The Hypersensitive Response : A Case Of Cell Death Induction In Plants

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Abstract

Recognition of a diverse range of pathogens, followed by an appropriate defense response, is crucial for the survival of plants. It is conditioned by initial recognition events between host plant and pathogen, which lead to activation of various host defense responses, including a specialized type of programmed cell death known as a Hypersensitive Response (HR). HR may play in plants the same role as certain programmed cell deaths in animals with respect to restricting pathogen growth. In addition, the HR could regulate the defense responses of the plant in both local and distant tissues. HR is commonly regulated by direct or indirect interactions between avirulence gene products from pathogen and resistance gene products from plant and it can be the result of multiple signalling pathways. Ion fluxes and the generation of reactive oxygen species (ROS) commonly precede cell death, but a direct involvement of the latter seems to vary with the plant-pathogen combination. ROS and ion fluxes are proximal response probably required for the HR and finally the potential elements of the signal transduction pathways leading to the activation of various mechanisms of ROS production followed by cell death. It seems likely that cell death within the HR acts more as a signal to the rest of the plant rather than as a direct defense mechanism. Exciting advances have been made in the identification of cellular protective components and cell death suppressors that might operate in HR. In this review, the physiological, biochemical and molecular machineries of the HR will be discussed.

Keywords : hypersensitive response, reactive oxygen species, ion flux, programmed cell death, oxidative burst, salicylic acid, benzoic acid, ethylene, PR proteins

Abbreviations : HR, hypersensitive response; ROS, reactive oxygen species; BA, benzoic acid; SA, salicylic acid; LOX, lipoxygenase; LRR, leucine rich repeat; NBS, nucleotide binding site; MAP, mitogen activating protein; PAL, phenylalanin ammonialyase; PGIP, polygalacturonase inhibiting protein; SOD, super oxide dismutase

1. Introduction

Plants have evolved sophisticated and efficient mechanisms to prevent the invasion of their tissues by pathogens, and disease rarely occurs. One common feature of disease resistance is the rapid development of cell death at and immediately surrounding infection sites, called the Hypersensitive Response, or HR [1,2]. When non-pathogenic or an avirulent strain of a pathogen attacks the plant then elicitor molecule is secreted by the pathogen which elicits rapid collapse of the challenged host cells, so-called Hypersensitive Response [3] and deploys a battery of inducible defences including antibiotics (phytoalexins), oxidants, cell wall reinforcing substances, lytic enzymes and induction of 'defence-associated' gene expression and other antimicrobial proteins in the challenged cells and surrounding cells .

Hypersensitive response was a term first applied by Stakman (1915) to describe the rapid and localized plant cell death induced by rust fungi in rust-resistant cereals (Fig.1).

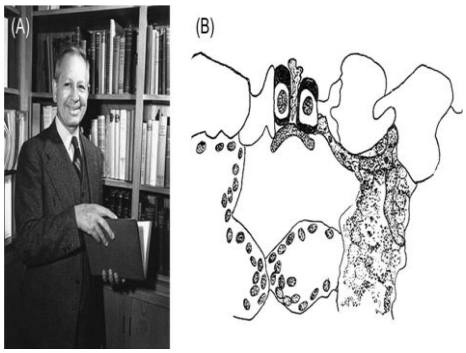

Fig. 1. EC Stakman and hypersensitiveness.
(A) EC Stakman (1883–1971) Photo courtesy of the University of Minnesota Archives. (B) An image from the original plate within Stakman (1915). The original legend stated 'Oats inoculated with *Puccinia graminis hordei*, four days after inoculation. Infection thread growing over cell and destroying chloroplasts; normal cells on left.'

The subsequent realization that such death was a common expression of disease resistance in plants, regardless of the type of inducing pathogen, led to its designation as the hypersensitive response, usually defined as 'the rapid death of plant cells in association with the restriction of pathogen growth [2]. The HR is generally recognized by the presence of brown, dead cells at the infection site and, depending on the pathogen, their number may vary from one to many. The HR may or may not be restricted to cells physically invaded by, or having direct contact with, the pathogen. A visible brown lesion may develop if sufficient cells die.

Hypersensitive cell death is commonly controlled by direct or indirect interactions between pathogen avirulence gene products and those of plant resistance genes and it can be the result of multiple signalling pathways. The HR reaction is usually preceded by rapid and transient responses occurring mainly at the plant cell surface and based predominantly on the activation of pre-existing components. These include ion fluxes generation and release of reactive oxygen species, changes in protein phosphorylation patterns, changes in exocellular pH and in membrane potential, and oxidative cross-linking of plant cell wall proteins. Ion fluxes and the generation of reactive oxygen species commonly precede cell death, but a direct involvement of the latter seems to vary with the plant-pathogen combination.

