Journal of Experimental Botany, Vol. 59, No. 3, pp. 501–520, 2008
doi:10.1093/jxb/erm239 Advance Access publication 12 December, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

FOCUS REVIEW PAPER

The hypersensitive response; the centenary is upon us but how much do we know?

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Received 12 July 2007; Revised 22 August 2007; Accepted 30 August 2007

Abstract

With the centenary of the first descriptions of 'hypersensitiveness' following pathogenic challenge upon us, it is appropriate to assess our current understanding of the hypersensitive response (HR) form of cell death. In recent decades our understanding of the initiation, associated signalling, and some important proteolytic events linked to the HR has dramatically increased. Genetic approaches are increasingly elucidating the function of the HR initiating resistance genes and there have been extensive analyses of death-associated signals, calcium, reactive oxygen species (ROS), nitric oxide, salicylic acid, and now sphingolipids. At the same time, attempts to draw parallels between mammalian apoptosis and the HR have been largely unsuccessful and it may be better to consider the HR to be a distinctive form of plant cell death. We will consider if the HR form of cell death may occur through metabolic dysfunction in which malfunctioning organelles may play a major role. This review will highlight that although our knowledge of parts of the HR is excellent, a comprehensive molecular model is still to be attained.

Key words: Hypersensitive response, pathogen, programmed cell death, resistance.

What is the hypersensitive response?

The resistance phenomenon known as the hypersensitive response (HR) was first described by pioneering plant pathologists around 100 years ago. H Marshall Ward saw variable responses by wheat cultivars to leaf rust (*Puccinia dispersa*, a synonym for *Puccinia triticina*), whilst EC Gibson noted that varieties of *Chrysanthemum* which were resistant to *Uredo* (*Puccinia*) *chrysanthemi* hyphae exhibited a localized plant cell death. Similar observations were made by Dorothea Marryat in the wheat–*Puccinia glumarum* (leaf yellow rust) pathosystem (Ward, 1902; Gibson, 1904; Marryat, 1907). However, the actual definition of the HR arose from the work of Elvin C Stakman (Fig. 1A) who investigated the responses of various cereal crops to differing *formae speciales* of the black stem rust fungus, *Puccinia graminis*. He noted cases where 'host plants exhibit a considerable degree of resistance to the fungus. … the host plant in such cases is … hypersensitive to the fungus'. He further defined 'hypersensitiveness … to indicate the abnormally rapid host plant death when attacked by rust fungi' (Stakman, 1915; Fig. 1B). A very general definition of the HR could therefore be an area of cell death that forms at the point of attempted pathogen ingress and which correlates with the exhibition of resistance. This retains Stakman's (1915) focus on a phenotype-based description, rather than defences which have come to be associated with this cell death phenomenon; for example, a myriad of defence genes and the production of anti-microbial secondary metabolites such as phytoalexins (Dangl and Jones, 2001; Dixon, 2001; Truman *et al*., 2006). It is possible that HR cell death is the consequence of such defences and this question will be briefly considered at the conclusion of this review.

A well-characterized HR is elicited following the injection of *Arabidopsis thaliana* leaves with high titres of bacteria (Fig. 2A). This may be assumed to represent, on a grand scale, HR events that would normally be occurring at only a few cells; albeit, this type of inoculation procedure can be accused of artificiality. Nevertheless, a HR elicited under 'natural' infection

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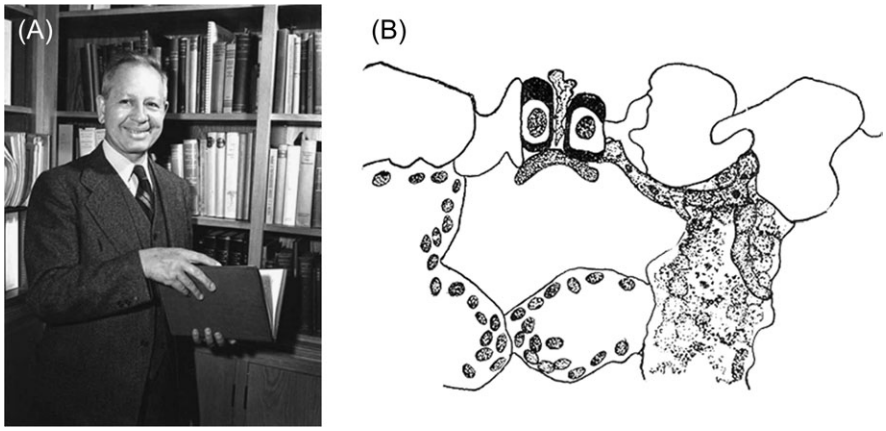

Fig. 1. EC Stakman and hypersensitiveness. (A) EC Stakman (1883–1971) Photo courtesy of the University of Minnesota Archives. (B) An image from the original plate within Stakman (1915). The original legend stated 'Oats inoculated with *Puccinia graminis hordei*, four days after inoculation. Infection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

conditions need not always be confined to a few cells. A prime example of a macroscopic-HR (macro-HR) is the lesions elicited by tobacco mosaic virus (TMV) in resistant tobacco (*Nicotiana tabacum*) cultivars (Fig. 2B). A distinctive feature of either macro- or micro-HR lesions is a sharp cellular delineation between the dead and surrounding living tissue (Fig. 2C, D). Despite these features, HR defies easy description. The HR forming during different plant–pathogen interactions can vary enormously in phenotype and timing at both macro and microscopic scales (Holub *et al*., 1994; Christopher-Kozjan and Heath, 2003; Krzymowska *et al*., 2007). Such variation only partially relates to differing infection strategies adopted by the various types of pathogen which elicit a HR, for example, oomycetes, fungi, bacteria or viruses, and undoubtedly reflects differences in underlying HR cell death mechanism(s). It may be misleading therefore to generalize between pathosystems and some significant variation between differing HR mechanisms will be referred to in this review. Further, some models for the HR are based on the use of cell death elicitors from necrotizing pathogens; for example, cryptogein from *Phytophthora cryptogea* and victorin from *Cochliobolus victoriae* which are, in fact, virulence factors. Although these could be thought to confuse HR studies there is clear evidence of overlap in necrotrophic pathogen-induced cell death and the HR (Gorvin and Levine, 2000). Therefore, although there will be bias towards studies employing HR-eliciting pathogens, data from necrotizing elicitors will also be included in this review as providing suggestive, if not definitive, insights into the HR mechanism(s).

Is the HR programmed?

It is almost axiomatic that the HR is an example of programmed cell death (PCD), but what is the nature of the programme? PCD occurs as a regulated process, mediated by the dying cell and frequently with contributions from surrounding tissue. It is perhaps more easily understood by contrast with non-programmed death, which classically results from cell lysis, for example, following mechanical damage, and where the biochemical and genetic status of the cell is irrelevant. In animal systems, the death programme can be initiated from the perturbation of survival factors, for example, disruption of the extracellular matrix or withdrawal of hormone growth factors (Stupack and Cheresh, 2003), and by the specific activation of the death programme as occurs with delivery of PCD-inducing granzymes into the targeted cell (Bots and Medema, 2006). Therefore, different forms of mammalian PCD are regulated by a myriad of death effectors and suppressors which are genetically encoded and expressed or activated within the dying cell.

Several early studies using inhibitors have demonstrated that the HR was dependent on active metabolism and protein synthesis (Nozue *et al*., 1977; Keen, *et al*., 1981; Aist and Bushnell, 1991). However, does the HR occur as a result of the withdrawal of survival factors and/or the initiation of death effectors? This question will be best addressed by identifying the components of the cell death programme, through which insight into the initiation and elaboration of the HR will be gained. A classic example of a death gene study was undertaken in the worm *Caenorhabditis elegans* where mutational approaches identified both effectors and suppressors (Hedgecock *et al*., 1983). Similar mutagenic approaches have been followed in *Arabidopsis* and mutants where the HR was compromised have been isolated. Although mutated genes have yet to be clearly associated with death effectors (see the Section, 'Towards a model for HR PCD'), these mutants could suggest that the perturbation of survival factors is a feature of the HR. On the other hand, large numbers of spontaneous death (SD) mutants have also

