

To Die or Not to Die – Is Cell Death Dispensable for Resistance during the Plant Hypersensitive Response?

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(Received: 15 December 2005; accepted: 20 January 2006)

One of the most typical and best studied type of plant resistance is the hypersensitive response (HR), a form of localized programmed cell death associated with restriction or even killing of pathogens that often leads to macroscopically visible localized tissue necrosis. It is generally assumed that cell death and resistance within the HR are physiologically and genetically linked. However, there has been considerable speculation about whether cell death is an absolute requirement for resistance conditioned by the HR. This review discusses the relation of cell death and resistance in the HR, in particular, the importance of cell death in this process. We intend to focus on the increasing amount of research evidence showing that in several plant–pathogen interactions, the two main components of the HR – resistance and cell death – can be physiologically, genetically and temporally uncoupled. In other words, HR should be considered as a combination of resistance and cell death responses, where cell death may be dispensable for the plant resistance response. The varying contribution of these two components (i.e. cell death and resistance) generates an array of defense strategies - ranging from extreme resistance to “systemic HR” - applied by resistant host plants in the battle against pathogen infections.

Keywords: hypersensitive response (HR), resistance, cell death, extreme resistance.

The ability to selectively recognize invading pathogens and to mount appropriate defense responses is a hallmark of eukaryotic immune systems. While plants lack the adaptive immunity found in mammals, they are able to defend themselves against a wide range of pathogens using sophisticated mechanisms of recognition and response. A typical form of plant resistance is activated when a plant resistance gene (*R*) product recognizes – directly or indirectly – a specific pathogen gene product encoded by an avirulence (*Avr*) gene (gene-for-gene disease resistance, see e.g. in Flor, 1971; Hammond-Kosack and Jones, 1996; Nimchuk et al., 2003). The final result of this recognition event is the hypersensitive response (HR), a form of localized programmed cell death associated with restriction of pathogens to the infection site that often leads to the appearance of macroscopically visible localized tissue necrosis (Klement, 1982; Goodman and Novacky, 1994; Greenberg and Yao, 2004).

It is generally assumed that cell death and resistance within the HR are physiologically and genetically linked. This would mean that cell death contributes to or is essential for resistance which makes sense when the invader is a biotrophic pathogen (i.e. it prefers

live host tissues) but seems difficult to interpret in the case of necrotrophic pathogens (i.e. the pathogen prefers dead host tissues). Therefore, it is possible that HR-type cell death occurs as a consequence of overactive defense responses during resistance (Király et. al., 1972). In fact, through the years there has been considerable speculation about whether cell death is an absolute requirement for resistance conditioned by the HR.

The purpose of this review is to discuss the relation of cell death and resistance in the HR, in particular, the importance of cell death in this process. We intend to focus on the increasing amount of research evidence showing that, at least in certain plant-pathogen interactions, the two main components of the HR – resistance and cell death – can be physiologically, genetically and temporally uncoupled.

Physiological uncoupling of resistance and cell death in the HR

As a result of non-self recognition, the HR is preceded by a series of biochemical and cellular signals, defense responses that include a burst of reactive oxygen species (ROS), NO, ion fluxes, increased salicylic acid content, the induction of kinase cascades and a specific set of so-called pathogenesis related and other genes (see e.g. Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001; Nimchuk et al., 2003). It is difficult to judge, however, which subsets of these processes are responsible for resistance and cell death, respectively, during HR. As mentioned previously, dead cells cannot provide nutrients and other growth substrates for biotrophic pathogens. On the other hand, dying cells might release some of the above-mentioned signals that are themselves antibiotics or disinfectants (Lamb and Dixon, 1997). This is supported by previous studies on the *N* gene-mediated resistance of tobacco to *Tobacco mosaic virus* (TMV). TMV particles could be found in cells surrounding the necrotic HR-lesion, even when lesion expansion had stopped (Da Graça and Martin, 1976). These results, however, also imply that pathogen localization (resistance) and cell death during a viral HR can be physiologically, temporally and spatially separated. Similar conclusions can be drawn from the early work of Farkas et al. (1960) which showed that treatment of leaves with antioxidant compounds (ascorbic acid, glutathione, etc.) markedly decreased the number of TMV-elicited HR-lesions, while virus titers did not change significantly. Furthermore, we have recently experienced that during a systemic TMV infection, virus titers remain high regardless of the absence or presence of HR-type cell death generated by ROS-treatments (Király et al., 2005).

