by nectrotrophic pathogen can restrict the spread of a biotrophic pathogen, but has an opposite effect against necrotrophic pathogens.

Furthermore, previous studies have provided some evidence that the HR is not always required for gene-for-gene resistance and SA synthesis. Examples have been reported with *Avr*-specific resistance genes that do not provoke macroscopic HR during the restriction of pathogen growth (Goulden and Baulcombe, 1993). Recent evidence that HR cell death and defense gene activation can be uncoupled comes from their apparent separation by the *dnd1* mutation (Yu et al., 1998; Clough et al., 2000) and protease inhibitor studies (del Pozo and Lam, 1998). Although these do not rule out the possibility that HR plays an important role in resistance, it suggests that disease resistance may be activated by a number of mechanisms, and in some cases, a subset of defense mechanisms would be sufficient to stop the growth of particular pathogens in the infected tissue.

HR cell death appears more tightly correlated with viral resistance as compared to resistance against bacterial pathogens. For example, the interaction between tobacco mosaic virus (TMV) and tobacco harbouring the *N* gene is a classic model system for studying gene-for-gene interaction and disease resistance (Holmes, 1938). Recently, Baker and coworkers systemically investigated the precise role of the *N*-encoded TIR, NBS, and LRR domains in conferring TMV resistance by the construction and analysis of a series of deletion and amino acid substitution mutant alleles of *N* (Dinesh-Kumar et al., 2000). Their deletion analysis suggests that TIR, NBS, and LRR domains each play an important role in the induction of resistance response against TMV. Moreover, they found that amino acid residues conserved among the TIR domain and NBS-containing proteins play critical roles in *N*-mediated TMV resistance. Some loss-of-function *N* alleles, such as the TIR deletion mutant and others with point mutations in the NBS region, apparently can interfere with the wild-type *N* function and behave like dominant negative mutations. Interestingly, many amino acid substitutions in the TIR, NBS, and LRR domains of *N* lead to a partial loss-of-function phenotype in which transgenic tobacco plants can mount a delayed HR compared with the wild-type plants but fail to contain the virus to the infection sites.

In animal cells, the ability of many viruses to replicate and spread is dependent on the production of inhibitors of apoptosis such as the p35 protein and Inhibitor of Apoptosis Protein (IAP) of baculovirus that act as inhibitors to caspases, a family

of cysteine proteases that serve as the crucial switch for many forms of PCD in animal cells (Green, 2000). Recent evidence from inhibitor studies and biochemical approaches suggests that caspase-like proteases may also be involved in PCD control in plants (Lam and del Pozo, 2000) (also see Section 5.3.4). Evidence for the functional significance of PCD as a plant defense response against viruses is apparent from studies in which baculovirus *p*35 was expressed in transgenic tobacco plants and then challenged with TMV (del Pozo and Lam, 2003). Infection of *p*35–expressing transgenic tobacco plants with TMV also can result in systemic spreading of the virus within a resistant background. Transgenic tobacco plants expressing mutant versions of the p35 protein that are defective in caspase inhibition did not show this phenotype. A striking characteristic of these plants is that TMV is able to escape from the primary inoculated leaves and systemically infect the plant in spite of the presence of the *N* resistance gene. Thus, in this particular plant–virus interaction, timely induction of HR cell death is necessary for restricting the pathogen to the primary infection site.

5.2 Approaches for the Characterizaton of the Response

5.2.1 Differential Gene Expression

The processes that determine the outcome of an interaction between plants and pathogens appear to be complex. Identification of genes differentially expressed in the compatible and incompatible interaction would allow a greater understanding of the molecular mechanism of HR cell death. To address this problem, differential library screening has been frequently used in earlier work. For example, Marco et al. (1990) reported the identification of two classes of genes (*str* and *hsr*) that are activated during the HR of tobacco in response to an incompatible isolate of *Pseudomonas solanacearum*, but not in response to an *hrp* mutant of the same bacterial isolate. Among these genes, activation of the tobacco gene *hsr203J* is rapid, highly localized, and specific for incompatible plant–pathogen interactions (Pontier et al., 1994). Its expression is also strongly correlated with PCD occurring in response not only to diverse pathogens but also to various cell-death-triggering extracellular agents (Pontier et al., 1998). On the other hand, using a synchronous HR-inducing system with TMV and resistant tobacco cultivars, Seo et al., (2000) isolated the cDNA of tobacco DS9 the transcript level of which specifically decreased three hours after TMV infection. The DS9 gene encodes a chloroplast-targeted homolog of bacterial FtsH protein, which serves to maintain quality control of some cytoplasmic and membrane proteins. The authors clearly demonstrated that reduced levels of DS9 protein in TMV-infected tobacco leaves accelerate the HR, suggesting that accumulation of damaged protein in the plastids may act as a signal for HR induction (Seo et al., 2000).