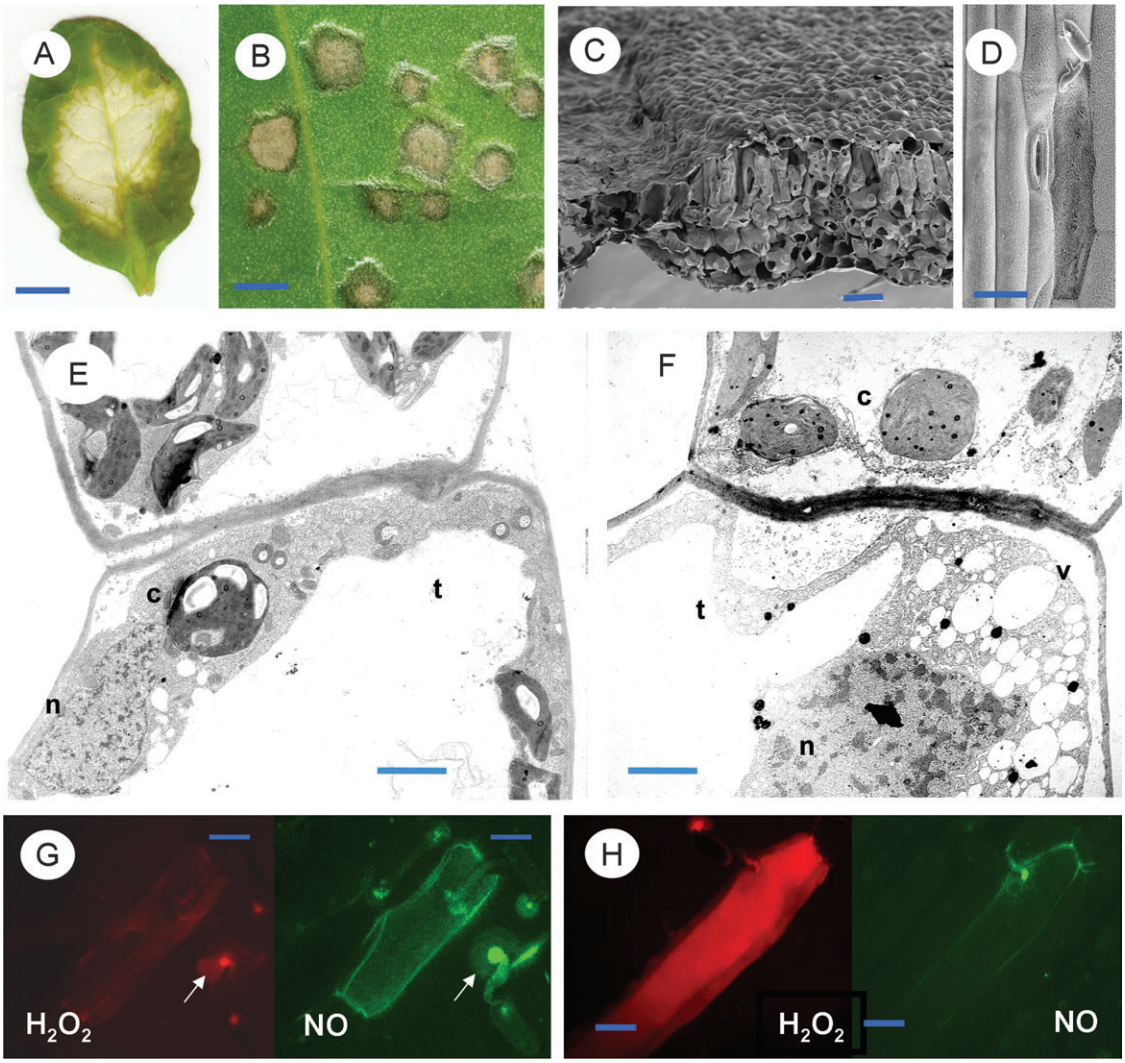

Fig. 2. Hypersensitive responses in various pathosystems. (A) A HR in *Arabidopsis thaliana* 24 h following infiltration with 1×10^6 cells ml^{-1} with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1*. Bar=1 cm (Image LAJ Mur). (B) Tobacco Mosaic Virus (TMV)-induced HR lesions forming on *Nicotiania tabacum* cv. Samsun NN at 72 h after inoculation. Bar=0.5 cm. (Image: LAJ Mur). (C) Scanning electron micrograph (SEM) of freeze-fractured TMV lesion at 72 h following inoculation. The demarcation between living and death tissue is clearly to be seen. Bar=100 μm. (Image, Darrell Gray and Iolo ap Gwynn, UW Aberystwyth, UK). (D) SEM of a collapsed cell in barley cv. P0-2 at 30 h after challenge with an avirulent strain of *Blumeria graminis* f.sp. *hordei* CC1 (*Bgh*). Bar=10 μm (figure taken from Prats *et al*., 2006, and is reprinted by kind permission of Oxford University Press). Transmission electron micrographs (TEM) of (E) uninfected tobacco leaf mesophyll cells and (F) equivalent cells at 48 h following inoculation with TMV within regions which were just exhibiting a loss in turgidity, which is an early macroscopic sign of the HR. Bars=5 μm (Image, Darrell Gray and Iolo ap Gwynn, UW Aberystwyth, UK). Indicated on both (E) and (F) are the (n) nucleus; tonoplast membrane (and arrowed), and chloroplasts (c). In (F), note the increased presences of large vesicles (v) and rounding of the chloroplasts with reduced white starch granules compared to (E). In (F), the mitochondria are dilated (not shown). In both (E) and (F) the central vacuole appears to be intact. Simultaneous imaging of H_2O_2 and NO in barley P0-1 at (G) 12.30 h and (H) 13.30 h following challenge with *Bgh*. H_2O_2, and NO associated with papilla-formation are arrowed. Samples are stained with DAF-2DA and Amplex Red (Bar = 10 μm).

been isolated, suggesting that mutated genes could be PCD suppressor genes (reviewed Lorrain *et al*., 2003).

Characterization of one SD mutant, the *Arabidopsis lesions simulating disease 1* (*lsd1*), however, has revealed both positive and negative PCD components associated with the HR. The *lsd1* mutant exhibits a runaway cell death when inoculated with HR-eliciting bacteria. This implicates LSD1 in the HR mechanism and contrasts with many other SD mutants (for example, *acd5*; Greenberg *et al*., 2000) where the HR is apparently unaffected. One role for LSD1 is to activate the expression of Cu/Zn superoxide dismutase (SOD) genes and this is correlated with the superoxide (O_2^-) sensitivity of the SD phenotype in *lsd1* (Jabs *et al*., 1996; Kliebenstein *et al*., 1999). LSD1 also acts negatively on at least two proteins via physical interaction to suppress cell death. A small gene family of *LSD-One-Like* (*LOL*) genes exists in *Arabidopsis* and LOL1 appears to be involved in promoting cell death via an unknown mechanism. The SD phenotype in *lsd1* was abolished when introgressed into a *lol1* background and,

crucially, HR type cell death was increased in LOL1 over-expressing plants (Epple *et al*., 2003). LSD1 also interacts with the AtbZIP10 transcription factor to prevent its translocation to the nucleus. Although over-expression of AtbZIP10 was only able to induce cell death in *lsd2* and not in non-mutant lines, the HR against *Hyaloperonospora* (formerly *Peronospora*) *parasitica* was suppressed in *atbzip10* mutants (Kaminaka *et al*., 2006). Therefore, these data have implicated LSD1 as a key 'switch' in the HR PCD mechanism, regulating both suppressor and effector functions. Further studies on how LSD1 responds to HR-inducing signals to release LOL1 and AtbZIP10 and possibly modify its activation of SOD expression are awaited with interest.

How far is the HR a plant version of apoptosis?

PCD has a long evolutionary history being described for cyanobacteria (Ning *et al*., 2002); bacteria (Rice and Bayles, 2003), invertebrates (Bergmann *et al*., 1998), and vertebrates, naturally including humans (Hengartner, 2000). In 'higher' organisms such as mammals, cell death could be seen as a quality control mechanism sculpting tissues and eliminating abnormal cells which are infected or potentially cancerous (Bergmann *et al*., 1998). Given the cross-kingdom prevalence of PCD, several plant scientists have seen parallels between the HR and the well-characterized animal PCD known as apoptosis; although it is ~1.5 billion years since the divergence of ancestral animal and plant cells (Wang *et al*., 1999).

Apoptosis is characterized by a series of hallmark cytological changes (Kerr *et al*., 1972) which the HR must resemble to some degree before the two processes could be considered to be influenced by conserved mechanisms. Apoptosis is marked by cellular shrinking, chromatin condensation, and margination and ruffling of the plasma membrane. Ruffling can lead to the packaging of cytoplasm and chromatin into apoptotic bodies which are phagocytosed by neighbouring cells. Phagocytosis does not always occur, in which case the cells will lyse in a process known as apoptotic necrosis (Majno and Joris, 1995). Besides apoptosis, other forms of mammalian cell death exist, each with distinctive morphologies (Kroemer *et al*., 2005). Apoptosis is often contrasted with non-programmed necrosis where there is cellular and organelle swelling and eventual lysis. Recently, authors refer to oncosis as non-programmed cell death and reserve the term necrosis for a post-mortem stage (Van Cruchten and Van den Broeck, 2002; Fink and Cookson, 2002). During oncosis there is a dramatic reduction in ATP production which may arise from the DNA repair enzyme poly (ADP)-ribose polymerase (PARP) depleting NAD levels in response to massive DNA damage (Pieper *et al*., 1999). During ATP-requiring apoptosis, PARP is specifically digested to avoid this effect (Shiokawa *et al*., 1997). However, oncosis need not be a totally unprogrammed event as increased intracellular calcium levels activates enzymes likely to derive signalling molecules, for example, phospolipase A_2, as well as proteolytic enzymes (Trump and Berezesky, 1996; Liu *et al*., 2004; Cummings *et al*., 2000; Golstein and Kroemer, 2007). Cell death can also be the end result of the autophagy process which is typified by the degradation of cellular compartments within vacuoles. Excellent reviews on the molecular events associated with autophagy have been recently published (Thompson and Viestra, 2005; van Doorn and Woltering, 2005). These include descriptions of the different types of autophagic processes broadly designated microphagy and macrophagy. In microphagy, the cytoplasmic portion to be consumed interacts with the vacuole, whereas in macrophagy the targeted cytoplasm is encapsulated within a double membraned autophagosome which then will merge with the vacuole (Yoshimori, 2004).