It seems apparent from the studies mentioned above that in some cases of virus-elicited HR cell death is not necessarily required for resistance. For plant–fungus interactions, this was first demonstrated by one of the previously mentioned laboratories (Király et al., 1972). HR-type necrotic lesions were induced in potato tubers and wheat or bean leaf tissues infected with compatible fungal races (*Phytophthora infestans*, *Puccinia graminis* and *Uromyces phaseoli*, respectively), when pathogen growth in host tissues was selectively inhibited by chemical or heat treatments. It was concluded that HR-type cell and tissue death is a result of inhibition of pathogen growth. In other words, cell and tissue death seemed to be a mere

consequence, not the cause, of resistance in the plant-pathogen interactions studied. The physiological uncoupling of resistance and cell death in a fungus-elicited HR was confirmed by later studies of barley resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*) governed by the *Mlg* resistance gene. This type of resistance is characterized by a subcellular, highly localized cell wall reinforcement at sites of attempted penetration (effective papillae) and a rapid death of attacked epidermal cells (HR). It turned out that *Mlg*-associated leaf epidermal HR is dispensable for growth arrest of powdery mildew in papillae, because inhibition of HR by the transcriptional inhibitor cordycepin was not accompanied by a release of fungal growth arrest and did not result in susceptibility (i.e. formation of haustoria). Also, growth arrest of the same powdery mildew race on *Mlg* coleoptiles occurred in the absence of detectable HR-type cell death (Schiffer et al., 1997). Gene dosage experiments further showed that heterozygous *Mlg mlg* plants, in comparison to the *Mlg Mlg* genotype, exhibit a drastic reduction of HR cell death without a proportional increase of haustorium formation (Görg et al., 1993). Taken together, these results provide evidence that in *Mlg*-specified resistance HR-type cell death is not the cause of resistance, only a consequence of fungal growth arrest. It is important to point out, however, that separate experiments from the above-cited work of Schiffer et al. (1997) show that *Mla*-mediated resistance to powdery mildew is destroyed by inhibition of HR cell death. This implies that in certain plant-pathogen interactions cell death is essential for successful resistance.

The physiological uncoupling of resistance and cell death is also possible during bacterial HR. An early work has shown that the number of bacteria in a hypersensitively resistant tobacco remained on a restricted level even if HR-type necrotization was fully inhibited by albumin (Király et al., 1977). It is known that induction of HR is dependent on calcium influx in plant cells. Ott and coworkers (2003) demonstrated that lanthanum, a calcium channel blocker inhibits cell death during HR of tobacco inoculated with the bacterium *Pseudomonas savastanoi* pv. *phaseolicola*. However, lanthanum only partially blocked HR-associated resistance. Thus, it was revealed that resistance to this bacterium during HR has both cell death-associated and cell death-independent components.

Consistent with the physiological separation of disease resistance and cell death are the effects of very low oxygen pressure and high humidity that can inhibit the HR cell death in response to certain pathogens without compromising resistance (Mittler et al., 1996; Hammond-Kosack et al., 1996). However, the existence of independent resistance and cell death responses within an HR does not exclude the possibility that cell death has a role in reinforcing or stimulating the induction of defenses and pathogen localization (Da Graça and Martin, 1976; Dangl et al., 1996; Alvarez et al., 1998).

Genetic separation of resistance and cell death in the HR

As mentioned previously, the HR is preceded by biochemical and cellular signals executed in part by changes in the expression of different types of plant genes. In a study to assess the function of such genes in disease resistance in *Nicotiana benthamiana*, 79 genes were found that were responsible for HR cell death during resistance mediated by the *Pto* resistance

gene against *Pseudomonas syringae* pv. *tomato* (Lu et al., 2003). However, only six out of 79 genes were able to also confer suppression of bacterial growth. Therefore, different sets of genes might participate during HR in the execution of cell death and resistance, respectively.

In past years several resistance genes have been cloned and it has become possible to transfer them to other plants while retaining their role in elicitation of HR against avirulent isolates of bacteria, viruses and fungi. For example, *Pseudomonas syringae* pv. *tabaci* strains that express *avrPto* will elicit HR in tobacco that has been transformed with the tomato *Pto* gene (Thilmony et al., 1995). Similarly, transgenic tomatoes that express the tobacco *N* gene develop HR against *Tobacco mosaic virus* (TMV) (Whitham et al., 1996), and transgenic tobacco and potato plants that express the tomato *Cf-9* gene develop HR when inoculated with avirulent races of *Cladosporium fulvum* (Hammond-Kosack et al., 1998).