Early attempts to document global changes in defense-associated gene expression were limited by the difficulty of identifying the significant genes and their products using differential screening or differential display methods. Although the

above studies introduced here provide the identification of some interesting factors that may be involved in HR cell death, many aspects of the response to infection remain uncharacterized. Improvements in technology such as the generation of expressed sequence tag (EST) collections for various plant species and the complete sequencing of the *Arabidopsis* genome offer the potential for a global understanding of the transcriptional response during HR activation. DNA microarrays are powerful tools for a wide range of areas in plant molecular biology and can provide information on the expression patterns for thousands of genes in parallel (Zhu and Wang, 2000; Ahanoni and Vorst, 2001; Kazan et al., 2001). DNA microarrays are currently fabricated and assayed by two main approaches, involving either *in situ* synthesis of oligonucleotides (oligonucleotide microarray) or deposition of presynthesized DNA fragments (cDNA microarray) on solid surfaces (see recent review by Aharoni and Vorst, 2002). The application of this technology is being used to comprehensively profile gene expression networks during the plant defense response that is triggered when a plant encounters a pathogen or an elicitor molecule (Maleck et al., 2000; Schenk et al., 2000; Chen et al., 2002; Scheideler et al., 2002). In addition to identifying new genes induced during various defense responses in a global scale, these studies are providing new insights into the complex pathways governing defense gene regulation.

5.2.2 Biochemical, Pharmacological and Physical Approaches

One of the earliest responses activated after host plant recognition of an Avr protein or nonhost specific elicitor is the oxidative burst, in which levels of ROI rapidly increase (Lamb and Dixon, 1997). Earlier pharmacological and physiological evidence using an inhibitor of the neutrophil NADPH oxidase, diphenylene iodonium (DPI), indicated that DPI can block the oxidative burst in plant cells (Doke, 1983; Doke and Ohashi, 1988; Levine et al., 1994; Auh and Murphy, 1995; Levine et al., 1996). Activation of the oxidative burst is governed by a phosphorylation/dephosphorylation poise because the protein phosphatase 2A inhibitor cantharidin can enhance ROI production in soybean cells in response to avirulent bacteria or elicitor (Levine et al., 1994; Tenhaken et al., 1995). In contrast, the serine/threonine protein phosphatase inhibitor okadaic acid inhibits the oxidative burst and HR cell death induced by TMV (Dunigan and Madlener, 1995). The inhibitor of eukaryotic ribosomes, cycloheximide, can inhibit the oxidative burst of soybean cells in response to avirulent pathogen (Shirasu et al., 1997) and suggests that *de novo* protein synthesis is required for this process. Lastly, mastoparan, a specific activator of G-proteins in mammal, induces H_2O_2 accumulation in soybean cells in the absence of elicitor (Legendre et al., 1992; Chandra and Low, 1995).

Another early signaling event induced in plants during recognition of an invading pathogen is thought to be the enhanced flow of ions across the plasma membrane. This response involves an inward flux of calcium and protons, combined with

outward fluxes of potassium and chloride (Atkinson and Baker, 1989). The involvement of ion fluxes in the induction of HR signal transduction pathway was suggested by direct physiological measurement of the particular ion concentrations (Nürnberger et al., 1994; Jabs et al., 1997), as well as by different pharmacological studies (Jabs et al., 1997; Zhou et al., 2000a). In parsley, inhibition of elicitor-stimulated ion fluxes by ion channel blockers prevented ROI production, defense gene activation, and phytoalexin biosynthesis, while artificial induction of ion fluxes, in the absence of the elicitor, stimulated these responses (Jabs et al., 1997). In tomato, treatment with fusicoccin, an activator of the plasma membrane H^+-ATPase pump, was found to cause the acidification of the apoplast and the induction of SA biosynthesis and PR-gene expression (Schaller and Oecking, 1999). Fusicoccin, as well as treatment with a low-pH buffer, was also found to enhance HR cell death in barley (Zhou et al., 2000b). Ca^{2+} influxes also play a crucial role in the execution of the HR. Blocking Ca^{2+} ion channels using calcium channel blocker La^{3+} was shown to inhibit HR in tobacco, *Arabidopsis* and soybean systems (Atkinson et al., 1990; He et al., 1993; Levine et al., 1996; Mittler et al., 1997b). Treatment of plant cells with a Ca^{2+} ionophore can also induce HR-like cell death (Levine et al., 1996). Calcium signals appear to be at least in part mediated through protein phosphorylation and such activity has been implicated in cell culture response to the bacterial nonspecific HR elicitor protein harpin (Pike et al., 1998). However, the genes encoding the channels that mediate these fluxes *in vivo* have not been identified, and no direct genetic evidence currently exists for the involvement of ion fluxes in the induction of the HR.

Salicylic acid (SA) plays a key role in the activation of SAR and gene-for-gene resistance. SA levels increase after pathogen infection, which, in turn, leads to the induction of a number of PR genes (Malamy et al., 1990; Métraux et al., 1990). SAR can also be modulated by treatment with SA and chemical inducers such as 2,6-dichloroisonicotinic acid (INA; Métraux et al., 1990) and benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Friedrich et al., 1996). Depletion of endogenous SA levels in *Arabidopsis* and tobacco by overexpression of the bacterial gene *nahG*, encoding the enzyme salicylate hydroxylase, results in a breakdown of SAR and gene-for-gene resistance (Ryals et al., 1996). INA and BTH do not increase SA concentration in the plant and can activate SAR in both wild-type and NahG plants, suggesting these synthetic analogues of SA act independently or downstream of SA in the SAR signaling pathway (Friedrich et al., 1996; Ryals et al., 1996).