Perhaps surprisingly, cytological comparisons suggest that the HR exhibits features that are typical of each of the major forms of animal cell death (Table 1). A shrinking plant protoplast has been frequently reported during the HR (Aist and Bushnell, 1991; Heath, 2000*a*) which could

Table 1. *Comparison of mammalian cell death hallmarks and the plant hypersensitive response*

Hallmarks	Animal cell death[a]			Plant HR
	Apoptosis	Oncosis	Autophagy	
Cytoplasmic shrinkage	+[b]	–[c]	–	+[2]
Cytoplasmic swelling	–	+	–	–[2]
Nuclear shrinking	+	–	–	–[3]
Chromatin condensation	+	–	–	+[4]
DNA laddering	+	–	–	–[4]
Mitochondrial swelling	–	+	–	+[5]
Loss in $\Delta\Psi_m$	+	–	–	+[6]
ATP dependence	+	–	–	–[7]
Cytochrome *c* release	+	–	–	+[8]
Chloroplast disruption	NA[d]	NA	NA	+[9]
Caspase activation	+	–	–	–[10] +[11]
Cysteine protease	+	–	+	+[12]
Membrane blebbing	+	–	–	?[3]
Apoptotic bodies	+	–	+	–[13] +[12]
Vacuolization	–	–	+	+[14]
Two-membrane vesicles	–	–	+	+[15]

[a] Descriptions of mammalian cell death programmes are complex and somewhat controversial. These features given here are based on reviews by Fink and Cookson (2002); Kroemer *et al*. (2005); Bredesen (2007); Golstein and Kroemer (2007). Apoptosis, autophagy, and necrosis/oncosis are also referred to as Types I, II, and III cell death, respectively. Various plant studies of the HR are indicated; [2] Heath (2000*a*); [3] Mittler *et al*. (1997); [4] Ryerson and Heath (1996); [5] Bestwick *et al*. (1995); [6] Yao *et al*. (2004); [7] Xie and Chen (2000); [8] Kiba *et al*. (2006); [9] Stakman (1915); Seo *et al*. (2000); Boccara *et al*. (2007); [10] del Pozo and Lam (1998); [11] Hatsugai *et al*. (2004); [12] Van der doorn and Jones (2004); [13] Levine *et al*. (1996); [14] Bestwick *et al*. (1995); Mittler *et al*. (1997), Liu *et al*. (2005); [15] Liu *et al*. (2005).

[b] + = the consensus suggests that the feature is characteristic of the cell death process.

[c] – = the consensus suggests that this feature is not characteristic of the cell death process.

[d] NA = not applicable.

be reminiscent of apoptotic cytoplasmic collapse. In mammalian cells, cytoplasmic contortions during apoptosis are the result of cytoskeletal rearrangements and these are also features of the HR. Thus, a rapid cessation of cytoplasmic mobility (Naton *et al*., 1996) has been linked to cytoskeletal depolymerization during the HR (Chen and Heath, 1991; Gross *et al*., 1993; Kobayashi *et al*., 1994; Shimmen and Yokota, 2004). The use of microfilament (but interestingly, not microtubulin) assembly inhibitors have shown that rearrangements are important to the HR (Skalamera and Heath, 1998; Yun *et al*., 2003). However, in apoptosis, the ultimate fate of the shrunken protoplasm is packaging within apoptotic bodies, but, except for some isolated reports (for example, Levine *et al*., 1996), there seems to be little evidence of the formation of apoptotic bodies at the end of the HR process. The absence of apoptotic bodies has been linked to the presence of the plant cell wall. However, retraction of the membrane from cell walls and cytoplasmic disassociation into large bodies has been noted in *Streptomyces antibioticus* during PCD (Miguelez *et al*., 1999) and, therefore, the absence of similar bodies with the HR suggests a distinctive mechanism. Hence the role of cytoskeletal rearrangements during the HR is unclear, but could involve promoting the assembly of defence-associated protein complexes and/or regulating the movement of cytoplasmic vesicles/organelles (reviewed by Takemoto and Hardham, 2004).

Cytological analysis has also revealed other non-apoptotic features. Several groups, including ours, have noted the increased formation of large vesicles within the cytoplasm prior to HR-linked cellular collapse (Fig. 2E, F; and, for example, Bestwick *et al*., 1995; Liu *et al*., 2005). Working on a TMV-induced HR, Mittler *et al*., (1997) noted that these vesicles contained chloroplastic remnants with fragmented nuclear material remaining within the collapsing nucleus. Vesicles appeared to have mostly originated from the vacuolar membrane although invagination of the plasma membrane suggested that this could be another source. Vacuolation is symptomatic of autophagy and, more particularly, microphagy, since the central vacuole tonoplast is apparently intact until plasma membrane lysis (Mittler *et al*., 1997; Fig. 2F). As an alternative, Jones (2001) saw parallels between the HR and a 'programmed' oncosis, both being typified by considerable electrolyte leakage and organellar swelling (Xu and Mendgen, 1991; Bestwick *et al*., 1995).

Do apoptotic molecular markers feature in the HR?

If cytological studies are failing to provide definitive parallels between the HR and mammalian cell death, it could be argued that divergent evolution has blurred the cytological outputs of a conserved underlying (apoptotic?) death programme. In such a case, it would be expected that molecular apoptotic markers would be conserved to some degree during the HR. Chromatin condensation and processing by specific nucleases are widely considered to be distinctive markers for apoptosis. This may be visualized as a 'DNA ladder' on agarose gels; due to the nucleosome protecting ~180 bp sections of DNA against nuclease activity, or by Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling (TUNEL) labelling of free 3′ DNA ends. TUNEL staining during HR were first reported by Ryerson and Heath (1996) within their cowpea–rust pathosystem. There, TUNEL-positive cells were detected at 15 h following challenge; only some 3 h following haustorial formation. Both DNA laddering and TUNEL labelling were detected in oats (*Avena sativa*) and eggplant (*Solanum melongena*) challenged with both avirulent and virulent pathogens (Yao *et al*., 2002; Kiba *et al*., 2006) whilst cell death induced by Cucumber Mosaic Virus D Satellite RNA in tomato exhibited TUNEL positive DNA fragmentation (Xu and Roossinck, 2000). Further, TUNEL-positive cells and laddering were observed in tobacco cultures after treatment with the necrotizing elicitor elicitin (Sasabe *et al*., 2000). However, Che *et al.* (1999) found evidence of TUNEL-labelled nuclei but not laddering in the incompatible interaction between *Pseudomonas avenae* and rice seedlings. Mittler *et al.* (1997) similarly could find no evidence of laddering during a HR elicited in tobacco by TMV. However, at later stages, TUNEL-positive nuclei were detected, acting, at least in part, through the action of a calcium-dependent, nucleus-located endonuclease NUCIII (Mittler and Lam, 1997; Mittler *et al*., 1997). Taken together, it would appear that chromatin processing in the HR does show apoptotic hallmarks, albeit with pathosystem-specific variation.

Many recent studies on HR have focused on the mitochondrion as this organelle plays an important role in mammalian apoptosis (Lam *et al*., 2001; Greenberg and Yao, 2004). In animal cells, upon receipt of a pro-apoptotic signal, for example, Bax proteins, permeability transition pores (PTP) form which disperse the membrane potential ($\Delta\Psi_m$). PTP formation correlates with the release of a large number of proteins including cytochrome *c* and an immature version of the cysteine-dependent aspartate protease (caspase)–procaspase 9. Cytochrome *c* interacts with procaspase 9 and a chaperone protein, Apaf1, to form an apoptosome complex: via an ATP-dependent mechanism the apoptosome matures caspase 9 to initiate a proteolytic cascade (Green and Kroemer, 1998; Grűtter, 2000; Lalier *et al*., 2007; Bao and Shi, 2007). Hence, although the apoptotic mitochondrion is malfunctioning, apoptosis requires ATP generation which is achieved by maintaining electron flow through respiratory complex IV. During the HR, there is early mitochondrial swelling (Bestwick *et al*., 1995) and a rapid loss of mitochondrial function, as noted in parsley (*Petroselinum crispum*) cells challenged with a HR-eliciting strain of *Phytophthora infestans* (Naton *et al*., 1996). In a recent more detailed

analysis, Yao *et al*. (2004) examined mitochondrial changes in transgenic *Arabidopsis* protoplasts encoding the HR-eliciting bacterial avirulence gene, *avrRpt2*, that was regulated by an inducible genetic switch. Following transgene induction there was a rapid loss in $\Delta\Psi_m$ which could be slightly suppressed by the PTP inhibitor cyclosporin A. This provides firm evidence of pore formation in mitochondria within cells undergoing a HR. However, unlike apoptosis, there appears to be little maintenance of ATP generation during the HR (Xie and Chen, 2000). Addition of the bacterial elicitor harpin to tobacco cell cultures also induced a rapid loss in cellular ATP levels prior to cell death, most likely by loss of cytochrome pathway respiration (Xie and Chen, 2000). Several laboratories have also described cytochrome *c* release before plant cell death; for instance, following the application of harpin or victorin (Curtis and Wolpert, 2002; Krause and Durner, 2004) and during a HR elicited by *Pseudomonas syringae* pv. *tabaci* in eggplant (*Solanum melongena*; Kiba *et al*., 2006). However, these latter observations need not indicate an apoptotic step in the HR, as there is no evidence for an apoptosome-like complex in plants. Further, new studies in animal systems are suggesting that many of the proteins released by the mitochondrion have little role in the initiation of apoptosis and may simply mark a non-programmed phase of cell death. (Ekert and Vaux, 2005). In the case of plants therefore, cytochrome *c* release could simply reflect a final phase of mitochondrial dysfunction.