The studies mentioned above have provided evidence that the genetic machinery required for the elicitation of HR is conserved between certain plant species and confirmed that a single resistance gene is sufficient to trigger the cell death and resistance responses that comprise an HR. However, there are several examples of gene-for-gene resistance in nature without HR-type cell death. For instance, barley resistance against the leaf scald fungus *Rhynchosporium secalis* mediated by the *Rrs1* resistance gene is characterized by an inhibition of hyphal growth and degradation of fungal structures in the absence of HR cell death (Lehnackers and Knogge, 1990). Also, certain alleles of the *Rx* gene in potato condition resistance to *Potato virus X* (PVX) without activation of HR cell death (Bendahmane et al., 1999). Thus, it is likely that resistance and cell death responses that result in the HR can be genetically uncoupled.

Genetic separation of disease resistance and HR-type cell death was first achieved in a plant-bacterium pathosystem with the *Arabidopsis thaliana dnd1* (defense no death) mutant. Yu et al. (1998) have shown that *dnd1* mutants lost the capacity to respond to *Pseudomonas syringae* pv. *glycinea* Race 4 with an HR yet still exhibited resistance to Race 4. This mutant also shows stunting (dwarfing), constitutively high levels of salicylic acid, pathogenesis-related gene induction, and increased resistance to virulent pathogens. These characteristics are abolished in *dnd1* mutants transformed with the *nahG* gene that degrades salicylic acid and is known to eliminate constitutively active defenses. Interestingly, however, the *nahG* transgene only partially abolished stunting and loss of HR cell death in *dnd1* plants (Clough et al., 2000). This implies that salicylic acid-independent defense pathways are also activated in these plants. The protein product of the *Dnd1* gene (DND1) encodes a cation channel of the cyclic nucleotide gated ion channel family (Clough et al., 2000). Recently, a closely related cyclic nucleotide gated ion channel mutant, *hlm1* (Balagué et al., 2003) and the allelic mutant *dnd2* (Jurkowski et al., 2004) were found to give phenotypes similar to *dnd1*. However, the mutant phenotypes of homozygous *dnd1* or *dnd2* plants that carry the wild-type allele of the other *dnd* locus suggest that the DND1 and DND2 proteins are not functionally redundant. This is supported by the observation that in *Dnd1/dnd1*, *Dnd2/dnd2* double heterozygote plants the simultaneous reduction of gene dosage at both loci is not sufficient to cause the obvious “defense no death” phenotype (Jurkowski et al., 2004). Taken together, these results indicate that the wild-type DND1 and DND2 proteins function as positive regulators of HR-type cell death but do not influence the resistance component of HR.

On the pathogen side, studies of mutant viral *Avr* genes have also shown that elicitation of resistance can be genetically uncoupled from HR-type cell death. For example, the coat protein of TMV elicits HR in tobacco that carry the *N'* gene. Mutations within the coat protein gene will alter this *Avr* gene product such that the virus will elicit a “systemic HR” (i.e. systemic necrosis) (Culver and Dawson, 1989). Similarly, a single amino acid change within the *Cucumber mosaic virus* 2a polymerase results in systemic necrosis in cowpea, whereas the wild type virus elicits an HR that restricts the movement of the virus to the inoculated leaf (Kim and Palukaitis, 1997). These mutagenesis studies of viral *Avr* genes have also led to the suggestion that different plant genes might be involved in eliciting HR-type cell death and resistance.

A natural example for the partial genetic separation of resistance and cell death within HR is provided by the *Turnip crinkle virus* (TCV)/*Arabidopsis* pathosystem. Genetic analyses revealed that HR development is conferred by a single dominant gene termed *HRT* (HR to TCV) (Kachroo et al., 2000). However, *HRT* alone may not be sufficient for complete resistance because all F₁ plants and 75% of the HR-positive F₂ progeny from crosses between resistant and susceptible plant ecotypes became susceptible after HR-development. Kachroo and coworkers (2000) showed that TCV resistance is also influenced by a second recessive gene, designated *rrt* (regulates resistance to TCV). Therefore, in the *Arabidopsis*-TCV interaction that results in HR, resistance is governed by two separate genes (*HRT* and *rrt*), while cell death is determined by only one of these genes (*HRT*).

