Reactive oxygen species and hormonal control of cell death

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The accumulation of reactive oxygen species (ROS) is involved in regulating cell death. Pathogen- and ozone-induced processes have become important models for the study of cell death regulation by ROS. Hydrogen peroxide and superoxide have emerged as the two key ROS and recent studies have addressed their sources and control of their production. ROS signals interact directly or indirectly with several other signaling pathways, such as nitric oxide, and the stress hormones salicylic acid, jasmonic acid and ethylene. The interaction and balance of these pathways determines whether the cell lives or dies.

Reactive oxygen species (ROS) are central to the regulation of programmed cell death (PCD) [1,2] and have previously been addressed in several reviews [3–5]. Recent studies have further refined our understanding of the sources and control of ROS production, as well as some of the factors that act together with ROS in cell death regulation. The focus of this article is the role of ROS and hormonal control in cell death propagation. The model systems examined are pathogen-induced cell death, termed the hypersensitive response (HR), and the ozone (O_3) response, both of which are unified in their induction of apoplastic ROS production (the oxidative burst) and cell death. Because the O_3 response is similar to the HR, O_3 has become established as a model system for studying the role of ROS in cell death regulation [6–10].

The cell death associated with the HR is regulated genetically and is a form of PCD, as illustrated by maize, tomato and *Arabidopsis* mutants that spontaneously trigger cell death in the absence of pathogens. These lesion-mimic mutants can be separated into two classes [11]: the initiation mutants develop spontaneous lesions of determinate size that are similar in appearance to normal HR-lesions triggered by pathogens; by contrast, the propagation mutants exhibit spreading cell death. *Arabidopsis lsd1* and *rcd1* mutants [12,13] are typical of propagation mutants, and extracellular superoxide radical ($O_2^{\cdot-}$) is necessary and sufficient to trigger spreading cell death. The existence of these two mutant classes suggests that genetically distinct processes are involved in lesion formation: first, the initiation of cell death and, second, the spread of cell death to a limited number of surrounding cells. This implies a dialog of signals between dying cells and healthy neighboring cells that determines lesion size [11]. A question thus arises about the nature of the processes that propagate or halt the spread of cell death and hence regulate the extent of lesion propagation.

Ozone as a model of cell death regulation

In contrast to stratospheric O_3, which protects plants from harmful ultraviolet radiation, tropospheric O_3 is a potent toxin [9]. In sensitive plants, O_3 causes the formation of lesions that have many characteristics in common with the HR. These include induction of an oxidative burst, deposition of autofluorescent phenolic compounds, pathogenesis-related (PR) protein expression and both micro- and macro-scale cell death and the associated local and systemic-induced pathogen resistance [6,7,10]. Thus, O_3-induced cell death is believed to be the result of deleterious firing of the HR program by the ROS formed from the degradation of O_3 in the apoplast. These ROS seem to act primarily as signal molecules, not as directly damaging agents. This has been widely accepted in the literature [7,14,15].

Oxidative burst

The oxidative burst is a common response to virtually every biotic and abiotic stress [16] including the HR and the O_3 response. For example, in the O_3-sensitive Bel-W_3 tobacco and the O_3-sensitive *Arabidopsis* accession Cvi-0, and in the *rcd1* and *jar1* mutants, a biphasic O_3-induced oxidative burst and prolonged ROS accumulation result in the activation of cell death [13,17,18]. When tobacco, seven tomato cultivars, 12 *Arabidopsis* accessions, two *Rumex* and one *Malva* species were assayed for ROS accumulation and O_3 lesions, a clear spatial and quantitative correlation was found between, depending on the species, either H_2O_2 or $O_2^{\cdot-}$ accumulation and O_3 damage [19]. In tobacco [17], tomato [19] and birch (*Betula pendula*) [8,20], there is a clear correlation between H_2O_2 and the later-appearing lesions, with no $O_2^{\cdot-}$ accumulation detectable, even though diphenylene iodonium (which, among other effects, inhibits production) considerably decreased both H_2O_2 accumulation and lesion formation in these species. In *Arabidopsis*, *Rumex* and *Malva*, however, $O_2^{\cdot-}$ was the ROS responsible for cell death [12,13,18,19]. Furthermore, those *Arabidopsis* accessions that showed the highest $O_2^{\cdot-}$ accumulation after a short O_3 exposure, before visible lesion formation, were also the most sensitive to O_3 [19]. This raises the questions of why, in some species, cell death

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and H_2O_2 are correlated when, in others, $O_2^{-}\cdot$ seems to be the ROS that can be detected, and of what is the primary source of the ROS.