Activation of caspases, which act to potentiate the death process, is another hallmark for mammalian apoptosis (Chipuk and Green, 2005). In plants, responses to pathogens have long been recognized to involve the activation of proteases (Pautot *et al*., 1993; Avrova *et al*., 1999; Solomon *et al*., 1999) and inhibitor studies have clearly indicated a role for these in the HR, particularly the papain-class of cysteine proteases (Belenghi *et al*., 2003; van der Hoorn and Jones, 2004; Sanmartin *et al*., 2005). However, papain proteases are not caspases, which other studies have suggested are active during the HR. These studies have employed tetrapeptides which are active, site-specific competitive inhibitors of mammalian caspases; most often tyr-val-arg-asp (YVAD; a caspase 1 inhibitor) or asp-glu-val-asp (DEVD; a caspase 3 inhibitor) to demonstrate the contribution of caspase-like proteases to the HR (del Pozo and Lam, 1998). It should be noted that these do not suppress all forms of HR. In tobacco, a HR elicited by *Pseudomonas syringae* pathovar (*P. s.* pv.). *tabaci* but not *P. s.* pv. *maculicola* could be suppressed by DEVD and YVAD inhibitors (Krzymowska *et al*., 2007). Such pharmaceutical approaches are bedevilled by questions concerning specificity (Rozman-Pungercar *et al*., 2003), and this is especially the case here, as no plant gene exhibiting high homology to caspases has been identified. As a solution to this conundrum, two plant alternatives to caspases have been proposed, metacaspases and vacuolar processing enzymes (VPE), both of which exhibit some homology to caspases (reviewed by Watanabe and Lam, 2004; Sanmartin *et al*., 2005).

Pathogen-induced expression of metacaspases has been reported (Hoeberichts *et al*., 2003); however, to date, no role during the HR has been established. Based on sequence and structure, nine *Arabidopsis* metacaspase genes have been described (Watanabe and Lam, 2004). AtMC4 and AtMC9 have been shown to be arginine/lysine-specific cysteine proteases which did not cleave caspase-specific target sites (Vercammen *et al*., 2004). If this specificity is shared by all metacaspases, given the results of using DEVD and YVAD inhibitors, it is difficult to see a role for metacaspases in the HR. By contrast, VPEs have emerged as major effectors of HR cell death. Hatsugai *et al*. (2004) observed that a TMV-elicited HR was inhibited by both VPE and mammalian caspase I inhibitors. Suppressing VPE expression through a gene silencing approach greatly compromised HR cell death and associated DNA laddering, caspase-like activity and also vacuolar collapse. Rojo *et al*., (2004) focused on an *Arabidopsis* γVPE knock-out mutant which exhibited a less-dramatic but still significant reduction in cell death and caspase activity when challenged with an HR-eliciting bacterial pathogen. Besides indicating that VPE are the source of much of the HR–associated caspase-like activity, these data implicate vacuolar processing and therefore autophagy, as an important feature of the HR (Jones, 2001; Yamada *et al*., 2004). Seay and Dinesh-Kumar (2005) saw a possible parallel between the carboxypeptidase Y (CPY) pathway in yeast and VPE action during the HR. Both γVPE and CPY accumulate in small precursor vesicles which are translocated to vacuoles within which they are N-terminally cleaved to activate proteolytic activity. The targets of VPE activity remain to be established, but an ultimate consequence could be tonoplast rupture leading to the release of hydrolytic enzymes.

A complementary study has indicated that autophagy also plays a role in defining the sharp edge around the HR (Fig. 2C, D). A high-throughput gene silencing screen in *Nicotiana benthamiana* found that lines suppressed in *ATG6*/*Beclin1* exhibited runaway cell death around a HR lesion elicited by TMV or bacteria pathogens. *ATG6*/*Beclin1* is a protein involved in vesicle nucleation and autophagosome formation in yeast and autophagosomes were observed around the edge of the HR (Liu *et al*., 2005). Clearly, one role for autophagosomes could be the neutralization of death-eliciting signals emerging from the HR lesion. Induction of *ATG6*/*Beclin1* gene expression was also observed within the HR prior to lesion formation. This could suggest that *ATG6*/*Beclin1* aids in the pronounced vacuolation seen during the HR (Fig. 2E; Seay and Dinesh-Kumar, 2005) although it must be noted that, in *ATG6*/*Beclin1* suppressed lines, the actual HR

lesions (if not the surround) were apparently normal. At various stages *ATG6/Beclin1* are likely to interact with other autophagy components which appear conserved between yeast and *Arabidopsis*. Homologues of yeast macrophagy components; TOR kinase and the ATG proteins which are involved in vesicle expansion and autophagosome enclosure have been detected within the *Arabidopsis* genome. Tantalizingly, although some knock-outs or RNA interference lines, other than for *ATG6/Beclin1* lines, have been generated, the responses of these lines to pathogens have yet to be reported (Thompson and Vierstra, 2005; Patel *et al*., 2006).

Considering all of these different types of data together (Table 1), the HR does indeed have some apoptotic features but also autophagic ones and, indeed, some are reminiscent of oncosis. In this, the HR has interesting parallels with PCD in non-chordate phyla. The famous *Dictyostelium discoideum* transition from the free-living unicellular form to a stalked multicellular structure is accompanied by PCD within the stalk. This form of PCD is characterized by vacuole formation, chromatin condensation, but no DNA-fragmentation (Levraud *et al*., 2003). Vacuolation is also a feature of PCD in cyanobacteria (Ning *et al*., 2002) and unicellular eukaryotes (Ameisen, 2002). However, it could be a mistake to see the HR as basal form of PCD shared amongst non-animal phyla. There is no reason that would prevent plants evolving a cell death programme as complex as apoptosis. It may, therefore, be more appropriate to consider HR to be a distinctive form of cell death; a view which will surely facilitate the integration of cytologicals aspects with other distinctive features of the HR. In drawing together the various pieces of knowledge relating to the HR, it will become clear that we have good understanding of only parts of the jigsaw, with our appreciation of how they fit together being very limited (Fig. 3).