Host cell death can also be caused by pathogen-produced phytotoxic compounds that function as key virulence determinants. Necrotrophic phytopathogenic fungi synthesize a wide range of phytotoxic compounds, including the sphinganine analog mycotoxins, which are produced by at least two unrelated groups of fungi, *Alternaria* and *Fusarium* spp (Gilchrist, 1998). Fumonisin B1 (FB1) is one of several related sphinganine analog mycotoxins produced by *F. moniliforme* and elicits an apoptotic form of PCD in both plants and animal cell cultures (Wang et al., 1996a,b). Ausubel and coworkers have recently established a relatively simple pathogen-free system in *Arabidopsis* involving FB1 that can be used to study

the signal transduction events involved in pathogen-elicited cell death (Stone et al., 2000). FB1-induced lesions in *Arabidopsis* are similar to pathogen-induced lesions in many aspects, including deposition of phenolic compounds and callose, production of ROIs, accumulation of the phytoalexin camalexin, and induction of defense-related gene expression. The authors also showed that FB1 can be used to select directly for FB1-resistant mutants, some of which display enhanced resistance to a virulent strain of *P. syringae*, suggesting that pathogen-elicited PCD of host cells may be an important feature for certain compatible plant-pathogen interactions.

In the past several years, indirect evidence from biochemical and physiological studies has pointed to the involvement of proteases as a key player in the activation of HR cell death. For example, in cultured soybean cells, synthetic protease inhibitors effectively suppressed PCD triggered by oxidative stress or by infection with avirulent pathogens (Levine et al., 1996). It is noteworthy that only a subset of the tested protease inhibitors (PMSF, AEBSF, and leupeptin) partially block PCD. No inhibition and in some cases even increased cell death were observed with the serine protease inhibitors TLCK and TPCK, suggesting the stabilization of certain positive factors for HR activation (Levine et al., 1996). On the other hand, it is widely known that caspases are conserved cysteine proteases that regulate animal PCD (White, 1996). The possible involvement of caspase-like protease activities during HR cell death was also implicated by using specific inhibitors and substrates (del Pozo and Lam, 1998; D'Silva et al., 1998) (see also Section 5.3.4). In mammalian systems, cysteine proteases including caspases are major executors of PCD, but other classes of proteases, such as cathepsin D, aspartate proteases, metalloproteases, calcium-dependent proteases (calpain) and the ubiquitin/proteasome system have also been found to be involved in PCD (Beers et al., 2000). It is currently unclear whether these classes of proteases may also be involved in the activation of HR cell death (Heath, 2000). Recent studies with the tomato *Cf-2* resistance system has identified the locus *Rcr3*, which encodes a papain-like cysteine protease, as a specific and critical mediator for elicitation of the HR by Avr2 expressing races of the fungus *Cladosporium fulvum* (Kruger et al., 2002). Although its mode of action remains to be determined, Rcr3 serves as the first clear genetic evidence that a dedicated protease is involved in the HR induction process.

The importance of the mitochondrion in the expression of HR-associated PCD in plants comes from studies of the alternative oxidase (AOX), the mitochondrial enzyme localized in the inner membrane (reviewed in Lam et al., 1999a). AOX can control the generation of reactive oxygen species (ROS) from the mitochondrial electron transport chain when oxidative phosphorylation is inhibited. For example, when activity of the mitochondrial electron transport chain is inhibited by antimycin A treatment, AOX expression is induced and ROS generation is kept to a minimum so that little cell death is activated (Maxwell et al., 1999). Overexpression of AOX has the reverse effects, suggesting that plant mitochondria have an important role as a signal generator for HR-induced cell death, perhaps by generation of ROS derived from electron-transfer intermediates in the inner

mitochondrial membrane (Maxwell et al., 1999). Furthermore, Chivasa and Carr (1998) has shown by using an inhibitor of AOX salicylhydroxamic acid, that inhibition of AOX activity causes the inhibition of SA-induced resistance to TMV in tobacco, and antimycin A and KCN also induced AOX transcript accumulation and resistance to TMV. Induction of AOX has also been observed under several stress conditions and a recent study in *Arabidopsis* showed that rapid localized AOX induction by avirulent bacterial pathogens requires SA (Simons et al., 1999). Thus, these features strongly suggest that AOX may act as a safety valve for the control of HR activation and are consistent with its enhanced expression during the latter phase of the HR. AOX is not found in animal cells, thus it may be a specialized regulator to control cell death activation in plants (Lam et al., 1999a). In addition to the above observation, the importance of mitochondria in the expression of HR cell death comes from studies of the mode of action for host-selective toxin, victorin, which is required for pathogenesis and induces rapid cell death in susceptible, toxin-sensitive oat genotype (reviewed in Wolpert et al., 2002).