NADPH oxidase as ROS source

Inhibitor studies in several species that primarily accumulate H_2O_2 in the apoplastic oxidative burst [17,19–22] have suggested that, at least partly, the primary source of the H_2O_2 in these species is $O_2^{-}\cdot$ produced by the NADPH oxidase complex. Accordingly, in tobacco, O_3 exposure upregulated two homologs of the NADPH oxidase [19]. This suggests that H_2O_2 is formed from $O_2^{-}\cdot$ by spontaneous or enzyme-catalysed dismutation. Thus, the dismutation rate on one hand and the $O_2^{-}\cdot$ production rate on the other hand could determine the ROS that is accumulating ($O_2^{-}\cdot$ or H_2O_2). When the dismutation rate remains high, no $O_2^{-}\cdot$ accumulation can be detected even when the production rate at the cell level is increased.

What, then, are the subcellular sources of the ROS in plants undergoing ROS-dependent cell death? Several different mechanisms have been suggested as the source of the apoplastic oxidative burst [23]. A NADPH oxidase homologous to that of activated mammalian phagocytes and neutrophils ($gp91^{phox}$) has been considered as a likely source for the apoplastic $O_2^{-}\cdot$ generation [24–26]. From the complete sequence of the *Arabidopsis* genome, ten NADPH oxidase genes have been identified, of which three (*RESPIRATORY BURST OXIDASE HOMOLOGS AtRBOHD–AtRBOHF*) are expressed in leaf and root tissues [24–27]. Antisense knockouts of the tobacco *NtrbohD* gene [28], analysis of the *Arabidopsis rbohD* and *rbohF* mutants [27], and virus-induced gene silencing of *NbrbohA* and *NbrbohB* in *Nicotiana benthamiana* [29] have confirmed the role of these proteins as the source of ROS in the oxidative burst that regulates cell death. Intriguingly, the outcome of lowered ROS accumulation in *Arabidopsis rboh* mutants differed in different circumstances. In some cases, reduced ROS compromised cell death induction whereas, in others, it actually enhanced cell death [27]. This indicates that ROS derived from the RBOH proteins interact in a complicated manner, presumably with other ROS, NO or defense pathways, in the regulation of cell death. Disruptions in the balance between these interactions results in seemingly unpredictable results. The elucidation of these pathways with the tools that are now available is an exciting challenge for future studies.

Nitric oxide (NO) is another important player that is required for ROS-induced cell death [30]. NO might be key to our understanding of how H_2O_2 and $O_2^{-}\cdot$ regulate cell death. In soybean suspension cells, the ratio of NO to H_2O_2 determined when cell death was activated [31]. Furthermore, it was shown that peroxynitrite, formed in a reaction between $O_2^{-}\cdot$ and NO, did not induce cell death in these cells as it does in animals. Thus, in this system, excessive $O_2^{-}\cdot$ or NO production might serve to scavenge the other and prevent the accumulation of NO and H_2O_2 required for cell death activation. However, urate, a scavenger of peroxynitrite, can reduce cell death induced by treatment with exogenous peroxynitrite or an avirulent pathogen in *Arabidopsis* [32]. Thus, the inability of peroxynitrite to induce cell death might be specific to soybean and requires further clarification. The mechanism proposed above, in which H_2O_2 and $O_2^{-}\cdot$ have opposing roles, underscores the importance of $O_2^{-}\cdot$ dismutation in cell death regulation. NADPH oxidases that synthesize $O_2^{-}\cdot$ coordinately with superoxide dismutase will produce H_2O_2, which acts as a cell death inducer. It remains to be elucidated whether the balance between all these processes determines, for example, why different *rboh* mutants had opposite effects on cell death [27].