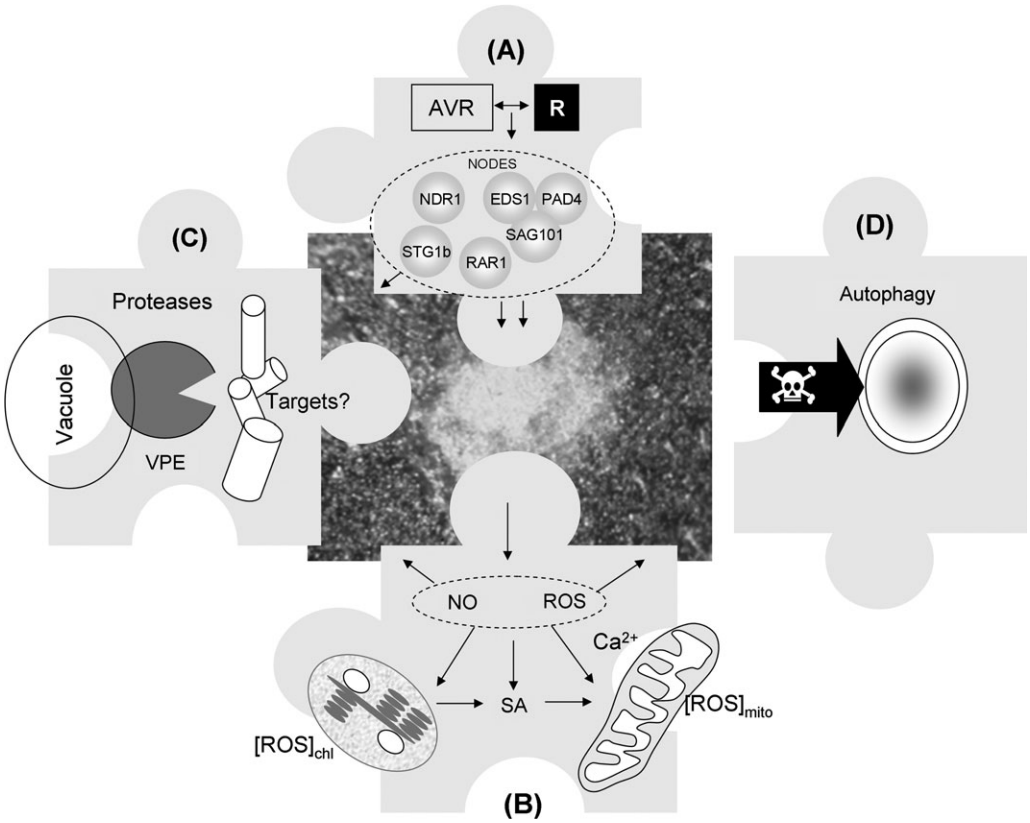

Fig. 3. Assembling the Hypersensitive Response jigsaw. Current investigations into the HR, mainly employing bacterial pathogens, are following different approaches leading to a detailed knowledge of certain aspects but the interaction is currently obscure. This situation is here depicted as pieces of a jigsaw, yet to be developed as a coherent whole. (A) The interaction of pathogen avirulence gene (AVR) with plant resistance gene (R) gene initiates the HR and also a large number of associated defences. Primarily, genetic approaches have identified signalling nodes (NDR1, EDS1-PAD4-SAG101, STG1b, RAR1) which are variously used by many R gene products to initiate defences and the HR. (B) Via mechanisms which are currently obscure, R-avr gene interaction leads to the persistent production of reactive oxygen species (ROS), nitric oxide (NO), salicylic acid (SA), and calcium (Ca^{2+}) fluxes. Effects on the organelles resulting in the increase of mitochondrial ($[ROS]_{mito}$) and chloroplastic ($[ROS]_{chl}$) are highlighted. (C) R-avr interaction and ROS and NO generation initiate the activity of caspase-like proteases which are likely to be vacuolar processing enzymes (for details see main text). The mechanism of activation of VPEs is currently obscure as are their proteolytic targets. VPE activity may ultimately lead to tonoplast rupture. (D) Recently, the containment of cell death around the HR-lesion periphery has been associated with the formation of double membraned autophagosomes which presumably contain death eliciting factors (Liu *et al*., 2005). The background image is a TMV-induced HR lesion at 72 h following inoculation of tobacco (*Nicotiana tabacum*) cv. Samsun NN.

Towards a model for HR PCD

The first piece of the jigsaw: gene-for-gene interactions

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 (Flor, 1956). Subsequently, a large number of *R* genes have been cloned and can be broadly classified into five classes (Martin *et al*., 2003). 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 Martin *et al*., 2003), 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 (Zhou *et al*., 1995, 1997; Tang *et al*., 1996). 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 (Shirasu and Schulze-Lefert, 2003). RAR1 proteins have two cysteine and histidine rich domains (CHORD), one of which binds to the HSP90, a molecular chaperone (Takahashi *et al*., 2003). The levels of RPM1 are reduced in *rar1* and *hsp90 Arabidopsis* mutants (Hubert *et al*., 2003) 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 (Austin *et al*., 2002; Takashashi *et al*., 2003; Bieri *et al*., 2004) and also with two components of the E3 ubiquitin ligase; SKP1 and CUL1 (Azevedo *et al*., 2002). The function of E3 enzymes is to add ubiquitin to specific proteins and thereby target them for degradation with the 26S proteasome (Vierstra, 2003). 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 (Aarts *et al*., 1998). One node is dependent on EDS1 and PAD4 which is required for the function of the TIR-NBS-LRR class of *R* genes (reviewed by Wiermer *et al*., 2005). 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 (see below). The alternative regulatory node is utilized by the CC-NBS-LRR *R* gene class and passes via NDR1 (Century *et al*., 1997; Coppinger *et al*., 2004). 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 (Day *et al*., 2006).

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 (Baker and Reddy, 1998). No such intimate association with, for example, proteases, has been described for RGP.

A metabolic piece of the jigsaw, chemical 'death' signals

As a result of impressive efforts by many groups we now know a great deal about particular chemical signals that are preferentially produced during the HR. In a crucial link with the RGP, the production of several signals is influenced by associated signalling modules; EDS1-PAD4-SAG101 being perhaps the best example (Wiermer

et al., 2005). However, a mechanistic understanding of how this occurs still eludes us. Indeed it may be such that chemical signals are, in fact, the effectors through which cell death is initiated (Fig. 3B).

An early study showed a rapid alkalinization of the apoplasm via H^+ influx/K^- efflux and Ca^{2+} influxes in soybean cultivars on application of the syringolide product of avrD (Atkinson *et al*., 1996). Calcium influxes in particular have been associated with cell death in animal cells and also plants. Measurements of calcium changes in *Arabidopsis* demonstrated a persistent rise in cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) that was specific to HR-eliciting bacteria (Grant *et al*., 2000). Sustained increases in $[Ca^{2+}]_{cyt}$ during a HR have been reported in cowpea challenged by a rust fungus (Xu and Heath, 1998) and in *Nicotiana plumbaginifolia* cells treated with cryptogenin (Lecourieux *et al*., 2006) as well as cell death in mammal tissues (Orrenius *et al*., 2003). Such are clearly important to the HR as application of the calcium channel blocker, $LaCl_3$, suppressed cell death in soybean cultures inoculated with avirulent bacteria (Levine *et al*., 1996) and also *in planta* with the cowpea-rust fungus pathosystem (Xu and Heath, 1998). More recently, the use of a calcium cyclic nucleotide gated channel (CNGC) blocker suppressed a HR in *Arabidopsis*. Previously, mutation of the CNGC channel in the *defence not death* (*dnd1*) mutant had been shown to abolish HR, so that taken together, these data demonstrate the centrality of calcium in HR cell death (Clough *et al*., 2000; Ali *et al*., 2007).

Perhaps the most heavily investigated death signals associated with the HR are partially reduced species of oxygen (ROS), which have long been recognized to orchestrate the HR (Levine *et al*., 1994). The link with the HR was first made by the Doke group who noted superoxide (O_2^-) production prior to a HR elicited by *Phytophthora infestans* on potato tubers and TMV in tobacco (Doke, 1983; Doke and Ohashi, 1988). Such an 'oxidative burst' has now been described during the early stages of many forms of HR (Lamb and Dixon, 1997) and occurs prior to cell death (Heath, 2000*b*). The majority of workers (e.g. Doke and Ohashi, 1988) have noted that ROS detoxification by enzyme scavengers was an effective method to delay the HR. Correspondingly, tobacco plants with reduced levels of ascorbate peroxidase were 'hyper-reactive' to challenge by avirulent bacteria (Mittler *et al*., 1999). In a crucial link with calcium influxes, the generation of ROS was found to be influenced by Ca^{2+}, via a reciprocal control mechanism (Levine *et al*., 1996; Grant *et al*., 2000).

The oxidative burst has been linked to an alkalinization-responsive peroxidase or amine oxidase (Bolwell *et al*., 2002; Allan and Fluhr, 1997) and the contribution of organellar ROS is now being increasingly appreciated (see below). However, most workers investigating the apoplastic oxidative burst have focused on NADPH oxidases as the major generating system. Plant versions of the gp91phox component of mammalian NADPH oxidase have been extensively characterized in *Arabidopsis* (respiratory burst oxidase homologues, *rboh*) *AtrbohA–AtrbohF*; and also in *Nicotiana benthamiana*, *NbrbohA,* and *NbrbohB* (Torres *et al*., 2002; Yoshioka *et al*., 2003). These studies have suggested that NADPH oxidases are major sources of ROS during the HR. However, it should be noted that, in mammalian neutrophils, the primary function of NADPH oxidases is not to generate ROS but, in conjunction with other ions fluxes, to increase the pH of the neutrophil phagosome (Segal, 2005). Plant NADPH oxidases could fulfil a similar role in the apoplast, thereby initiating ROS indirectly via alkalinization–responsive peroxidases (Bolwell *et al*., 2002).

If ROS are indeed major PCD effectors the mechanism of their action requires characterization. Perhaps the most obvious mechanism for cell death is lipid peroxidation. Hydroxyl radicals ($^{\cdot}OH$) will readily abstract a proton from, for example, phospholipids to initiate a lipid radical–lipid hydroperoxide (LOOH) chain reaction ($LH+OH \rightarrow L+H_2O$; $L^{\cdot}+O_2 \rightarrow LOO^{\cdot}$; $LOO^{\cdot}+LH^{\cdot} \rightarrow LOOH+L^{\cdot\cdot}$etc). The peroxidation of non-saturated groups within acyl chains [also known as polyunsaturated fatty acids (PUFA)] in a membrane would severely disrupt its integrity. Significantly, several authors have measured lipid peroxidation during a HR (fKeppler and Novacky, 1986; May *et al*., 1996; Kenton *et al*., 1999). However, LOOH need not only derive from oxidative damage but also from lipoxygenase (LOX) action and relative importance of each mechanism to the HR is attracting considerable interest (Göbel *et al*., 2001; Jalloul *et al*., 2002; Montillet *et al*., 2002, 2004). The major plant phospholipid acyl chains are C18:3 and (in species such as *Arabidopsis*), C16:3 (primarily localized in the chloroplast). The derivation of either 7 or 11-LOOH from C16:3 and 9 or 13-LOOH from C18:3 PUFA reflects the LOX-driven proton abstraction at the unsaturated ω-7 carbon position. Such specificity will not be shown by ROS-dependent peroxidation and hence, the accumulation of other LOOH forms (i.e. 10 and 14-LOOH in C16:3 or 12 and 14-LOOH in C18:3) are markers for oxidative events (for an excellent description of the underlying chemistry, see Montillet *et al*., 2002). During the HR elicited by *Xanthomonas campestris* pv. *malvacearum* in cotton, Jalloul *et al*. (2002) found that 9-LOOH were the predominant PUFA-derivative formed leading them to question ROS as a major source of LOOH. However, later work by Montillet *et al*. (2002, 2004, 2005) examining a HR elicited by *P. s.* pv. *syringae* in tobacco found that ROS-derived LOOH predominated in the light but only LOX-derived 9-LOOH was observed in the dark. Hence, the contribution of ROS-derived LOOH to the HR is probably pathosystem and environment dependent. By contrast, LOX-derived LOOH appears to be a more

general feature of the HR, and may be more central to its elaboration. The transcriptional up-regulation of LOX has been frequently reported during the HR (Porta and Rocha-Sosa, 2002), and has been correlated with increased activity of phospholipase A_2, an enzyme which will supply PUFA to LOX from the membrane phospholipids (Göbel *et al*., 2001). The importance of LOX products to the HR was demonstrated by Rancé *et al.* (1998) who observed reduced cell death in LOX-suppressed lines following challenge with an avirulent race of *Phytophthora parasitica* var. *nicotinia*. A more recent study specifically reduced 9-LOX gene transcripts in potato, but this had little effect on a *P.s.* pv. *maculicola* elicited HR. However, interestingly, there seemed to be a compensatory increase in oxidative and 13-LOX-derived LOOH, suggesting that the source of LOOH may not be important to the HR and also that their relative production is under mutual regulation (Göbel *et al*., 2003). Within a cell killing mechanism, LOX could simply act to augment membrane damage (Croft *et al*., 1990), but they are also involved in the production of a range of defence products, some of which are cytotoxic (Knight *et al*., 2001; Alméras *et al*., 2003).