5.2.3 Genetic Dissection of the Key Factors Involved in HR

It has been difficult to assess experimentally the utility of cell death in gene-for-gene disease resistance because cell death is usually a central feature of this response. A mutational approach was used to shed further light on the relationships between HR cell death and pathogen growth arrest. The *dnd* (defense, no death) class of mutants, including *dnd1*, *dnd2*, and *Y*15, were identified by their reduced ability to produce the HR in response to avirulent *P. syringae* that express avrRpt2, and were isolated in a screen designed to discover additional components of the *avrRpt2-RPS2* disease resistance pathway in *Arabidopsis* (Yu et al., 1998). Among these mutants, the *dnd1* are defective in HR cell death but retain characteristic responses to avirulent bacteria, such as induction of PR gene expression and strong restriction of pathogen growth. Interestingly, progeny lines derived from the *dnd1* mutant also failed to produce an HR in response to *P. syringae* strains expressing avirulence genes *avrRpm1* or *avrB* (Kunkel et al., 1993). Since two separate resistance genes (*RPS2* and *RPM1*) control responsiveness to these three separate avirulence genes (*avrRpt2*, *avrRpm1*, *avrB*), it appears that DND1 is a common component of the plant defense response shared by distinct signal initiators. Recent identification of the *Dnd1* gene by positional cloning revealed that DND1 shows homologies to cyclic nucleotide-gated ion channels and confirmed to have ion channeling activity when expressed in yeast and animal cells (Clough et al., 2000). However, its mode of action *in planta* remains to be defined.

The highly localized nature of the HR suggests that mechanisms must exist to keep cell death contained. A large class of mutation exists in maize that is characterized by the spontaneous formation of discrete or expanding lesions of varying size, shape, and color in leaves (Johal et al., 1995). Because lesions associated with some of these mutants resemble symptoms of certain diseases of maize, they have been collectively called disease lesion mimics. To date, more than 40 independent lesion mimics, both recessive (designed *les*) and dominant

(designed *Les*), have been identified in maize (Johal et al., 1995). More recently, systematic screening of similar mutants in *Arabidopsis* yielded a number of mutants called *acd* (accelerated cell death), *lsd* (lesion stimulating disease resistance), and *cpr* (constitutive expresser of PR genes) (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995; Bowling et al., 1997). The expression of lesions in these mutants, generally designated as disease lesion mimics, can be developmentally programmed and is often affected by the environment or genetic background of the plant (Johal et al., 1995; Dangl et al., 1996). In some cases, lesion mimic mutants exhibit SAR and show high, constitutive levels of PR gene expression. Recently, some recessive lesion mimic genes have been cloned from three plant species: *Arabidopsis*, barley, and maize (Büschges et al., 1997; Dietrich et al., 1997; Gray et al., 1997; Hu et al., 1998). For example, the *LSD1* gene of *Arabidopsis*, which encodes a zinc finger protein, may negatively regulate cell death (Dietrich et al., 1997). Likewise, the *Mlo* gene of barley appears to encode a membrane protein whose function may be to negatively regulate both cell death and the disease resistance response (Büschges et al., 1997). The maize *Lls1* gene inhibits cell death, although apparently by degrading a phenolic mediator of cell death (Gray et al., 1997). Also, several recent studies have revealed that genetic disruption of biosynthesis pathway of tetrapyrroles (chlorophylls and heme) causes lesion formation that can lead to the induction of a set of defense response including activation of SAR (Hu et al., 1998; Molina et al., 1999; Ishikawa et al., 2001). Tetrapyrrole biosynthesis is highly regulated, in part to avoid the accumulation of intermediates that can be photoactively oxidized, leading to the generation of ROI and subsequent photosensitized damages. In this case, a light-sensitive ROI cascade mediated by the accumulated tetrapyrrole intermediate can apparently mimic the oxidative burst seen in plant defense response.

5.2.4 Lessons Learnt from Transgenes Which can Activate HR-Like Symptoms

Spontaneous formation of HR-like lesions in the absence of a pathogen has also been reported in a number of transgenic plants that express foreign or modified transgenes. Several transgenes that can activate or affect different components of a signal transduction pathway involved in pathogen recognition or defense response activation have been reported (reviewed in Dangl et al., 1996; Mittler and Rizhsky, 2000). Moreover, activation of HR-like cell death by transgene expression is viewed as important evidence for the existence of PCD pathways in plants.

The induction of proton and ion flux across the plasma membrane during plant-pathogen interaction was found to be one of the primary events that occurs during activation of the HR and other defense mechanisms. However, the genes encoding the channels that mediate ion fluxes *in vivo* have not been identified, and no direct molecular evidence currently exists for the involvement of ion fluxes in the induction of the HR. We have previously shown that expression of the gene encoding the bacterial proton pump bacterio-opsin (*bO*) in transgenic tobacco and potato plants resulted in the induction of multiple defense mechanisms with a

heightened state of resistance against pathogen attack (Mittler et al., 1995; Abad et al., 1997). In the absence of a pathogen, *bO*-expressing plants developed lesions similar to HR lesion, accumulated PR protein, and synthesized SA. Furthermore, our recent study using different mutant forms of bO provided direct molecular evidence that passive leakage of protons through the bO proton channel is likely the cause of the lesion mimic phenotype in transgenic tobacco plants (Pontier et al., 2002). The activation of defense mechanisms by *bO* expression supports a working hypothesis that enhancing the proton flux across the plasma membrane may mimic the presence of a pathogen, similar to the situation that occurs in a number of disease lesion mutants (Dangl et al., 1996, Mittler and Lam, 1996).