Surveillance in the plant is the collective duty of a complex array of constitutively expressed *R* genes (for resistance). Race-specific pathogen recognition is hypothesized to result from the direct or indirect interaction of the product of a dominant or semi dominant plant resistance (R) gene with a product derived from the corresponding dominant pathogen avirulence (*avr)* gene [4,5]. Subsequent signal transduction events are assumed to coordinate the activation of an array of defense responses. Individual *R* genes have narrow recognition capabilities and they trigger resistance only when the invading pathogen expresses a corresponding '*avr* gene' (for avirulence). The simplest mechanistic model is that the avr gene encodes a ligand that is recognized by the product of the matching R gene which then triggers the HR and disease resistance [6]. In addition, molecules from the pathogen called elicitors are able to trigger HR [7]. Plant receptors are also thought to be involved in recognition of these elicitors [8,9]. Subsequent to recognition, biochemical and metabolic plant modifications are well conserved among different plant microbe interactions [10]. Following pathogen recognition, constitutively expressed signal transduction pathways are engaged.

Furthermore, a large set of inducible genes, commonly known as defence related genes are expressed. They include enzymes involved in the synthesis of anti-microbial compounds phytoalexins, structural proteins incorporated into the cell wall [11], and the pathogenesis related (PR) proteins.

This review aims to focus on the physiological, biochemical and molecular machineries of the HR.

2. Definitive features of the HR

The HR encompasses both cell death and 'defence gene' expression. However, it is important to note that disease resistance and all of the inducible defence responses currently associated with the HR can occur in the plants in the absence of cell death. Moreover, pathogens may also cause cell death and trigger defence responses while successfully growing in susceptible tissue. A further complication is the fact that genetic defects, or treatments unlikely to resemble those causing cell death during a natural HR, can cause a cell death that mimics the HR morphologically and in the induction of defence responses [12,13,14]. As a result, there are no features that currently can unequivocally identify the HR in the absence of a plant-pathogen interaction.

Attempts to rectify this problem have resulted in the discovery of two genes in tobacco (with related genes in tomato), *HIN1* and *HRS203J*, that have the potential for being early marker genes for the HR [15,16], and which have been used to distinguish hypersensitive cell death from natural leaf senescence [17]. However, HSR203J is also expressed during cell death caused by successful pathogenesis [16] and the expression of both genes is induced by heavy-metal salts [16,17] which do not trigger features typical of hypersensitive cell death in other systems [18,19]. Therefore, it is a matter of research to tell whether or not unique marker genes for the HR exist.

3. Morphological changes in the HR

In most studied pathosystems, pathogen infection is nonsynchronous . Several systems are utilized to describe the development of HR in living plant tissues where individual infection events can be followed. One well characterized system is the interaction between the biotrophic fungus *Uromyces vignae* and cowpea. At 15 h after inoculation during an incompatible interaction, [20] observed the following sequence of cytological events:

1. Migration of the nucleus to the site of fungal penetration and intense cytoplasmic streaming.
2. Cessation of cytoplasmic streaming, Brownian motion of the organelles, condensation of the nucleus, accumulation of granules at the periphery of the cytoplasm, shrinkage of the protoplast, and
3. Collapse of the cytoplasm and death of the infected cell.

Similar cytological changes were observed in the interaction between *Erysiphe graminis* f.sp *hordei* and barley plants carrying the Mla12 resistance gene [21].

As yet there is no specific molecular or cytological marker in plants which would allow clear discrimination between necrosis and the HR. Levine et al (1996) detected plasma membrane blebbing, cell shrinkage, condensation of both the cytoplasm and nucleus, and structures that might be interpreted as apoptotic bodies during the HR triggered by bacterial pathogens, but not in susceptible tissues. However, they did not detect DNA laddering. Although cell death in plants could functionally play the same role as in animals, it may be that the mechanisms underlying this process evolved differently [22].

4. Genetic control of the HR

Resistance is generally, but not always [23], controlled by single, parasite-specific resistance (*R*) genes. HR requires the pathogen to have an avirulence (*avr*) gene that 'matches' the *R* gene in a 'gene-for-gene relationship'. *R* and *avr* genes appear to have a more complex relationship for bacterial pathogens, with single *R* genes 'matching' more than one *avr* gene [10]. Whether the HR expressed in non-host plants has the same type of genetic control is controversial [23].