An alternative oxidative death mechanism could be to modify the levels or redox status of antioxidant pools, for instance of glutathione (GSH). Levels of GSH have been noted to increase during the HR (May *et al*., 1996; Vanacker *et al*., 1998) where they may exist primarily in the oxidized form (May *et al*., 1996). It is certainly the case that co-application of reduced glutathione with bacterial pathogens can be an effective method to suppress the HR (Mur *et al*., 2005; Kiba *et al*., 2006). Further, initiation of cell death in the *lsd1* mutant was influenced by glutathione levels, if not by redox status (Senda and Ogawa, 2004). GSH often functions in association with ascorbate; and it may be relevant to the HR that the *Arabidopsis* reduced ascorbate mutant *vtc1* exhibits a limited SD phenotype (Pavet *et al*., 2005). Such data suggest the existence of redox sensitive cellular components playing a role in the HR. The possibility that these include the mitochondrion will be explored below.

Interestingly, although the *Arabidopsis AtrhobD* knockout mutants exhibited reduced levels of ROS, they showed increased cell death when introduced into an *lsd1-1* background or challenged with avirulent bacteria (Torres *et al*., 2005). One interpretation of these data was that the stoichiometry of ROS and nitric oxide (NO) levels governs whether cell death is initiated or suppressed (Delledonne *et al*., 2001). The generation of NO from soybean cultures inoculated with avirulent bacteria was first noted by Delledonne *et al*. (1998) and was coincident with ROS generation. Our own observations with the HR in barley conditioned by *Mla1* against powdery mildew (*Blumeria graminis*) indicate complete spatial correlation between NO and oxidative bursts, although NO production does appear to be initiated first (Fig. 2G, H). The importance of NO in the HR was established in seminal works where transgenic plants expressing either bacterial nitric oxide dioxygenases or flavohaemoglobins were used to reduce NO levels have shown that this plays a vital role in the development of the HR (Zeier *et al*., 2004*a*; Bocarra *et al*., 2005). It remains to be established, however, how far NO and ROS act in isolation or synergistically to confer cell death. The work of de Pinto *et al*. (2002), for example, using tobacco BY-2 cells suggested that synergistic NO and H_2O_2 was an important factor in the initiation of cell death. NO alone did not induce cell death and, unlike H_2O_2, had minimal effects on ascorbate and glutathione antioxidant systems. However, the simultaneous generation of NO and H_2O_2 resulted in cytological and biochemical features which were reminiscent of the HR. A more recent investigation focusing on gene expression in whole tobacco plants has suggested that NO and H_2O_2 effects may overlap to a great extent (Zago *et al*., 2006). This study analysed cDNA-amplified fragment polymorphisms (AFLPs) to indicate gene expression in wild type and catalase-deficient (and therefore H_2O_2-accumulating under elevated light conditions) transgenic tobacco plants with and without treatment with the nitrosylative donor, sodium nitroprusside (SNP). Of 214 differentially expressed transcripts only 16 required both NO and H_2O_2, whilst 152 could be modulated by either NO or H_2O_2. It may be significant that no death-associated genes such as LOLI and LSD1 were detected in this study, leaving open the possibility that NO-ROS synergy is more important in the cell death process. Attempts to address such questions would clearly be aided if the mechanism(s) of NO production could be defined so that mutants could be generated. Unfortunately, the mechanisms of NO generation have yet to be clearly defined and this, along an over-use of pharmaceutical agents designed against mammalian nitric oxide synthase enzymes has inhibited progress in the plant NO field. However, notable advances have been made in establishing S-nitrosylation of proteins and glutathione as a significant regulatory component in plant defence (Feecham *et al*., 2005; Rusterrucci *et al*., 2007). Interestingly, the metacaspase AtMC9 is S-nitrosylated at its active site cysteines to suppress autoprocessing of the pro form as well as proteolytic activity. However, mature forms of AtMC9 were not susceptible to such modification and regulation (Belenghi *et al*., 2007). If AtMC9 has a role in the HR, it may be that *denitrosylation* is an early event, following which the mature protease would be impervious to NO suppression.

The mitochondrion during a HR; a death signal integrator?

Recently, highly compelling models have suggested that mitochondria could play a central role in integrating the effects of multiple death signals (Fig. 4; Jones, 2001;

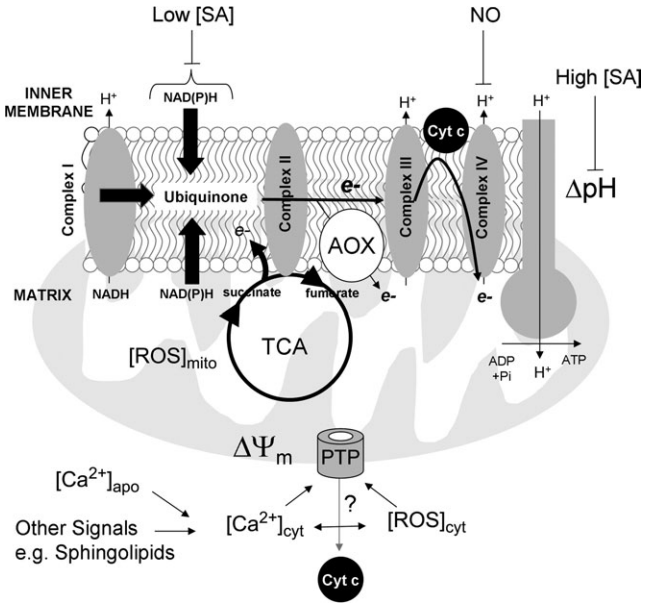

Fig. 4. Mitochondrial responses during the hypersensitive response. The alteration in respiratory electron transport and the formation of permeability transition pores (PTP) are highlighted (see main text for references). Influxes of calcium from apoplastic sources and other signals such as sphingolipids will increases in cytoplasmic calcium ($[Ca^{2+}]_{cyt}$. It is likely that plant bax-inhibitors (BI-I) act to maintain $[Ca^{2+}]_{cyt}$ homeostatis and their action is either swamped or suppressed during the HR. Increases in $[Ca^{2+}]_{cyt}$ and reactive oxygen species $[ROS]_{cyt}$ initiates depolarization of the mitochondrial membrane ($\Delta\Psi_m$) which is intimately associated with the formation of PTPs. Increases in $[Ca^{2+}]_{cyt}$ are also initiated by sphingolipids such as ceramides. The electron transport chain functions to form a pH gradient (ΔpH) across the membrane to generate the proton motive force required for ATP generation. Nitric oxide (NO) will inhibit complex IV and low levels of salicylic acid (SA) will suppress electron donation from NADH dehydrogenases. Both will lead to over-reduction of cytochrome components leading to the production of mitochondrial ROS ($[ROS]_{mito}$) leading to oxidative damage. SA also induces the expression of alternate oxidase (AOX) which will divert electrons from the ubiquinone pool to form H_2O with the excess energy being lost as heat. AOX can therefore prevent cytochrome over-reduction and has an anti-oxidant effect. Higher levels of SA can act as an uncoupler to dissipate ΔpH. The release of cytochrome *c* (possibly via the PTP) is a feature of many of these death effectors and, by further contributing to interrupted electron flow, adds to the generation of $[ROS]_{mito}$. Defence-associated signals will therefore catastrophically affect mitochondrial integrity, ATP generation, and associated generation of NAPH via the TCA cycle. It is hypothesized that this catastrophic loss in organelle function is a key event in HR type cell death.