Regulation of the NADPH oxidase

The early induction of rapid ion fluxes across the plasma membrane is involved directly in the induction of the oxidative burst. Plant *RBOH* proteins contain Ca^{2+}-binding EF-hands and do not have the separate cytoplasmic subunits of the complex that the prototype mammalian NADPH oxidases have [25]. Thus, Ca^{2+} in particular has been shown to play an important role in the activation of $O_2^{-}\cdot$ production [22]. Anion fluxes are also involved. Inhibition of Cl^{-} fluxes by different anion channel blockers prevented the oxidative burst induced by osmotic stress in tobacco suspension cultures [33], and treatment of tobacco cell suspensions with the elicitor cryptogein induced cell death, which could be blocked by inhibition of NO_3^{-} efflux [34].

In addition to ion fluxes, other important regulators are involved in the activation of the NADPH oxidase in mammalian and plant systems, including protein kinases and phosphatases, small GTPases and phospholipases. In plants, a Rac-family small GTPase has been implicated in ROS production and cell death [35]. Similarly, a GTPase activating protein that regulates the activity of Rop (Rho-like small G-protein of plants) is required for ROS signaling in the anoxia response [36].

Mitochondria as ROS sources

Another intracellular source of ROS is the mitochondria [37], which are also thought to be an integral component in PCD regulation [22,38,39]. The mitochondrial electron transport chain can produce significant quantities of ROS, primarily because of the presence of the ubisemiquinone radical, which can transfer a single electron to oxygen and produce $O_2^{-}\cdot$. When the subcellular compartmentalization of O_3-induced H_2O_2 formation was studied in birch [20], increased ROS accumulation, temporally coinciding with cell death was observed in the mitochondria.

The mitochondrial alternative oxidase (AOX) catalyses the O_2-dependent oxidation of ubiquinol, limiting the mitochondrial generation of ROS. Lack of AOX induction caused increased ROS production [40]. Consistent with this, tobacco cells lacking AOX had increased PCD in response to H_2O_2 and tobacco plants overexpressing AOX developed smaller HR lesions in response to virus infection [41,42]. H_2O_2 treatment of *Arabidopsis* cells and H_2O_2 accumulation in catalase-deficient tobacco lead to induction of antioxidant defenses and increased AOX levels in the mitochondria [22,43]. The normal function of the mitochondria could be perturbed during oxidative stress through the early accumulation of ROS in other

Box 1. Hormonal interactions regulating ROS-dependent cell death

Activation of the NADPH oxidase (RBOH) requires ion fluxes and protein kinases [22,34]. The action of reactive oxygen species (ROS) is amplified and death is induced through salicylic acid (SA) at the sites of lesion initiation. During the initial cell death, jasmonic acid (JA) signaling is suppressed by salicylic acid and ethylene (ET). From these initial sites, a burst of ethylene production spreads to surrounding cells and induces competence for programmed cell death (PCD) (Fig. I). In tomato, ethylene signaling was required for salicylic acid accumulation and cell death spread during symptom formation after virulent pathogen attack [91]. This model is consistent with the cooperative action of salicylic acid and ethylene seen during O_3 and pathogen symptom development [48,57]. But to which signal do ethylene- (and salicylic acid-) primed cells become competent? $O_2^-\cdot$ is actively produced in lesions and then, during spreading cell death, in a row of cells in advance of cell death spread. This pattern suggests that $O_2^-\cdot$ is the death-inducing signal to which ethylene and ethylene-dependent salicylic acid prime cells. Thus, ethylene and $O_2^-\cdot$ conspire in a feedforward chain reaction responsible for spreading cell death. The $O_2^-\cdot$ signal is passed forward rows of cells at a time from dying cells. Cell death results in the production of jasmonic acid, which triggers the jasmonic acid-dependent lesion containment. Jasmonic acid can antagonize lesion spread in several ways, for example, through the suppression of salicylic acid biosynthesis and signaling. Also, attenuation of ethylene sensitivity by jasmonic acid contributes to halting cell death spread.