Ca^{2+} signal is also essential for the activation of plant defense responses, but downstream components of the signaling pathway are still poorly understood. Calmodulin (CaM) is known to be a universal Ca^{2+}-binding signal mediator in eukaryotes. Specific CaM isoforms of soybean, SCaM-4 and SCaM-5, are activated by infection or pathogen-derived elicitors, whereas other SCaM genes encoding highly conserved CaM isoforms did not show such response (Heo et al., 1999). Constitutive expression of either isoforms in tobacco resulted in spontaneous lesion formation, constitutive PR gene expression and enhanced resistance against virulent oomycete, bacterial and viral pathogens. Surprisingly, in contrast to SA-dependent activation of these pathogen-induced markers in wild-type tobacco, their lesion formation and PR gene activation in these transgenic tobacco plants did not require SA, suggesting that specific CaM isoforms are components of an SA-independent signal transduction pathway leading to disease resistance (Heo et al., 1999) (see also Section 5.3.1).

GTP-binding proteins (G-proteins) act as molecular signal transducers whose active or inactive states depend on the binding of GTP or GDP, respectively, in the regulation of a range of cellular processes-including growth, differentiation, and intracellular trafficking. In animals and fungi, cholera toxin (CTX) can activate signaling pathways dependent on heterotrimeric G-proteins. Transgenic tobacco expressing a gene encoding the A1 subunit of cholera toxin (CTX) showed greatly reduced susceptibility to the bacterial pathogen *Pseudomonas syringae* pv *tabaci*, accumulated high levels of salicylic acid (SA) and constitutively expressed PR genes, suggesting that CTX-sensitive G-proteins are important in inducing the SAR (Beffa et al., 1995). Furthermore, expression of *rgp1*, a gene encoding a Ras-related small G-protein, in transgenic tobacco was shown to increase resistance to TMV infection through SAR activation pathway (Sano and Ohashi, 1995). Shimamoto and coworkers recently reported that expression of a constitutively active derivative of monometric G-protein Rac of rice (OsRac1) activated ROS production and phytoalexin levels, developed symptoms of HR-like lesion, increased resistance against virulent fungal and bacterial pathogens, and activated cell death with biochemical and morphological features similar to apoptosis in mammalian cells (Kawasaki et al., 1999; Ono et al., 2001). Conversely, a dominant-negative OsRac1 was shown to suppress elicitor stimulated ROI production and pathogen-induced cell death in transgenic rice.

Expression of some metabolism-perturbing transgenes in plants is thought to result in the alteration of cellular homeostasis and the generation of a signal that activates the PCD signaling pathways (Dangl et al., 1996; Mittler and Rizhsky, 2000). For example, tobacco plants expressing yeast-derived vacuolar and apoplastic invertases develop spontaneous necrotic lesions similar to the HR caused by avirulent pathogens; uncontrolled expression of these genes can drastically alter the metabolic balance of cells due to changes in hexose transport or metabolism (Herbers et al., 1996). In animal systems, many perturbations in cellular metabolism were shown to activate an apoptosis-signaling pathway (Bratton and Cohen, 2001). Since infection of plants with avirulent pathogens such as bacteria and viruses is likely to cause general alterations in the metabolic balance of cells (Dangl et al., 1996; Mittler and Lam, 1996), mutation of general housekeeping genes involved in plant cell metabolism can result in PCD in some cases, but not in others.

5.3 Current Mechanistic Understanding of the Response

5.3.1 Recent Studies Related to Calcium and its Homeostasis as Signal for HR

Transient influx of Ca^{2+} constitutes an early event in the signaling cascades that trigger plant defense responses. Since Ca^{2+} signaling is usually mediated by Ca^{2+}-binding proteins such as calmodulin (CaM), identification and characterization of CaM-binding proteins elicited by pathogens could provide insights into the mechanism through which Ca^{2+} regulates defense responses including the HR. Very recently, an interaction between CaM and Mlo proteins was found by screening a rice cDNA expression library in *Escherichia coli* with the use of soybean CaM1 conjugated to horseradish peroxidase as a probe (Kim et al, 2002a). Rice Mlo homologue (OsMlo) has a molecular mass of 62 kDa and shares 65% sequence identity and predicted topology with barley Mlo, a seven-transmembrane-helix protein known to function as a negative regulator of broad spectrum disease resistance and plant cell death (Büschges et al., 1997). These research groups also showed that barley Mlo can bind CaM (HvCaM3) using the above *in vitro* assays, and *in vivo* expression assays using both the yeast split-ubiquitin technique and transient expression system in barley epidermal cells by biolistic methods (Kim et al., 2002b). The significance of barley Mlo-CaM interaction *in vivo* in pathogen defense was also shown by transient expression assays in which Mlo activity is shown to depend on its specific binding to CaM. Likewise, gene suppression of HvCaM3 by RNA interferance (RNAi) in an *Mlo* background quantitatively lowered the susceptibility seen in Mlo wild-type leaves, which is consistent with an enhancing function for CaM in Mlo-mediated defense suppression. Resistance suppression by CaM3 required the presence of wild-type MLO because its expression in the mutant *mlo* background did not influence the resistant phenotype (Kim et al., 2002b). Taken together, these results provide strong evidence that CaM has

an activator role for Mlo-mediated defense suppression and places CaM activity upstream of, or coincident with, the action of Mlo. However, the precise connection between a change in cellular Ca^{2+} concentrations and this novel interaction between CaM and Mlo for HR regulation remains to be defined.