A complete genetic uncoupling of virus resistance and cell death that comprise an HR was shown for the first time by Cole et al. (2001). *Cauliflower mosaic virus* (CaMV) strain W260 elicits an HR in leaves of *Nicotiana edwardsonii*, an interspecific hybrid derived from a cross between *N. glutinosa* and *N. clevelandii*. Interestingly, *N. glutinosa* is resistant to W260, but responds with local chlorotic lesions rather than necrotic (HR) lesions. In contrast, *N. clevelandii* responds to W260 with systemic cell death. The reactions of the progenitors of *N. edwardsonii* to W260 infection indicate that each parent species contributes a factor toward the development of HR. Two lines of evidence showed that resistance and cell death, that comprise the HR elicited by W260, can indeed be uncoupled. First, the non-necrotic resistance response of *N. glutinosa* could be converted to HR when these plants were crossed with *N. clevelandii*. Second, cell death and resistance segregated independently in the F₂ population of a cross between *N. edwardsonii* and *N. clevelandii*. It was concluded that during HR of *N. edwardsonii* to W260 infection resistance is conditioned by a gene derived from *N. glutinosa*, whereas cell death is conditioned by *ccd1* (CaMV cell death), a gene derived from *N. clevelandii* (Cole et al., 2001; Király et al., 1999) (Fig. 1). Further evidence that cell death can be uncoupled from resistance during HR could be seen in a cross between *N. edwardsonii* and *N. bigelovii*. The F₁ hybrid plants that resulted from this cross were resistant to W260 infection, but the *ccd1*-governed cell death reaction was suppressed and plants displayed chlorotic local lesions instead of an HR (Cawly et al., 2005). The suppression of HR-type cell death was specific to CaMV, as TMV was able to elicit a normal HR in the plants. These studies clearly showed that in the CaMV/*Nicotiana* pathosystem, the HR is comprised of genetically separate resistance and cell death components.

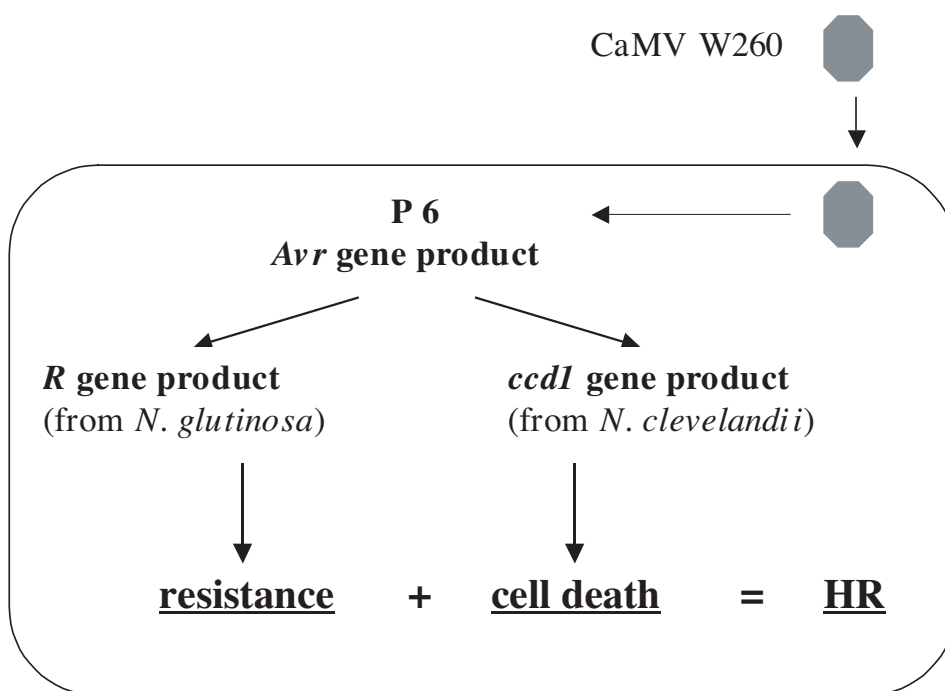


Fig. 1. A possible model for the genetic uncoupling of resistance and cell death within the hypersensitive response (HR) in *Nicotiana edwardsonii*. During HR elicited by *Cauliflower mosaic virus* (CaMV) strain W260 resistance is conditioned by the product of a gene derived from *N. glutinosa*, whereas cell death is conditioned by the product of the gene *ccd1* (CaMV cell death) derived from *N. clevelandii*. Elicitation of HR is initiated by the CaMV avirulence gene product (P6) that interacts with products of the resistance and cell death genes present in *N. edwardsonii* (based on Cole et al., 2001 and Cawly et al., 2005)