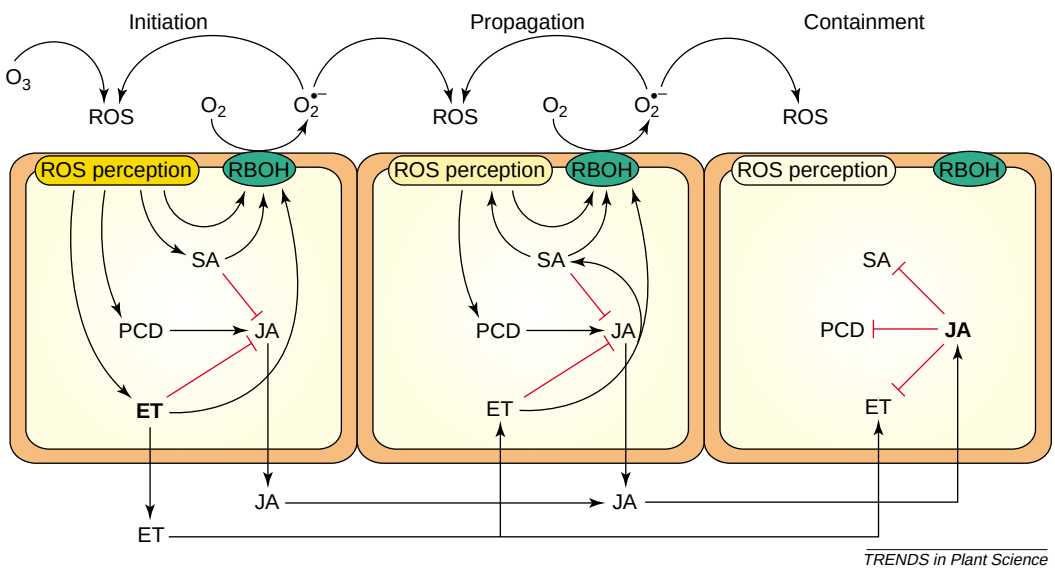

Fig. 1.

subcellular compartments or changes in the plant hormone ethylene (discussed below), leading to increased production of ROS in the mitochondria.

Hormonal regulation of ROS-dependent cell death

A picture of the integral role of plant hormones in the regulation of ROS-dependent cell death is now emerging. To facilitate their initial characterization, hormone signal transduction pathways have necessarily been conceptualized as linear and independent. However, as more details of these signaling pathways have become available, so their interconnected nature has become increasingly evident. The three hormones ethylene, salicylic acid and jasmonic acid are of particular importance in ROS-dependent cell death. These pathways do not operate independently but rather are linked together in a complex web of interactions, as indicated by, for example, the *hrl1* mutant [44]. It has been suggested that the overall sensitivity of the plant cell to a given hormone is at least partially established in the interplay of multiple hormones [45].

Oxidative cell death cycle

The extent of HR and O_3-lesion propagation seems to be under hormonal control, with different hormones and their interactions regulating ROS production and the competence of the cell to perceive and react to ROS signals (Box 1). Regulation of the ROS-dependent cell death in the oxidative cell death cycle has been proposed based on work with plants undergoing HR [46] and later modified based on O_3-induced oxidative cell death [13]. In this model (Fig. 1), which is further supported by the results reviewed here, ROS, salicylic acid and cell death function in a self-amplifying feedforward loop in the regulation of cell death. Ethylene is required for the continuation of ROS accumulation, which drives cell death. This attributes a previously unknown role to ethylene and ROS as positive co-regulators of cell death and expands the role of ethylene as a cell death regulator [47,48]. Jasmonic acid is involved in the containment of the lesion propagation.

Salicylic acid

Salicylic acid and ROS have been proposed to be on a positive feedback loop that amplifies signals leading to defense responses and cell death [46,49]. This salicylic acid-dependent signal potentiation loop has been proposed to be negatively regulated by LSD1 [50], which also explains the runaway cell death phenotype of the *lsd1* mutant. Cell death and the accumulation of salicylic acid are intimately associated. This is supported by the many