5.3.2 Transcriptional Mediators and Lipid Metabolism that are Involved in HR Activation

In animal cells, PCD is controlled through the expression of a number of conserved genes. Some gene products activate PCD, such as caspases, whereas others are inhibitors, such as some members of the Bcl-2 family. In addition to their role in cell-cycle regulation, recent studies have suggested a new role for MYB proteins as regulators of cell survival and/or cell death through the regulation of a new MYB target gene, Bcl-2 (Frampton et al., 1996; Solomoni et al., 1997). A *MYB* gene from tobacco is induced in response to TMV activated HR and it can bind to a consensus MYB recognition sequence found in the promoter for the *PR-1a* gene (Yang and Klessig, 1996). Furthermore, Daniel et al., (1999) have shown that expression of *Arabidopsis MYB30* is closely associated with the initiation of cell death. This gene is thought to be a strong candidate for a component of a regulatory network controlling the establishment of cell death.

Genetic approaches in *Arabidopsis* have been used to identify signaling components involved in HR control. Recent findings have strongly suggested that specific regulation of lipid metabolism may closely associate with HR activation (Falk et al., 1999; Jirage et al., 1999; Brodersen et al., 2002). The *EDS1* gene was cloned by transposon tagging and found to encode a protein that has similarity in its animo-terminal portion to the catalytic site of eukaryotic lipases (Falk et al., 1999). The PAD4 gene was cloned by map-based positional cloning and found to encode another member of the L-lipase class of plant proteins that include EDS1 (Jirage et al., 1999). EDS1 and PAD4 were shown to be required for SA accumulation upon avirulent pathogen and their mRNA levels are upregulated by applications of SA, although EDS1 and PAD4 function upstream of SA accumulation. It should be noted that EDS1 appears to be involved in signaling pathway for specific types of TIR-NBS-LRR resistance genes (Liu et al., 2002; Peart et al., 2002). The recessive *acd11 Arabidopsis* mutant exhibits characteristics of animal apoptosis and defense-related responses that accompany the HR (Brodersen et al., 2002). The *acd11* phenotype is SA dependent, as *acd11* is rescued by NahG gene, and application of BTH to *acd11/nahG* restores cell death. This SA-mediated death pathway requires both functional PAD4 and EDS1, as the *acd11* phenotype is suppressed by the *pad4-2* and *eds1-2* mutations. Molecular cloning, complementation, and biochemical analyses revealed that ACD11 encodes a homolog of mammalian glycolipid transfer protein and has sphingosine transfer activity (Brodersen et al., 2002). Furthermore, it was shown that a putative lipid transfer protein (DIR1) is involved in SAR signaling in *Arabidopsis* (Maldonado et al., 2002). Lipid molecules such as jasmonic acid, phosphatidic acid and N-acylethanolamines are synthesized or released from membranes upon pathogen or insect attack. Some act as second

messengers in plant defence signaling (Wasternack and Parthier, 1997; Chapman, 2000; Munnik, 2001). A role for DIR1 and ACD11 in disease resistance signaling would be consistent with the observation that some mammalian lipid transfer proteins act as lipid sensors or are involved in phospholipase-C-linked signal transduction (Wirtz, 1997). Therefore, these molecular genetic studies using *Arabidopsis* mutants implicate the involvement of a lipid derived signal component for HR and SAR signaling and, ACD11 and DIR1 could act as a translocator for release of the mobile signal into the vascular system and/or chaperone the signal through the plant.

5.3.3 *Reactive Oxygen Species Generation and Cellular Energy Status as a Rheostat*

Many studies document the detection of O_2^- and/or its dismutation product, H_2O_2, during the HR (Lamb and Dixon, 1997). *Arabidopsis lsd1* mutants exhibit impaired control of cell death in the absence of a pathogen and could not control the spread of cell death once it was initiated (Dietrich et al., 1994). Jabs et al. (1996) showed that treatment with superoxide, but not H_2O_2, triggers cell death in *lsd1* mutants. DPI, an inhibitor of neutrophil NADPH oxidase, reduced cell death in the *lsd1* genetic background. This suggests that superoxide is necessary and sufficient to propagate lesion formation in an *lsd1* background, accumulating before the onset of cell death and subsequently in live cells adjacent to spreading *lsd1* lesions. LSD1 encodes a zinc finger protein with homology to mammalian GATA-type transcription factors and it may function either to suppress a pro-death pathway component or to activate a repressor of plant cell death (Dietrich et al., 1997).