Amirsadeghi *et al*., 2006). In particular, it may be that changes in the redox status of this organelle are important for cellular metabolic dysfunction leading to the HR. Treatments with harpin or avirulent *P. syringae* strains in *Arabidopsis* demonstrated that there was a rapid (~1.5 h) production of mitochondrial ROS ($[ROS]_{mito}$) that slightly precedes PTP formation, the dissipation of $\Delta\Psi_m$, and a loss in ATP generation (Møller, 2001: Yao *et al*., 2002; Yao and Greenberg, 2006). Such events will have catastrophic effects on mitochondrial function and whole cell viability as disruption of mitochondrial electron flow is sufficient to initiate plant DNA laddering (Vanlerberghe *et al*., 2002). Based on current evidence, it seems likely that the initiation of $[ROS]_{mito}$ occurs due to many of the death signals already described (Fig. 4). In mammalian mitochondria, PTP formation is intimately associated with the calcium fluxes (Crompton, 1999) which act, at least partially, through the initiation of $[ROS]_{mito}$ (Kowaltowski *et al*., 1996). Calcium influxes will also initiate NO biosynthesis (Ali *et al*., 2007) which also promotes $[ROS]_{mito}$ through inhibition of Complex IV (cytochrome *c* oxidase) to promote PTP formation further. Although not discussed in this review, sphingolipids are now being seen as cell death elicitors and these will also act to disrupt mitochondrial function (Yao *et al*., 2004; Townley *et al*., 2005). Cytochrome *c* release appears to be dependent on $[ROS]_{mito}$ and will further disrupt electron transport, perhaps presenting a mechanism through which ROS effects are potentiated within the mitochondrion (Amirsadeghi *et al*., 2007).

Into the gang assault by death signals on the mitochondria, the contribution of SA must be considered. Although classically associated with systemic acquired resistance (SAR; Ryals *et al*., 1996), many authors have observed that the application of salicylic acid (SA) to plants initiated cell death and that phenotypes of many SD mutants are associated with elevated SA accumulation or associated signalling (Naton *et al*., 1996; O'Brien *et al*., 1998; Lorrain *et al*., 2003). Within the context of HR, SA may act mainly by promoting the production of ROS (Lamb and Dixon, 1997). One mechanism through which SA increases ROS involves the inhibition of mitochondrial ATP synthesis via a disruption of the electron flow through the respiratory transport chain (Xie and Chen, 2000). A more extensive analysis demonstrated that, at <1 mM concentrations, SA acted as a respiratory uncoupler, i.e. suppressing ΔpH and thus ATP generation, whilst at >1 mM concentration SA blocked electron flow from the NADH dehydrogenases to the ubiquinone pool (Norman *et al*., 2004). SA-elicited respiratory dysfunction correlated with the induction of alternate oxidase (AOX) which interrupts electron flow at the ubiquinone pool to reduce oxygen, with the excess energy being lost as heat. The AOX pathway allows high rates of mitochondrial respiration to be maintained under conditions of stress and functions to suppress $[ROS]_{mito}$ production by preventing over-reduction of components such as ubiquinone (Yip and Venlerberghe *et al*., 2001). Therefore, SA-associated AOX induction may be an antioxidative strategy in the wake of respiratory disruption, which is presumably unsuccessful during the HR. Significantly, antisense AOX lines exhibited cell death when the cytochrome pathway was down-regulated (Vanlerberghe *et al*., 2002).

Another strand of data could also implicate the mitochondrion as an important player in the cell death process. In mammalian cells, both *Bcl-2* and *Bcl-xl* function by preventing the formation of PTP in response to pro-apoptotic factors, for example, by the protein Bax (Green and Reed, 1998). It is of great interest therefore,

that when mammalian *Bcl-xl* and *C. elegans ced9* were expressed in tobacco the HR elicited by TMV was suppressed (Mitsuhara *et al*., 1999). Putative plant equivalents of Bcl-2 have been found, the so-called BAX inhibitor (*BI-1*) genes. Plant *BI-1* gene suppressed PCD in yeast and human cells (Kawai *et al*., 1999; Bolduc *et al*., 2003) and down-regulation of tobacco *BI-1* accelerated abiotic induced cell death (Bolduc and Brisson, 2002) whilst over-expression of an *Arabidopsis BI-1* in rice reduced cell death induced by a fungal elicitor (Matsumura *et al*., 2003). A greater mechanistic understanding of *BI-I* has arisen following the work of Ihara-Ohori *et al*. (2007). These authors established that *BI-I* failed to suppress Bax-cell death induced in yeast mutants believed to be perturbed in transmembrane ion fluxes mediated via Ca^{2+} ATPases. In plants, over-expression of *AtBI-1* attenuated cytosolic calcium accumulation following treatment with H_2O_2. Further, BI-I was shown to interact with the calcium signal component, calmodulin. Hence, BI-I plays a role in calcium homeostasis and signalling and therefore, unlike mammalian Bcl-2 (Foyouzi-Yossefi *et al*., 2000), is likely to act only indirectly on mitochondrial-elicited cell death (Fig. 4).

The chloroplast: throwing light on the HR?

A growing body of data is implicating the chloroplast as well as the mitochondrion as a significant contributor to the HR; mostly likely via the generation of ROS. At least some forms of HR appear to be influenced by light and much ROS-dependent lipid peroxidation during the HR may be light dependent (Zeier *et al*., 2004*b*; Montillet *et al*., 2005). It seems likely that there are two linked mechanisms through which chloroplasts could contribute to ROS production during the HR (Fig. 5). One involves the photoproduction of ROS when photon intensity is in excess of that required for CO_2 fixation; i.e. excess excitation energy (EEE; reviewed by Karpinski *et al*., 2003; Szabo *et al*., 2005; Asada, 2000). The second mechanism is the generation of ROS associated with the release of porphryin ring-containing chlorophyll products from the reaction centres as part of catabolic processes. Figure 5 outlines the generation of EEE and some important dissipatory mechanisms.

Examination of a bacterially elicited HR in *Arabidopsis* showed that a reduction in chlorophyll fluorescence (F_v/F_m), which was indicative of PSII damage, occurred at ~ 9 h (Alméras *et al*., 2003). In a TMV-inoculated tobacco a decrease in photosynthetic electron flow has been noted by 4 h following a shift to conditions which allowed the elicitation of a HR (Seo *et al*., 2000). A more recent study has suggested even earlier photosynthetic effects during a HR. Boccara *et al*. (2007) examined chloroplast responses in *Nicotiana* sp. following treatment with harpin using optical coherence tomography in conjunction with confocal laser scanning microscopy. Within as little as 30 min chloroplast membrane changes were observed which were consistent with a state transition (Fig. 5A) which implied early conditions of EEE.

The most comprehensive analysis of photosynthetic performance during cell death has not been carried out with a HR but in *lsd1* (Mateo *et al*., 2004). Stomata were observed to be closed in *lsd1* which would lead to EEE due to a rapid fall in internal CO_2. As this mutant has reduced expression of SOD (Epple *et al*., 2003) and also catalase levels, this resulted in increased, presumably peroxisomally located, ROS. Hence, the *lsd1* mutant was compromised in photorespiratory dissipation of EEE (Fig. 5B) and this was linked to the SD phenotype (Mateo *et al*., 2004). Further, the reduced levels of CuZn SOD in *lsd1* may also compromise the ability of the water–water cycle to reduce O_2^- production at PSI (Fig. 5C). Mateo *et al*. (2004) also noted that SA reduced stomatal opening, suggesting that compromised EEE acclimation could also feature during the HR. Our studies have identified both open and closed stomatal configurations during a HR against, respectively, mildew and rust fungi in cereals (Prats *et al*., 2006, 2007). Hence, EEE associated effects during the HR may be pathosystem-specific.

Photosensitization mediated by porphyrin is a well-established process in which singlet oxygen ($^1O_2$) is generated via triplet chlorophyll P680 ($^3P680^*$) (Fig. 5D). Illumination with light of an appropriate wavelength (λ <600 nm) initiates a short-lived singlet excited state within the chlorophyll porphyrin ring. Subsequently, in a diffusion-controlled process, the triplet state transfers its energy to the ground state of molecular oxygen to generate $^1O_2$ (Foote, 1994). Where this exceeds the capacity of $^1O_2$ quenchers, for example, carotenoids, to detoxify the radical, considerable oxidative damage will result. It is therefore unsurprising that several light-dependent SD mutants have been mapped to genes involved in porphyrin biosynthesis or catabolism. Early screens in *Arabidopsis* identified the accelerated cell death mutants, *acd1* and *acd2*, which represented lesions in the pathway of chlorophyll degradation (Lorrain *et al*., 2003; Tanaka *et al*., 2003). The ACD1 protein has been identified as the enzyme pheophorbide *a* oxygenase, which functions to oxidize pheophorbide, a toxic chlorophyll product whose accumulation leads to the *acd1* death phenotype (Fig. 5E). The maize *lls1* SD mutant has also been found to be due to a mutation in pheophorbide oxygenase (PaO; Pruzinska *et al*., 2003; Yang *et al*., 2004). The product of PaO activity is red chlorophyll catabolite (RCC), and its removal during normal leaf senescence is catalysed by RCC reductase which is mutated in the *acd2* mutant. SD mutants associated with the biosynthesis of chlorophyll have also been isolated. The maize *le22* mutant accumulates uroporphyrin III (Hu *et al*., 1998), whilst the Arabidopsis *lin2* mutant is mutated in coporphyrinogen III oxidase (Ishikawa *et al*., 2001) and *flu* accumulates protochlorophyllide