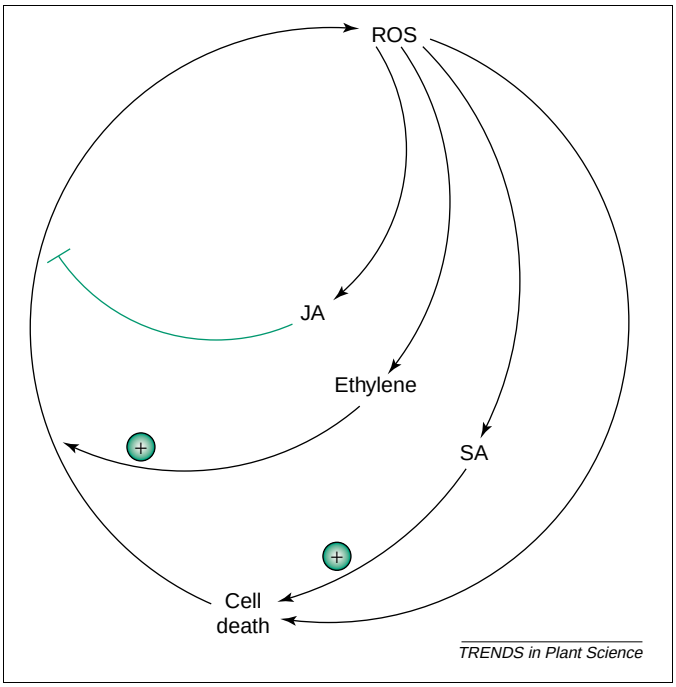

Fig. 1. Hormonal regulation of the oxidative cell death cycle. Increased reactive oxygen species (ROS) accumulation together with salicylic acid (SA) induces cell death. Ethylene is required for the amplification of ROS production, which results in a positive feedback cycle (+) that promotes the lesion spread (compare with Box 1). Increased accumulation of jasmonates, through either the activation of jasmonic acid (JA) biosynthesis by the ROS or increased substrate availability from the dying cells, acts as a negative regulator of the oxidative cell death cycle and can overcome the promoting effect of ethylene to ROS generation, resulting in the containment of lesion spread.

lesion mimic mutants that have constitutively elevated levels of salicylic acid. Transgenic plants engineered to degrade salicylic acid with the bacterial *NahG* gene are unable to induce cell death after pathogen attack [51]. However, it has recently been shown that the effects of *NahG* are not limited to salicylic acid depletion [52]. Catechol, the byproduct of salicylic acid degradation by *NahG* induces the accumulation of H_2O_2, which in turn results in the loss of resistance to a bacterial pathogen. It is not clear whether this has implications for the regulation of cell death by salicylic acid. However, conclusions that rely heavily on the phenotypes of *NahG* plants should be re-evaluated.

There is clear evidence of cell death signals such as ROS and NO being involved in the regulation of key steps in salicylic acid biosynthesis during pathogen infection [31,49]. An alternate pathway of salicylic acid biosynthesis involved in the establishment of systemic resistance has been elucidated by the cloning the allelic *sid2* (*salicylate induction deficient 2*) and *eds16* (*enhanced disease susceptibility 16*) mutants encoding the isochorismate synthase (*ICS*) gene. The normal induction of HR cell death in *sid2*/*eds16* plants argues against involvement of salicylic acid derived from the ICS pathway in ROS signal amplification and cell death activation.

Salicylic acid accumulates in O_3-exposed plants, in which high levels of salicylic acid accumulation correlate with lesion formation. The O_3-sensitive Cvi-0, *rcd1* and *jar1*, which hyperaccumulate salicylic acid upon O_3 exposure became markedly more O_3 tolerant when transformed or crossed with *NahG* or the salicylic acid signaling mutant, *npr1* [53] (J.K. *et al.*, unpublished). Similarly, expression of *NahG* in the tobacco cultivar Xanthi resulted in reduced lesion formation upon O_3 exposure [54]. Furthermore, when exogenous salicylic acid was added to $O_2^{-}\cdot$-treated Col-0, it significantly enhanced the induction of cell death [55]. Similarly, pretreatment of Col-0 with salicylic acid before O_3 exposure increased cell death significantly [53].

Salicylic acid is known to inhibit the activity of the last step in the ethylene biosynthesis pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase [56]. Given the cell-death-promoting role of ethylene, the antagonism of ethylene biosynthesis by salicylic acid might be a mechanism by which salicylic acid accumulation can contribute to the containment of lesion growth. By contrast, salicylic acid and ethylene have been shown to act co-operatively during symptom formation in O_3-treated [48] and virulent-pathogen-treated [57] *Arabidopsis*.