4.1. Gene-for-gene interaction

In integrating cytological and PCD elements into a plant HR model, the role of the resistance (R) gene

products (RGP) in initiating the HR process must be a major consideration. Harold Flor first described the dependence of the HR and resistance on R gene-interaction with pathogen encoded avirulence (avr) gene production, hence the term gene-for-gene interactions [24]. Subsequently, a large number of R genes have been cloned and can be broadly classified into five classes [25]. A near ubiquitous feature of RGP is the possession of variable numbers of leucine-rich repeats (LRR), and frequently nucleotide binding sites (NB). Those NB containing RGP that have either regions of homology to insect Toll or mammalian IL-1 receptors, the TIR domain, forming the TIR-NB-LRR R gene class. Another major class of R gene has a coil-coil motif instead of a TIR domain and is designated CC-NB-LRR. It is not relevant to this review to consider the minutiae of RGP domain function (for which the reader is directed to [25]), only how R–avr interactions could link with cell death mechanisms; and this is far from clear.

An impressive early study used yeast two-hybrid approaches to demonstrate the physical interaction between the avrPtoB avirulence gene product, the Pto RGP, and a companion NB-LRR protein, Prf which was also required to initiate a HR. Further, other Pto-interacting (Pti) genes included a serine–threonine kinase that is phosphorylated by Pto and an ethylene-associated ERBP transcription factor [26,27,28]. However, no interaction with obvious death effectors was found. A recent re-interpretation of RGP function suggests that RGP act to protect plant proteins against manipulation by pathogen-derived effectors. According to this hypothesis Pto, which is guarded by Prf, is the pathogenicity target of AvrPto, rather than a host resistance protein, and Prf is the host defence R protein that recognizes the AvrPto:Pto complex and initiates the HR. In substantiation of this model, Mackey et al. (2002) found RIN4, a protein that interacted with both AvrRpm1 and the RGPs, RPM1, and RPS2. This interaction was required to elicit a HR.

Guarded proteins such as RIN4 and Pto presumably represent RGP outputs through which the HR cell death is elicited. These outputs have also been targeted from mutant screens. For instance, analyses of rar1 and sgt1b mutants in barley and Arabidopsis have revealed one convergence point between both the CC-NB-LRR and TIR-NB-LRR class RGPs [29]. RAR1 proteins have two cysteine and histidine rich domains (CHORD), one of which binds to the HSP90, a molecular chaperone .

The levels of RPM1 are reduced in rar1 and hsp90 Arabidopsis mutants [30] and it may be that RAR1 together with HSP90 functions to stabilize RGP and promotes the formation of an active configuration to allow interaction with the guarded host protein. Upon interaction with the avirulence gene product, the HR appears to be effected through the SGT1b protein. SGT1b is a conserved adaptor protein which, in plants, has been shown to interact with RAR1, HSP90, and LRR domains of resistance gene products [31,32] and also with two components of the E3 ubiquitin ligase; SKP1 and CUL1 [33] . The function of E3 enzymes is to add ubiquitin to specific proteins and thereby target them for degradation with the 26S proteasome. It may be hypothesized that ubiquitinization targets cell death suppressors which are destroyed in the proteasome in order to initiate the HR. The search for SGT1b-E3 protein targets is ongoing in many laboratories. At another level, important signalling elements following R-avr interactions have been rationalized into two regulatory signalling nodes [34]. One node is dependent on EDS1 and PAD4 which is required for the function of the TIR-NBS-LRR class of R genes [35]. The eds1 mutant was isolated from a screen for enhanced disease susceptibility to H. parasitica whilst pad4 originated from a screen for phytoalexin deficiency. EDS1, PAD4, and a third component, SAG101, have been found to interact physically

and forms part of a MAPKinase 4 signalling module which is important for salicylic acid (SA) and reactive oxygen species (ROS) generation . The alternative regulatory node is utilized by the CC-NBS-LRR R gene class and passes via NDR1 [36,37]. In a major recent advance, NDR1 was observed to interact physically with RIN4 at the plasma membrane, suggesting that this forms part of the inductive switch leading to a HR [38]. To conclude this section, the distinctiveness of the RGP signalling modules needs to be emphasized. RGP signalling is very unlike any mammalian form of cell death that responds to external stimuli. For example, with pro-apoptotic mammalian Fas and TNF receptors cell death is initiated via caspases which are intimately associated with the receptor [39]. No such intimate association with, for example, proteases, has been described for RGP.