One source generating ROI in plants is thought to be produced by enzymatic machinery similar to the mammalian respiratory burst NADPH oxidase complex (Doke, 1985; Lamb and Dixon, 1997). Recently, homologues of $gp91^{phox}$ (respiratory burst oxidase homologue [*rboh*]), which is a plasma membrane localized component of neutrophil NADPH oxidase, was isolated from rice (Groom et al., 1996), *Arabidopsis* (Keller et al., 1998; Torres et al., 1998), and tomato (Amicucci et al., 1999). They can encode a protein of about 105 kDa in size, with a C-terminal region that shows pronounced similarity to the 69 kDa apoprotein of the $gp91^{phox}$ and a large hydrophobic N-terminal domain that is not present in mammalian $gp91^{phox}$. This domain contains two Ca^{2+}-binding EF hand motifs and has extended similarity to the human Ran GTPase-activating proteins. A recent mutant study provides strong genetic evidence that *Arabidopsis rbohD* and *rbohF* are required for accumulation of ROIs in the plant defense response (Torres et al., 2002). The *AtrbohD* gene is required for most of the ROI observed after inoculation with avirulent bacteria, whereas *AtrbohF* apparently has a limited contribution. In contrast, the *atrboh* mutants exhibit enhanced HR and less sporangiophore formation in response to the weakly avirulent fungi, *Peronospora parasitica*. Interestingly, although *atrbohF* exhibits minor suppression of ROI production, it exhibits strongly enhanced cell death phenotype. A double mutant combination of the two *Atrboh* genes dramatically suppresses the oxidative burst triggered by bacterial and fungal

pathogens (Torres et al., 2002). Using a novel activity gel assay, Sagi and Fluhr (2001) also confirmed that a putative plant plasma membrane NADPH oxidase can produce O_2^-. These studies show that pathways for ROI generation and their involvement in the HR can be quite complex.

5.3.4 Mechanism for Cell Death Activation

Identities of the key executioners in HR cell death remain elusive, whereas in animal systems a large number of caspases and their regulators have been defined in the past decade (Aravind et al., 1999). Caspase-like protease activity has been observed to be transiently activated in plants synchronized to undergo the HR (del Pozo and Lam, 1998). Peptide inhibitors of caspases can abolish HR cell death of tobacco induced by avirulent bacteria without affecting the induction of defense-related genes significantly. On the other hand, induction of proteolytic activity that may be relevant to cell death during the HR has also been studied in the cowpea rust fungus/cowpea pathosystem using a bovine poly(ADP-ribose) polymerase (PARP) as substrate (D'Silva et al., 1998). PARP is a well-characterized substrate for caspase-3 and was found to be endoproteolytically cleaved when added to extracts prepared from fungus-infected cowpea plants that were developing a HR, while no PARP cleaving activity could be detected in the presence of extracts from cowpea plants that were undergoing a susceptible interaction. The cleavage of PARP observed in this study could be partially suppressed using caspase inhibitors. Moreover, it was clearly shown that tetrapeptide caspase inhibitors can block or significantly diminish plant cell death associated with compatible plant-bacteria interactions which are activating an HR as part of pathogenesis and, in all cases when death is limited, bacterial multiplication is concomitantly reduced (Richael et al., 2001). These studies provide support for a caspase-like protease(s) in plants, the activity of which is correlated with the induction of HR cell death.

Using iterative database searches, Uren et al. (2000) identified potential relatives of caspases in the *Arabidopsis* genome, which they termed metacaspases. Their homology to mammalian caspases is not restricted to the primary sequence, including the catalytic diad of histidine and cysteine, but extends to the secondary structure as well. The plant metacaspases can be divided into two subclasses based on the sequence similarity within their caspase-like regions and their overall predicted domain structure. Type I plant metacaspases contain a predicted N-terminal prodomain which consists of a proline-rich region and a zinc finger motif that is also found in LSD1, a negative regulator of HR cell death (Dietrich et al., 1997). Type II plant metacaspases possess no obvious prodomain but have a conserved insertion of approximately 180 amino acids between the regions corresponding to the p20 and p10 subunits of activated caspases (reviewed in Lam and del Pozo, 2000). However, it remains unclear whether plant metacaspases are functionally equivalent to classical caspases in terms of their target specificities as well as their involvement in controlling the activation of cell death. Recently, a caspase-like protein has been identified in the budding yeast *Saccharomyces cerevisiae* (Yor197W) and is implicated in cell death induced by H_2O_2, acetic acid and ageing (Madeo

et al., 2002). Yeast caspase-1 (YCA1) is a member of the metacaspase family and like Type I plant metacaspases, it also has a proline-rich domain at its N-terminus. Overexpression of YCA1 enhances apoptosis-like death of yeast upon addition of H_2O_2 or acetic acid, whereas targeted ablation of YCA-1 dramatically improves survival. YCA1 protein also seems to undergo proteolytic processing in a manner that is dependent on its active-site cysteine, which is similar to mammalian caspases (Madeo et al., 2002). However, many features of the yeast metacaspase YCA1 remain to be clarified. These include direct demonstration of its protease activity, identification of its substrate specificity, and elucidation of its endogenous targets and regulators. It would be interesting to investigate whether plant metacaspases are functionally equivalent to YCA1 using the yeast *yca1* mutant (Madeo et al., 2002) as well as to define their possible roles in HR cell death using reverse genetic approaches.