Ethylene

Ethylene has vital roles in many aspects of plant growth and development. Ethylene evolution is also associated with wounding, pathogen attack, anaerobiosis, senescence, heavy metals and oxidative stresses such as O_3 [58]. Importantly, ethylene is involved in the regulation of PCD in several different developmental and inducible processes [59–61]. Ethylene -biosynthesis genes are present in large multigene families in which different genes or sets of genes respond to various developmental and environmental cues [58]. Ozone-induced leaf damage is preceded by a rapid increase in ACC synthase activity, ACC content and ethylene emission, which are required for ROS accumulation and lesion development [13,48,62–65].

Similarly in *Arabidopsis*, external addition of ethylene during cell death increased $O_2^{-}\cdot$ production and caused increased spreading cell death [13]. Accordingly, also the ethylene-overproducing mutants *eto1* and *eto3* are more sensitive to O_3 and display increased ROS-dependent cell death amplification [48] (J.K. *et al.*, unpublished). The results demonstrate a selective ozone response of ethylene biosynthetic genes and suggest a role for ethylene as a positive regulator of ROS production and regulation of the spread of cell death.

These results are consistent with the role assigned to ethylene in the regulation of the pathogen-triggered cell death. In plants challenged with a compatible bacterial pathogen, ethylene signaling was required for symptom development [66]. However, the HR triggered in an incompatible interaction developed fully in ethylene-insensitive *Arabidopsis* plants [66]. Thus, there seems to be a core cell death pathway that is ethylene independent. However, this does not exclude the possibility that ethylene-dependent cell death can contribute to lesion size in incompatible interactions; manipulation of ethylene signaling has been shown to alter lesion size in at least three different incompatible interactions in tomato and tobacco [42,67,68].

In O_3-exposed plants, ROS formation from the degradation of O_3 is not confined to a limited location as it is in the HR, O_3 enters the sub-stomatal cavities throughout

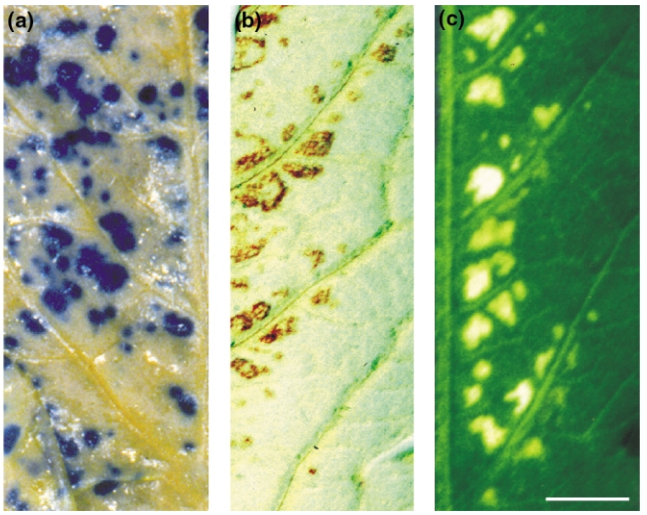

Fig. 2. Ethylene synthesis, H_2O_2 accumulation and cell death in transgenic plants in which the promoter of the tomato ACC oxidase gene (*LE-ACO1*) has been fused to the marker gene *uidA*. The GUS activity staining shows the localization of ethylene biosynthesis that is induced by ozone in tomato plants 1 h after the beginning of the ozone exposure (a). Hydrogen peroxide accumulation 7 h after the beginning of a 5 h ozone exposure has a similar spatial location as ethylene synthesis 6 h earlier (b). Cell death at 24 h has corresponding spatial localization close to the veins as hydrogen peroxide accumulation had (c). Inhibition of ethylene synthesis or perception also prevented H_2O_2 accumulation and subsequent cell death [65]. Reproduced, with permission, from Ref. [65]. Scale bar = 5 mm.