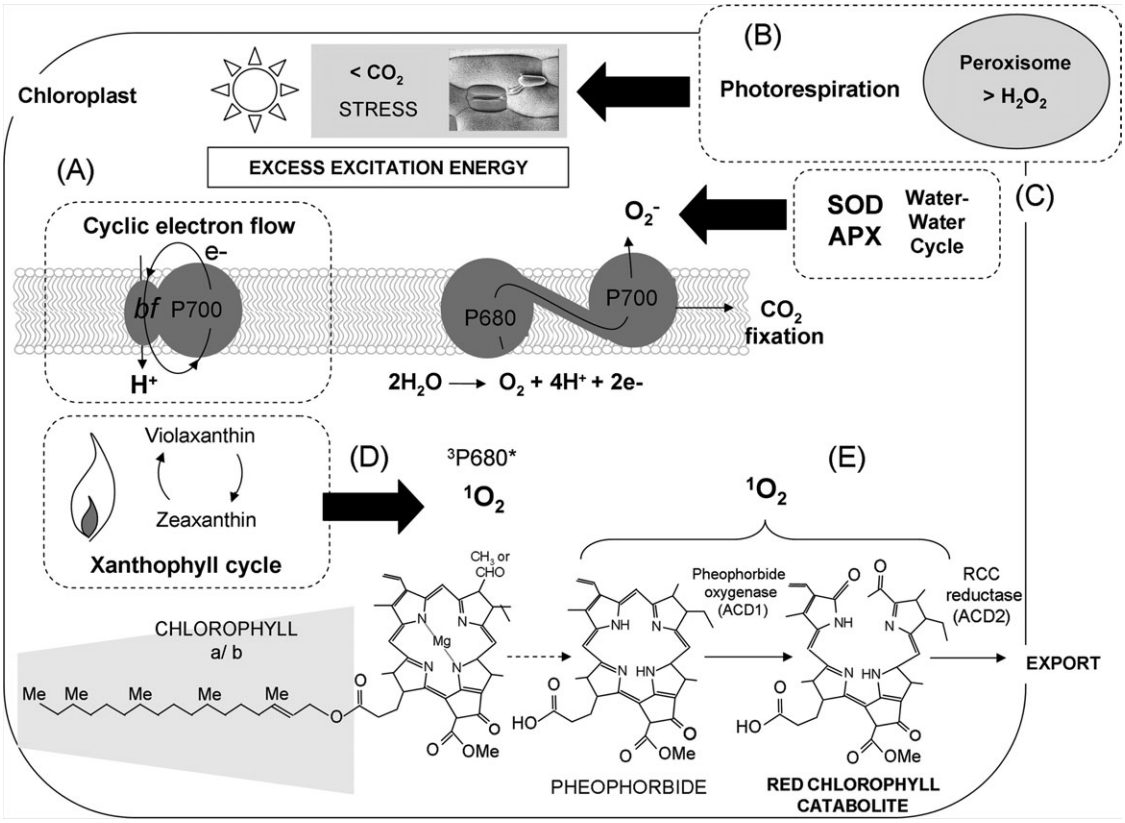

Fig. 5. Photosynthetic excess excitation energy and chlorophyll catabolites: sources of ROS during the HR? Excess excitation energy (EEE) occurs with elevated light levels and/or reduced CO_2 assimilation. EEE causes overreduction of photosystems which can be dealt with a variety of mechanisms. (A) 'state transitions' convert PSI into a solely proton–proton pumping/ATP generating complex via a reconfiguration of the light harvesting complexes, and an association of cytochrome *bf* with PSI. This results in light driven electron flow between these three components. (B) Photorespiration is the oxygenase reaction of Rubisco producing 2-phosphoglycerate and 3-phosphoglycolate. The latter is converted to glycolate and oxidized to glyoxylate by peroxisomally located glycolate oxidase, before ultimately forming 3-phosphoglyerate and entering the Calvin cycle. A by-product of the glycolate oxidase activity is H_2O_2 which is scavenged by catalase. (C) The water–water cycle (WWC) acts to maintain electron flow through the PSII and PSI to maintain a pH gradient. The WWC channels electrons from the water-splitting complex at PSII to the production of O_2^- at PSI$^-$ which is dismutated by CuZn superoxide dismutase (reviewed by Asada *et al*., 2000). (D) Under conditions of EEE, chlorophyll within the P680 reaction centre adopts the over-excited triplet state (^{3}P680*). ^{3}P680* will be converted back to ^{1}P680 by combination with $^3O_2$ which is produced by water oxidase in the lumen, but with the concomitant generation of singlet oxygen ($^3O_2 + {}^3P680^* \rightarrow {}^1P680 + {}^1O_2$) which is quenched by carotenoids. Under low pH, the violaxanthin is converted to zeaxanthin; this latter form allows a particularly fast thermal dissipation of excitation energy. (E) Release of chlorophyll catabolites from reaction centres uncouples $^1O_2$ generation from carotenoid scavenging leading to oxidative damage [the enzymic steps mediated by pheophorbide oxygenase and red chlorophyll catabolite (RCC) reductase, encoded by *ACD1* and *ACD2*, respectively, in *Arabidopsis* are indicated. See main text for details and references].

(Meskauskiene *et al*., 2001). As further evidence for the cytotoxicity of porphyrin compounds, application of protoprophyrin IX triggered cell death which induced chromatin-condensation, nicking as revealed by TUNEL, and mitochondrial dysfunction (Yao *et al*., 2004). Further, when the levels of the protoporphyrinogen oxidase (PPO) expression were suppressed by transgenic means in *Arabidopsis*, lines exhibited a SD phenotype (Molina *et al*., 1999). PPO is the last common enzyme in the haem and chlorophyll tetrapyrrole biosynthetic pathway and, interestingly, mutants in the haem pathway, *hy1* (in haem oxygenase) or *hy2* (phytochromobilin synthase) do not exhibit SD (Ishikawa, 2005). To relate these observations to a HR, the accumulation of pheophorbide was measured in *Arabidopsis* challenged with avirulent bacteria and it was found that it was co-incident with the first macroscopic indications of a HR. Further, in a stay-green *Arabidopsis* line, pheophorbide accumulation was not observed and the HR was delayed (LAJ Mur, H Ougham, unpublished data). Such data implicate chlorophyll catabolites as potentiators in the HR cell death process. If $^1O_2$ is a major player in the HR, it may act through EXECUTER 1, a chloroplastically located protein, mutation in which abolished $^1O_2$ induced cell death (Wagner *et al*., 2004). The responses of *Arabidopsis executer1* to pathogens have yet to be reported, but would be of great interest.

What is the HR programme and is the HR required for resistance?

To conclude this review certain key questions regarding our understanding of the HR need to be re-addressed. The

extensive comparison of HR with apoptosis in the first part of this review indicated how different these two death phenomena really are. For example, characterization of RGP signalling has not led to any intimate association with death effectors equivalent to mammalian caspases and there also appears to be no death complex equivalent to the mammalian apoptosome. Indeed, it may be that the role of RGP signal may be to initiate the deployment of non-HR defences, most likely via the production of so-called 'death signals'. It should be noted that the death signals, ROS, NO and SA, are effective initiators of resistance in the absence of a HR (Ryals *et al*., 1996; Mittler, 2002; Grün *et al*., 2006). However, each will also disrupt mitochondrial function so that HR death may, therefore, be a secondary effect of their production, albeit probably useful since it has been maintained throughout plant evolution. Hence the HR death programme may not be 'hard-wired' as is the case with apoptosis which involves dedicated death effectors and suppressors, but is part of a continuum of effects mediated by defence elicitors.

Against this, the LSD1 signal module may provide the most coherent argument for a HR death effector and suppressor gene programme. However, the main role for LSD1 could be homeostatic, for example, ROS detoxification and dissipation of EEE (Epple *et al*., 2003; Mateo *et al*., 2004). VPE activity and autophagic models could be taken as evidence of a hard-wired HR programme, but it should be noted that those autophagy genes have other roles and cannot be considered a dedicated programme. Given these observations, autophagy genes may kill as part of a mechanism which aims to clear dysfunctional cells.

This metabolic dysfunction model could suggest that the HR is incidental to resistance, and there is evidence to support this. In a significant recent paper, Al-Daoude *et al*. (2005) discovered another protein interacting with the RPM1 RGP, RIN13, whose over-expression in transgenic lines enhanced resistance to avirulent *P. s*. pv. *tomato*. However, the HR was abrogated so that the observed cell death matched virulent strains. Other data suggested that RIN13 was a positive effector of RPM1 function.

In 1915, Stakman speculated whether the HR represented 'real resistance or an extreme case of hypersensitiveness' (Stakman, 1915). Clearly, this latter point, namely the importance of cell death to resistance, remains to be resolved even today.

Acknowledgements

We gratefully acknowledge the efforts of Darrell Gray and Iolo ap Gwynn (UW Aberystwyth, UK) and Barry Thomas [Institute of Grassland and Environmental Research (IGER), UK] in deriving the electron micrographs. We appreciate the help given by the University of Minnesota Archives (USA) and Gwen Jenkins (UW Aberystwyth, UK) in the compilation of this review. Thanks are also given to Tim LW Carver and Howard Thomas (IGER) for useful comments on the text. The Mur Group's research is supported by funding from the BBSRC, the Royal Society, and the Aberystwyth University Research Fund. Further support is provided by the EU Human Resources Mobility Fund. The Ougham group's research is supported by BBSRC and industrial funding. The Prats Group research is funded by ERG and the Spanish Science and Technology Ministry.

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