The complete genetic separation of resistance and cell death during HR is also possible in bacterial infections as recently demonstrated by Gassmann (2005). A new bacterial avirulence gene (*hopPsyA*) was described in *Pseudomonas syringae* pv. *syringae* 61 that is able to trigger resistance in the absence of macro- and microscopic HR-type cell death in *A. thaliana*. In a cross between two *hopPsyA*-resistant plant accessions that differ in their resistance phenotypes (symptomless resistance vs. HR) HR-type cell death segregated as a recessive trait regulated by the locus *hed1* (HR regulator in EDS1 pathway). This implies that *hed1*, just like *ccd1* in the CaMV/*Nicotiana* pathosystem, is a positive regulator of cell death, while the dominant alleles of these genes should confer suppression of cell death (Cawly et al., 2005; Gassmann, 2005). Cloning of these cell death regulator genes in the future will enable the generation of isogenic, disease resistant plant lines that differ only in their ability to mount an HR-type cell death. This should allow a quantitative analysis of the possible, if any, contribution of cell death to disease resistance, e.g. by comparing resistance against pathogens with different lifestyles (biotrophic vs. necrotrophic).

Extreme resistance – the temporal uncoupling of resistance and cell death in the HR

The HR is characterized by pathogen arrest at or near necrotic lesions which means that resistance and cell death are activated more or less simultaneously. In contrast, lack of cell death is characteristic of the so-called extreme resistance against virus infections. An additional typical feature of extreme resistance is an almost total inhibition of virus accumulation in inoculated tissues. Examples of this resistance response include the response of potato to *Potato virus Y* (PVY) and *Potato virus X* (PVX) governed by the resistance genes *Ryadg* and *Rx*, respectively, and *RSvI*-mediated resistance of soybean to *Soybean mosaic virus* (SMV) (Valkonen et al., 1994; Tozzini et al., 1991; Hajimorad and Hill, 2001).

Since the elicitation of extreme resistance, as opposed to HR, is not associated with cell death and is not dependent on salicylic acid (Bendahmane et al., 2000), it was concluded that extreme resistance is a rapid process that develops independently of HR. Several lines of evidence suggest, however, that the HR is a slow, secondary response associated with extreme resistance. First, it is known that HR is elicited in *Rx*-expressing potato and *RSvI*-expressing soybean lines that are graft inoculated with PVX and SMV, respectively (Benson and Hooker, 1960; Hajimorad and Hill, 2001). Second, an HR in *Gomphrena globosa* is elicited by the same primary structure in the PVX coat protein as *Rx*-mediated extreme resistance in potato (Goulden and Baulcombe, 1993). Furthermore, the functionally identical *Rx1* and *Rx2* genes both show similarities with the nucleotide binding site, leucine-rich repeat (NBS-LRR) class of plant resistance genes that confer an HR (Bendahmane et al., 1999, 2000). Finally, Bendahmane and coworkers have showed in a series of elegant experiments that *Rx1* has indeed a potential to initiate HR-type cell death if the PVX avirulence gene product (coat protein, CP) is expressed constitutively as a transgene, rather than from the PVX genome. When the PVX CP was expressed from the viral genome, the early and rapid elicitation of extreme resistance prevented PVX accumulation and further production of the CP. In contrast, when the CP was expressed from a transgene, initial activation of extreme resistance did not block continued production of the CP, which lead to activation of HR-type cell death as a secondary response (Bendahmane et al., 1999). The potential of *Rx2* to elicit HR was also demonstrated by varying expression levels of the gene in transgenic plants. High level expression of *Rx2* from a strong, constitutive promoter resulted in extreme resistance to PVX, while lower level of *Rx2* expression from a weak promoter resulted in HR (Bendahmane et al., 2000).

The experiments mentioned above demonstrate that HR (i.e. resistance and cell death) is a slow, secondary response associated with extreme resistance (i.e. resistance without cell death). In order to conclusively prove that extreme resistance and HR are indeed temporally separate responses during virus infections, Bendahmane et al. (1999) inoculated transgenic *Nicotiana benthamiana* plants that expressed the HR-independent *Rx1* and HR-associated *N* resistance genes with a TMV construct harboring the PVX CP gene. Thus, a single virus genome expressed the elicitors of *Rx1*- and *N*-mediated (HR) resistance. The outcome of this infection was extreme resistance, rather than HR, demon-

strating that extreme resistance and HR – in other words, resistance and cell death – can be indeed temporally uncoupled. This implies that the same responses might be activated in *Rx1*- and *N*-mediated resistance but activation of the resistance response in the infection cycle is rapid or more pronounced in the *Rx1* background, as compared to *N*-mediated resistance. In the latter case, the virus can reach relatively high levels by the time the resistance response is activated and this leads to an HR-type necrotization in the infected host.