Mutants exhibiting HR-like phenotypes have been long described in many plant species, including corn [40,41], tomato [42], barley [43] and Arabidopsis [44]. These mutants, also known as lesion mimic mutants, are classified into initiation and propagation mutants; initiation mutants inappropriately induce PCD and form localized, necrotic spots, whereas propagation mutants can not stop it, once it has been initiated [45]. A forward genetic screen for mutants with HR-like lesions and characteristics of defense responses, including molecular and biochemical markers and enhanced disease resistance, revealed the lesion simulating disease resistance (lsd) class of mutants [46]. Two of these genes have been cloned: LSD4, an FtSH protease and the zinc-finger protein LSD1 [47], a negative regulator of superoxide-induced cell death [48]. LSD1 protects plants from ROS-induced stresses and consequently, lsd1 mutant plants are characterized by runaway cell death (rcd) [46,49]. Therefore, lsd1 can be regarded as a sensitized mutant with respect to cell death initiation, and it has been instrumental in identifying other components of the signaling pathway leading to programmed cell death. For example, EDS1 and PAD4 functions are required for lsd1 rcd induced by abiotic stress [49]. EDS1, PAD4 and NDR1 are also required for full lsd1 rcd in response to pathogen infection [50]. EDS1 and PAD4 regulate a ROS- and SA-dependent signal amplification loop, which in turn is modulated by LSD1 [50].

5. Inducers, effectors and regulators of the HR

Many reviews provide detailed information concerning the induction and signal transduction leading to disease resistance [10]. Thus, it is clear that atleast two steps are necessary to induce the HR: recognition of the pathogen and transduction of the perceived signal(s) to the effector (s) of cell death.

The specific pathogen recognition model suggests that the first event in trigerring the HR could be the direct recognition of the pathogen *avr* gene product by the corresponding plant R gene product. Recent evidence indicates that there is such a direct interaction between the tomato *Pto* resistance gene product and the product of the avirulence gene *avrPto* from *Pseudomonas syringae* pv tomato [51,28]. The analysis of the sequences of the different cloned resistance genes suggests that this possible type of direct interaction may not only happen in the plasma membrane but also in the cytoplasm [52] and in the nucleus [53,54].

Although it is generally assumed that *R* genes are constitutively expressed, there is one report that an *R* gene may be induced during the HR .

R genes that are not involved in *avr-R* gene complementarity do not resemble those. Thus, the *R* gene *HM1* of maize codes for an enzyme that detoxifies the host-selective toxin produced by the fungus *Cochliobolus carbonum* [55], thereby allowing the metabolism that leads to the HR (which is not controlled by *HM1*) to occur [23].

5.1. R proteins as guards of cellular machinery

The 'guard hypothesis' provides an intriguing conceptual framework for the action of disease effectors and the R-protein complex. It was put forward in an attempt to rationalize why Pto protein kinase requires the NB-LRR protein Prf to activate defence upon recognition of AvrPto. According to this model, Pto is a general component of host defence, perhaps in a pathway for response to nonspecific elicitors of phytopathogenic bacteria. The function of Avr Pto for *P. syringae* is to target Pto and suppress this nonspecific defence pathway. Prf is thus an NB-LRR protein that 'guards' Pto, detects its interdiction by AvrPto (or any other bacterial effector), and then activates defence.

5.2. Other genes involved in the HR

Mutation studies have revealed that the HR also depends on additional genes that presumably are present in both resistant and susceptible members of host species and which confer the ability of all plants to undergo an HR even in non gene-for-gene situations. These RDR (required for disease resistance) genes [56] may be different for different *R* genes, irrespective of the type of pathogen against which they act. In *Arabidopsis*, for example, mutations in *NDR1*, a gene that codes for a putatively membrane-associated protein, suppresses resistance mediated by the LZ-NB-LRR but not the TIR-NB-LRR class of resistance genes while the reverse is true for mutations in *EDS1*, a putative L-family lipase [57]. These data suggest that there may be several signalling pathways leading to hypersensitive cell death, and that the one activated depends more on the class of *R* gene than the type of inducing pathogen.

5.3. Specific elicitors

avr gene products might be expected to trigger the HR only in plants that contain a matching *R* gene, For viruses, specific elicitors have been identified as coat proteins, the helicase domain of a replicase gene, or a movement protein [58]. For fungi, specific elicitors are primarily peptides of unknown function [59] that are known [60] or assumed to be products of *avr* genes and are secreted only under specific conditions [60] or stages of development [20].