the leaf. However, in tomato, both H_2O_2 accumulation and ethylene biosynthesis were confined to distinct regions surrounding the vascular tissue, mainly in the parenchyma cells (Fig. 2) [65]. Furthermore, both ethylene synthesis and perception were required for active H_2O_2 production, which in turn was required for cell death. This restricted expression is of interest because not all cells seem to be responding to O_3 in the same way. Furthermore, this co-localization predicts that high concentrations of both ethylene and ROS occur in the same cells in a temporally coordinated manner. A similar pattern of cell death that is preferentially localized to cells close to the vascular bundles has also been seen in other O_3-exposed species [17,19,69] and in H_2O_2-overproducing catalase-antisense tobacco [22]. The spatial location close to the veins is similar to the location of ROS generation and so-called 'micro-HR' during the establishment of systemic resistance [70]. As discussed [17], the cells in the periveinal region might be predisposed to amplify ROS production and thus to act as ROS receptor cells that are predisposed to die upon a ROS signal.

In tomato, the genes encoding ethylene receptors were induced differently by O_3 [65] and during pathogen infection [71]. The increased synthesis of 'fresh' receptors, unoccupied by ethylene, has been proposed to decrease ethylene sensitivity and to be involved in the desensitization of plants to ethylene when the ethylene responses need to be shut down [72,73]. The increased ROS production in the mitochondria discussed above could also be perturbed through changes in ethylene synthesis and sensitivity. It has been shown that activation of AOX is ethylene dependent [74]. The lack of AOX induction caused increased ROS production [40]. Reduced ethylene sensitivity has also been shown to compromise the upregulation of cyanide detoxification, which clears this toxic byproduct formed during the oxidation of ACC into ethylene [69]. Thus, high ethylene synthesis, attenuated induction of the AOX or cyanide-resistant respiration, and defective HCN removal owing to reduced ethylene sensitivity might result in inhibition of the normal mitochondrial respiration by HCN and thus cause increased ROS production in the mitochondria.

Jasmonic acid

Jasmonic acid is a plant signaling compound with roles in both stress and development [75]. It is induced by a wide range of biotic and abiotic stresses, including O_3, but it is best known for its role in the wound response [14]. This cyclopentone compound is a derivative of the octadecanoid lipid pathway and is derived from linolenic acid. Several jasmonic acid-deficient mutants have been isolated, resulting in nearly complete definition of the jasmonic acid biosynthesis pathway [14,76]. Importantly, jasmonic acid is not the only biologically active molecule on the jasmonic acid biosynthesis pathway. Several intermediates are active in defense signaling [77,78]. Also, the volatile jasmonic acid derivative methyl-jasmonic acid (MeJA) is an important diffusible molecule involved in both intra- and interplant signaling [79]. It is now apparent that not only jasmonic acid but also many other related signal molecules, all derived from fatty acids, act together in signaling. This has led to the concept of the 'oxylipin signature', in which the full profile of lipid signals taken together determines the signaling outcome [14,76,80].

Gene expression analysis with jasmonic acid-inducible marker genes and jasmonic acid treatments have suggested that jasmonic acid could be a factor involved in the containment of the ROS-dependent lesion propagation [13,53]. The O_3 sensitivity of jasmonic acid mutants further supports this idea. The jasmonic acid-insensitive mutants *jar1* and *coi1*, and the jasmonic acid-biosynthesis-defective *fad3 fad 7 fad 8* triple mutant are all highly O_3 sensitive [13,53]. Furthermore, *jar1* exhibits a transient spreading cell death phenotype and a pattern of $O_2^{\cdot-}$ accumulation similar to that observed in *rcd1* [13]. Post-treatment of O_3-exposed *rcd1* with jasmonic acid halted spreading cell death, providing direct evidence of the role of jasmonic acid in lesion containment [13]. Similarly, pretreatment of tobacco or the O_3-sensitive Cvi-0 *Arabidopsis* accession diminished O_3 damage [53,54].

Lesion containment by jasmonic acid could be achieved through regulation of ethylene receptors; it has been shown that jasmonic acid induces genes encoding ethylene receptors [81]. As discussed above, increased receptor protein synthesis decreases ethylene sensitivity and desensitizes plants to ethylene. In this way, jasmonic acid could affect ethylene-dependent lesion propagation by reducing the ethylene-dependent ROS accumulation and thus result in halting of the lesion spread.