There are several additional examples of gene-for-gene resistance responses that seem to have temporally distinct HR-independent (extreme resistance) and HR-dependent (resistance and cell death) components. In other words, early and rapid activation of resistance is not associated with cell death, while HR (resistance + cell death) is the result of a later and slower activation of resistance. For example, the “defense no death” (resistance without cell death) phenotype of *dnd* mutants could result from the constitutive up-regulation of generalized defense responses that, in the background of a resistance gene, leads to earlier elicitation of resistance without cell death. *Cf-9*-mediated resistance in tomato to *Cladosporium fulvum* is usually characterized by an HR-independent arrest of hyphal growth activated early in the fungal infection cycle. However, if the elicitor of *Cf-9*-mediated resistance is expressed from a virus vector, a “systemic HR” (i.e. systemic necrosis) develops, indicating a late activation of resistance (Hammond-Kosack et al., 1995). Extreme resistance to *Turnip crinkle virus* (TCV) infection occurs in the absence of HR through overexpression of the *HRT* resistance gene in transgenic plants. In essence, no HR develops because the plant responds too rapidly for the virus to elicit HR. “Micro HR” (i.e. microscopically visible cell/tissue death) develops through moderate expression of *HRT* in transgenic plants, whereas a “systemic HR” develops in transgenic plants that express a low level of *HRT* (Cooley et al., 2000; Kachroo et al., 2000).

The above-mentioned examples show that resistance responses of plants have an extremely dynamic nature and display quantitative and qualitative variation. Factors affecting a resistance response include timing, the level of interacting components and the affinity between them. Therefore, the possible outcomes of plant-pathogen interactions represent variable points in a continuum of responses ranging from extreme resistance through “micro HR” and HR to “systemic HR” (Bendahmane et al., 1999; Cooley et al., 2000). In nature, these different types of resistance responses likely represent different stages in the constant “arms race” between disease resistant plants and their pathogens.

Concluding Remarks

An increasing amount of research evidence accumulating from the 1960s to present show that, at least in several plant–pathogen interactions, the two main components of the HR – resistance and cell death – can be physiologically, genetically and temporally uncoupled. The existence of independent resistance and cell death responses within an HR, however, does not exclude the possibility that cell death has a role in reinforcing or stimulating the induction of defenses and pathogen localization (Da Graça and Martin, 1976; Dangl et al., 1996; Alvarez et al., 1998).

The fact that elicitation of resistance can be genetically uncoupled from the elicitation of cell death during HR should further facilitate the molecular characterization and cloning of these cell death regulator genes in the future. Isogenic, disease resistant plant lines can be generated that either elicit or do not elicit HR-type cell death. Such a quantitative analysis of the possible, if any, contribution of cell death to disease resistance might be useful in comparing resistance against pathogens with different lifestyles (biotrophic vs. necrotrophic).

The temporal separation of resistance and cell death suggests that the rapid and effective HR-independent extreme resistance and the slower less effective HR are variations on the same theme that are governed by a common mechanism. This mechanism may include an oxidative burst, as shown for HR (e.g. Doke and Ohashi, 1988; Ádám et al., 1989; Hüeckelhoven et al., 2000). Analysis of extreme resistance in plants with an elevated antioxidant capacity would indicate if production of reactive oxygen species (ROS) is also required for this HR-independent type of resistance. Interestingly, it has been shown that in *Mla12*-mediated resistance of barley to powdery mildew a ROS, hydrogen-peroxide, can enhance both HR and HR-independent resistance depending on the time of application. If hydrogen-peroxide was applied after establishment of fungal infection (2 days after inoculation), the HR that normally appears during *Mla12*-mediated resistance was enhanced. However, the same treatment before the establishment of fungal infection (1 day after inoculation) resulted in a symptomless resistance response and pathogen inhibition (Hafez and Király, 2003). Therefore, ROS-compounds seem to have a role in both HR-independent resistance and HR-associated resistance with necrotization.

The dynamic nature of resistance responses in plants implies that resistance can be effective with or without cell death, depending on factors like timing and speed of the resistance response and the affinity of interacting plant and pathogen components. These possible outcomes of plant–pathogen interactions represent an array of defense strategies applied by resistant host plants in the constant evolutionary battle with their pathogens.

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