5.4. Non-specific elicitors

In addition to *avr* gene products, fungal and oomycete pathogens have a variety of components or secretory products, such as arachidonic acid, cell wall carbohydrates, glycoproteins and proteins, that can elicit plant defence responses and, in some cases, cell death [20]. Although proof of a role for these elicitors in the HR is generally lacking. Direct evidence comes from the case of transformants of the potato pathogen, *Phytophthora infestans*, in which the lack of INF1, a 10 kDa protein of the death-eliciting elicitin family, is associated with a loss of ability to trigger the HR in one of three non-host *Nicotiana* species [61].

The HR may cause pathogen arrest but may also occur as a consequence of the activation of other defence responses.

6. Induction of the hypersensitive cell death

Hypersensitive cell death and defence gene activation may involve separate signalling pathways, both initially activated by avr-R gene product interaction [62]. For fungal pathogens, the widespread sensitivity of plants to their non specific elicitors makes it quite likely that the latter induce defence responses in addition to any such induction by avr-R gene product interactions. Further evidence that hypersensitive cell death and defence gene activation are not mandatorily linked comes from their separation by mutation [63,64] and inhibitor studies [65]. Thus, the initial signalling pathway can fork and give rise to atleast two branches: one activates the synthesis of

phytoalexins and defence proteins while the other one specifically results in the cell death.

6.1. Ion fluxes appear to be an early step in the HR

Plants commonly respond to external stimuli, including microbial elicitors of cell death and/or defence responses, by calcium influx into the cell [66]. However, the use of kinetin to raise cytosolic calcium to levels equivalent to those seen prior to the HR in cowpea did not result in cell death [67] suggesting that additional signals are involved or that the calcium signal needs a specific feature, such as a periodicity 'signature' [68]. The elicitor from *P. sojae* and another from *C. fulvum* activate mitogen-activated protein (MAP) kinases in signalling pathways that seem independent of the oxidative burst [69,70]. For bacterial [66] and oomycete pathogens, one of the earliest signs of the HR is membrane dysfunction. In tomato suspension cells, the activation of a HC-ATPase by a *C. fulvum* specific elicitor has been suggested to be responsible for inducing the opening of plasma membrane calcium channels [71]. In contrast, non-specific elicitors of defence responses often inhibit HC-ATPase activity [71]. Interestingly, inhibitors or stimulators of proton -ATPase activate different defence pathways in tomato suspension cells [72], but the link between proton fluxes and cell death is still unclear. For example, mutant bacteria that cannot induce a HR in tobacco leaves or death in cell suspensions still elicit HC/KC exchange [73]. In the *C. fulvum*-tomato [71] and *Phytophthora infestans*-potato [74] systems, ion flux responses to elicitor receptor interaction seem to be mediated via hetero-trimeric G proteins; correspondingly, mastoparan, a G-protein activator, elicits cell death, extracellular alkalinisation, and an oxidative burst [75].

6.2. The role of reactive oxygen species

The production of reactive oxygen species probably plays a key role in plant defence [76,77,78]. Often the first response activated in many incompatible interactions. It may be the trigger that initiates the HR. Dolie and colleagues (1983, 1988) were the first to report that superoxide anions (O_2^{*}) were produced in incompatible interactions, initially between potato and *Phytophthora infestans* (late blight fungus) and then between tobacco and tobacco mosaic virus. The levels of ROS inside the cell are maintained at their lowest by the relevant protective mechanisms using compartmentalized isozymes of catalase, superoxide dismutase or peroxidise (Fig.2). In some cases, especially under stress conditions, this protective action is overridden by the oxidative burst.

These are the enzymes involved in ROS generation-

1- Plasma membrane bound NADPH and NADH oxidases

2- pH-dependent cell wall peroxidises

3- Exocellular germin like oxalate oxidases

4- Amine oxidases

5- Protoplastic ROS-generating systems

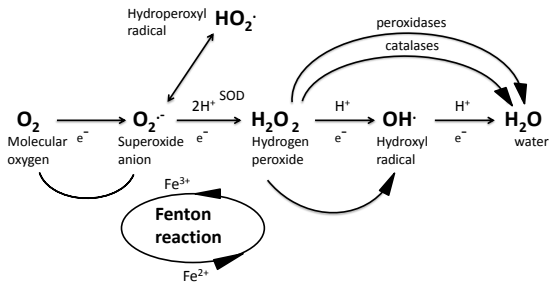

Fig 2. General scheme of ROS generation and their regulation (modified, Apel and Hirt, 2004).