In animal cells, mitochondria-mediated PCD acts through the proapoptotic Bax and its related proteins that associate with the outer mitochondrial membrane and can oligomerize to form an ion-conducting channel through which macromolecules and other metabolites can pass. This activity can be blocked by the anti-apoptotic proteins Bcl-2 and Bcl-$X_{L,}$ which play crucial functions to control PCD activation or suppression (Lam et al., 1999b; Martinou and Green, 2001). Recent comparative genomics revealed that no obvious homologue of mammalian Bcl-2 related proteins exists in *Arabidopsis*. Nonetheless, overexpression of human Bcl-2, nematode CED-9, or baculovirus Op-IAP transgenes can confer resistance to several necrotrophic fungal pathogens and a necrogenic virus in tobacco plants (Dickman et al., 2001). Likewise, in transgenic tobacco plants overexpressing Bcl-X_L, delay of HR cell death as well as UV-induced PCD, has been reported (Mitsuhara et al., 1999). Expression of Bax using a TMV vector triggers cell death in tobacco leaf cells in an *N* gene-independent manner (Lacomme and Santa Cruz, 1999). Bax also confers a lethal phenotype when expressed in yeast with typical hallmarks of Bax-induced PCD in animal cells despite the apparent absence of classical caspases or Bcl-2-related proteins in yeast. Thus, the expression of pro- and anti-apoptotic proteins in these heterologous systems has similar effects to those observed in animal cells. This is consistent with the speculation that a conserved cellular pathway for cell death control may exist in eukaryotes. It is possible that the metacaspase YCA-1 may mediate Bax-induced cell death in yeast. This can now be tested with the *yca1* strain (Madeo et al., 2002).

Two groups have used yeast to isolate suppressors of Bax-induced cell death (Greenhalf et al., 1999; Xu and Reed, 1998). One of these suppressors, Bax inhibitor-1 (BI-1), prevents cell death in yeast and animal cells, suggesting that BI-1 could be a distinct class of PCD regulator for pathways activated by Bax expression. Homologues of BI-1 isolated from *Arabidopsis* and from rice have also been shown to suppress Bax-induced PCD in yeast (Kawai et al., 1999; Sanchez et al., 2000). These BI-1 proteins contain six potential transmembrane helices and it has been proposed that they may form ion-conducting channels or modify the activity of existing channels formed by Bax. Expression of *AtBI-1* was rapidly up-regulated in plants during wounding or pathogen challenge. *AtBI-1* up-regulation

appears to be *R*-gene independent and is not remarkably affected by mutations required for specific classes of *R* gene, suggesting a ubiquitous role in responses for biotic and abiotic stresses (Sanchez et al., 2000). On the other hand, Kawai-Yamada et al. (2001) demonstrated that AtBI-1 overexpression could rescue transgenic plants expressing Bax gene from lethality, while Bax caused potent PCD symptoms, including leaf chlorosis, cytoplasmic shrinkage, and DNA laddering. Although this finding provides direct genetic evidence that Bax-induced cell death can be down-regulated by overexpression of AtBI-1 protein *in planta*, it remains unclear how AtBI-1 suppresses the activity of Bax, given that no obvious Bcl-2 family members have been found in yeast and plants. Surprisingly, it was recently reported by the same research group that AtBI-1 did not block Bax-induced cell death, but instead triggered apoptotic cell death in certain mammalian cultured cells (Yu et al., 2002). The unexpected apoptotic effect of AtBI-1 was shown to be blocked by the caspase inhibitor XIAP and antiapoptic protein Bcl-X_L, suggesting that the cell death caused by AtBI-1 is similar to that caused by Bax and that AtBI-1 caused apoptosis in this case through a caspase-dependent pathway. Yu et al. (2002) speculated that plant BI-1 may competitively interact with endogenous mammalian BI-1 or with a BI-1 target protein in certain cell types, thus interfering with its function and thereby triggering cell death.

5.4 Future Perspectives

The past few years have seen a steady increase in our knowledge of HR cell death in plants. In spite of all the information described above, research in the past years have added very little to our sparse knowledge of the actual control mechanism for HR cell death in clear molecular terms. In particular, no relative of any classical metazoan regulator of apoptosis (for example, Ced-3/caspases, Ced-4/ Apaf-1, Ced-9/Bcl-2) has been defined structurally and genetically so far. The apparent absence of caspases, which are considered to be the major executors of cell death in metazoans, has been a strong argument against a mechanistic and functional conservation of PCD between plants and animals (Lam et al., 2001). Plant genomic studies have produced large quantities of sequence information that await functional analysis. In particular, metacaspases and BI-1 related proteins could be likely candidates for plant cell death regulators at the present time. *Arabidopsis* contains at least nine possible metacaspase-encoding genes, one BI-1 homologue and two other BI-1-related homologues: AtBI-2 and AtBI-3. Furthermore, *Arabidopsis* contains a new gene family discovered by homology searches that we designated as ABRs (for AtBI-2 related proteins) (Lam et al., 2001). This gene family contains twelve putative genes that encode proteins with five or six predicted membrane-spanning helices, although most of the predicted amino acid sequences are unique for this family. Deployment of reverse genetic approaches such as PTGS/RNAi strategies (Wang and Waterhouse, 2001) and knockout screens using T-DNA or transposon insertion collections (Bouche and Bouchez, 2001), coupled with informatic approaches should help to speed up the first essential step of

identifying the important players involved in plant cell death activation. This approach would be complementary to forward genetic approaches that are revealing new regulators which may not have counterparts in other organisms. As a second step for studying the physiological function of these regulators, development of other functional genomic tools, such as global transcriptome profiling by DNA microarrays and proteome analyses, would be of importance if we are to take full benefit of resources generated from the rapidly developing model plant systems such as *Arabidopsis* and rice.

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