6.3. The role of benzoic acid and salicylic acid

lncompatible 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 [79]. 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 [80]. It is not clear whether SA biosynthesis is a cause or a consequence of the HR . As SA donates an electron to Compound I catalase, it is converted into the oxidized form (SA^*). This SA free radical (SA^*) could initiate lipid peroxidation and may also modify other macromolecules. However, the SA free radical (SA^*) does not inhibit peroxidases involved in lignin biogenesis.

Several other roles for SA and/or BA in plant defence have been proposed. Both compounds may be directly antimicrobial [79]. Furthermore, exogenous SA application induces the coordinated expression of a subset of PR genes in numerous plant species [81]. Elevated SA levels can also inhibit wound-induced gene expression by blocking jasmonic acid (JA) biosynthesis. Thus, at sites of *R-Avr* gene-mediated microbial incompatibility, elevated *SA* levels should ensure that defence responses required for the arrest of microbial growth are activated whereas those against chewing insects and migrating nematodes are not induced unnecessarily.

6.4. The role of ethylene in the HR

Although the processes of plant PCD share similarity to animal PCD, the control of cell death in plants involves plant-specific regulators. In addition to common suicidal cascades, it has been demonstrated in a number of experimental plant systems that the plant hormone ethylene plays an important role in programmed cell death and senescence. The role of ethylene in pathogen-induced cell death is evaluated in ethylene insensitive (never-ripe) NR-tomatoes. Following infection of these mutants, greatly reduced cell death is observed, indicating ethylene involvement in programmed cell death [82]. Ethylene signalling is found to play a role in the cell death induced by the mycotoxin Fumonisin B1 in Arabidopsis and Tomato [83,84,85]. In Oat mesophyll cells, the administration of inhibitors of ethylene biosynthesis and action – aminooxyacetic acid (AOA) and silver thiosulphate (STS) effectively inhibited victorin-induced PCD, involving RUBISCO cleavage, DNA laddering and changes in mitochondrial permeability [86]. Microarray study of AAL toxin-treated tobacco reveals that genes responsive to reactive oxygen species, ethylene and a number of proteases are among the earliest to be upregulated, suggesting that an oxidative burst, production of ethylene and proteolysis play a role in the activation of the cell death [87]. In *Taxus chinensis* cell suspension ethylene enhances cell death induced by a fungal elicitor from *Aspergillus niger* [88]. Overproduced ethylene correlates closely with expression of lethal symptoms and apoptotic-like changes in hybrid tobacco seedlings and the lethality can be suppressed by ethylene synthesis inhibitors AOA and aminoethoxyvinylglycine (AVG) [89].

Two partly overlapping cell death pathways are proposed. These comprise one pathway involving caspase-like proteases that requires low level of ethylene and one caspase-independent pathway operative at high ethylene levels. The latter pathway presumably acts through MAPK-like proteins that are not essential in PCD at basal ethylene concentrations [90].

7. Signalling mechanism(s) of the HR

It is envisaged that R proteins act as receptors to detect the microbial *Avr*-dependent signal and thus initiate downstream signalling (Fig.3 and Fig.4). 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 signalling pathways. Immediately downstream of pathogen perception, the activation of pre-existing protein kinases, phosphatases, and G proteins are the most likely next steps. 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 [91]. The extremely rapid induction of the oxidative burst and/or ethylene biosynthesis [92] 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 [93]. Once the earliest defence 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 (Fig.3**).** This hierarchy of signalling events probably provides the overall framework to induce co-ordinately the diverse array of defence responses in the various cellular compartments. Considerable amplification of specific defence responses then occurs, via either positive feedback or signal cross-talk.

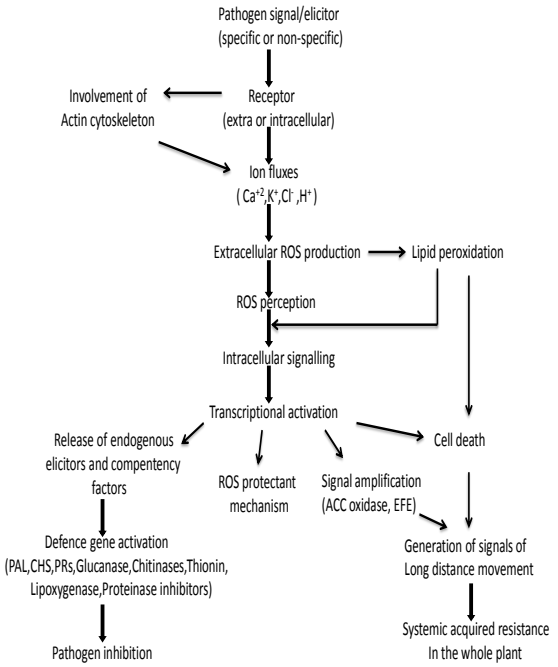

Fig.3 A hypothetical model of the signal transduction pathways leading to the HR (modified, Heath, 2000).

The activation of specific cellular protection mechanisms is likely to accompany the defence response. These mechanisms include upregulation of the cytoplasmic Halliwell-Asada cycle that minimizes the consequences of oxidative stress. Furthermore, increased transcription of specific SOD and catalases genes may occur to ensure that maximal enzymatic activity is maintained within the appropriate cellular compartments. For example, the expression of glutathione peroxidase, glutathione S-transferase, and polyubiquitin genes has been detected in incompatible interactions [94]. Glutathione peroxidase activity can block cell death in mammalian systems, whereas glutathione S-transferase detoxifies the products of lipid membrane peroxidation and other products of cellular oxidative stress. Polyubiquitin is required for the recycling of damaged proteins. BA, SA, and other phenolics may act as free radical scavengers that protect cells from oxidative toxicity. 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 [95]. Overall, precise temporal and spatial coordination of induced defence responses is required to successfully kill or contain the invading microbe while simultaneously minimizing the damage to host tissue (Fig.4). In the initially attacked cell(s), rapid responses may ultimately lead to cell death, whereas in the surrounding cells, induced defence 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 defence signalling pathways after the invading microbe has been successfully contained.

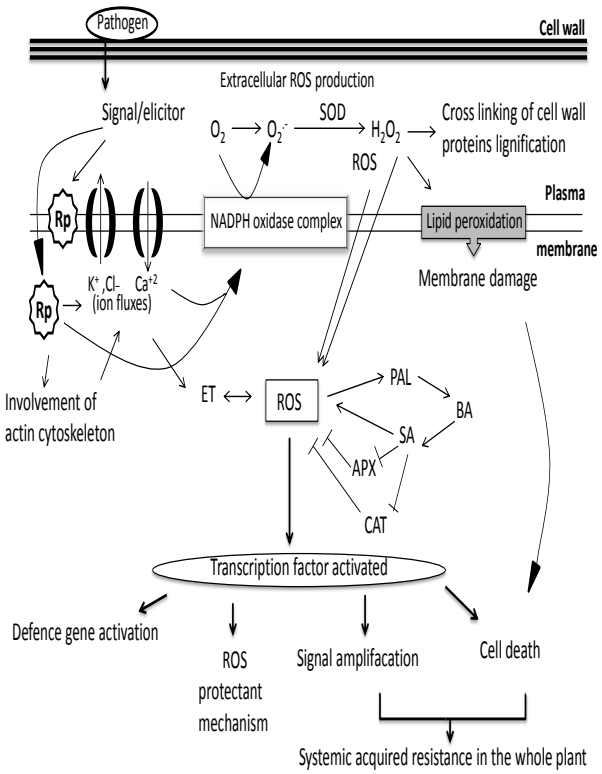

Fig.4 Showing the signalling mechanism of HR (modified, Morel and Dangle, 1997).

8. Protectant mechanisms of host against pathogen

The induction of HR involves several plant signals generated in the plasma membrane (ROS, ion fluxes). These signals seem to converge into a few genetically and pharmacologically seperable pathways. Subsequently defence genes, ROS protectant mechanisms and cell death can be induced via divergent pathways (Fig.5).

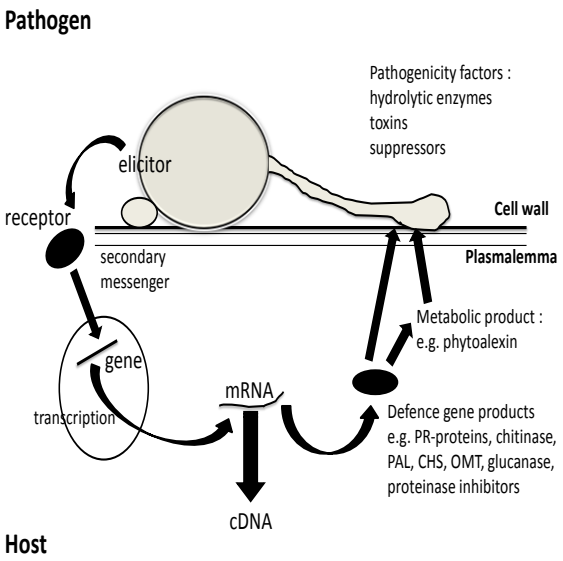

Fig.5 shows some protectant mechanisms of host against pathogen (modified, Agrios, 1988).

8.1. PR proteins and other defence-related proteins

Several PR proteins possess either antifungal or antibacterial activity *in vitro* and are now known to be chitinases, or glucanases, or to bind chitin [96]. 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.

8.2. Lipoxygenases (LOX)

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 co-ordinately amplify specific responses . LOX activity may also cause irreversible membrane damage, which would lead to the leakage of cellular contents and ultimately result in plant cell death [97]. Alternatively, LOX-catalyzed reactions can result in the production of toxic volatile and non-volatile fatty acid-derived secondary metabolites that could directly attack invading pathogens [98].

8.3. 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 [99] (e.g. Phaseolin in bean, Pisatin in pea, Glyceolin in soybean, Rishitin in potato ,Gossypol in cotton etc.).

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. 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.

8.4. ROS protectant mechanisms

Potential ROS protectant mechanisms include anti-oxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and polyubiquitin. Expression of these genes occurs concomitantly with cell death and H_2O_2 may play a role in their induction [94,100]. The induction of these protectant mechanisms, in contrast to the induction of defence genes and cell death, can be independent of Ca signalling [100]. This further suggests that the induction of defence genes, cell death and anti-oxidant protectant mechanisms are probably controlled by divergent pathways.

8.5. Cell wall fortification

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 *Bacillus 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. Polygalacturonases (PGs) are believed to contribute to cell wall softening by some necrotrophic fungi. Polygalacturonase-inhibiting proteins (PGIPs) inhibit PGs. PGlPs are induced in the bean-*Colletotrichum lindemuthianum* interaction with similar kinetics to pathogenesis-related (PR) proteins [101]. It has been hypothesized that PGlPs 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 defence responses. Alternatively, PGlPs may slow the rate of hyphal extension so that other components of the defence response can be more effectively deployed. For example, constitutive expression of the pear fruit PGlP in transgenic tomato plants enhanced resistance to colonization of ripe fruits by *B. cinerea* [102].

One type of cell wall fortification that occurs rapidly in response to fungal invasion is the formation of papillae. Papillae often form immediately beneath the penetration peg and are heterogeneous in composition [103], they are thought to physically block fungal penetration of host cells. Rapid callose deposition in cell walls is also frequently associated with sites of pathogen incompatibility. Callose deposition also occurs when plant cell cultures are challenged with pathogen-derived elicitors or when plant tissue is mechanically wounded [104]. Blockage of plasmodesmata with callose is an essential component of the defence response required to impede cell-to-cell movement of viruses. An additional but probably slower mechanism that renders cell walls more impermeable is the local elevation of their lignin content. 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 (*Trticum aestivum*) and the rust *Puccinia graminis* f.sp. *tritici.*

Conclusion and future prospects

The HR is an intrinsically programmed process. However, because of the great diversity of triggers [95] and morphologies of the cell deaths [103], there are probably several ways in which a cell may die. It is clear that there may be a rapid convergence of the initially activated *Avr-R* gene dependent signalling events into one or a few common pathways that coordinate the overall defence response. Whether these same pathways are also activated by nonspecific elicitors is not known.

Plant pathologists still need to establish criteria and find strict markers (if such exist) to differentiate between cell death resulting from environmental or metabolic perturbation and cell death resulting from the activation of the internal HR program. However, the morphological characterization of the HR may be difficult due to the rapidity at which the cellular modifications occur. Genetic approaches and cloning of plant genes (such as the genes responsible for the disease lesion mimic phenotypes and R gene suppressors) will shed new light on the mechanisms involved in regulating and executing the HR. Genetic dissection of the signal transduction leading to HR is underway and has already suggested that various signal pathways exist. These may or may not converge [36]. The HR also results from a complex interplay of signals from both the plant and the pathogen. The latter can sometimes interfere with these processes in order to successfully colonize the plant. It does not seem that HR is always necessary for resistance [43]. Rather coordination between the different induced mechanisms is required for successful resistance. Cell death during the HR appears to be part of a

continuous process where different pathways cross talk. Death associated with Disease symptoms and HR probably share common mechanisms and study of susceptibility will probably give us new insights into resistance mechanisms.

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