The timing and control of jasmonic acid biosynthesis suggests several ways in which jasmonic acid signaling might be modulated during the regulation of lesion growth. One level of control in jasmonic acid biosynthesis

Box 2. MAP kinases and ROS-dependent cell death

MAPK (mitogen-activated protein kinase) cascades are central signal transduction links and integration points in plants, yeast and mammals [85]. MAPK cascades are activated by a wide range of signals including abscisic acid (ABA), auxin, ethylene, reactive oxygen species (ROS) and pathogens [85]. The first complete MAPK cascade (MEKK1, MKK4 and MKK5, MPK3 and MPK6), which functions in *Arabidopsis* innate immunity signaling, has been identified [92]; MKK4 and MKK5 are associated with the regulation of H_2O_2 production and cell death [93]. Homologous MAPKs have been shown to be activated by H_2O_2 in tobacco [wound-inducible protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK)] and *Arabidopsis* (MPK3 and MPK6) [94,95]. An *Arabidopsis* nucleoside diphosphate kinase 2 (NDPK2) has been found to interact with MPK3 and MPK6, and knockout mutants of *AtNDPK2* have decreased tolerance to ROS, whereas overexpression of *AtNDPK2* leads to increased stress tolerance [96]. The same two tobacco MAPKs, WIPK and SIPK, are activated in O_3-exposed tobacco [95]. Gain-of-function and loss-of-function experiments with these MAPKs demonstrate that their balanced and tightly regulated activation is indispensable for O_3 tolerance (i.e. the regulation of cell death) [97]. Furthermore, the enhanced cell death phenotypes of the *mpk4* and *edr1* mutants further illustrate the importance of MAPKs in cell death regulation [84,98].

and/or signaling might be the sequestration of enzymes and substrates inside the chloroplast [82]. In this way, jasmonic acid biosynthesis and signaling will only be activated by the availability of substrate upon cellular decompartmentalization during wounding or cell death. Other studies suggest direct signaling pathways, associated with but not dependent on cell death, that lead to jasmonic acid biosynthesis and signaling. First, there is a requirement for the mitogen-activated protein (MAP) kinase WIPK for jasmonic acid accumulation [83]. Interestingly, several studies [84,85] have linked the modulation of jasmonic acid accumulation and signaling to MAP kinase cascades (Box 2). Further evidence comes from the *cet* (*constitutive expression of thionin*) mutants, which overexpress the strongly jasmonic acid-inducible thionin gene (*Thi2.1*) and form microlesions [86]. Microlesions were independent of COI1-mediated jasmonic acid signaling in all of these mutants, and independent of salicylic acid signaling in *cet2* and *cet4.1* but not in *cet3*. Significantly, in the double *cet3 NahG* mutant, *Thi2.1* expression was independent of lesion formation. This suggests that signals other than salicylic acid and jasmonic acid are involved in regulation of lesion initiation. These results clearly illustrate that jasmonic acid accumulation and signaling are intimately associated with death induction signals, but are not dependent on cell death itself. The cloning of these *cet* genes should help to illuminate the processes involved in stress perception leading to jasmonic acid accumulation.

In contrast to this protective role demonstrated above, jasmonic acid signaling is required for fumonisin-B1-induced cell death, which is also considered to be a model for HR-like cell death [87]. Furthermore, the bacterial toxin coronatine, which is a structural analog of jasmonic acid and mimics its action [75], induces chlorotic symptoms in plants. These findings are consistent with the role of jasmonic acid in promoting senescence [88], which is also a form of PCD.

Conclusions and future challenges

Powerful genetic strategies driven by the use of *Arabidopsis* have resulted in the elucidation of many hormone and other signaling pathways in plants. As illustrated by the studies reviewed here, the application of this knowledge and, in particular, the use of signaling mutants have allowed the delineation of signals involved in cell death regulation. Similarly, genetic approaches involving mutants have been key in identifying novel plant pathways, such as the MAP kinase cascades (Box 2) involved in the regulation of ROS responses and cell death regulation. The picture is also more complicated, considering that even more hormones (e.g. abscisic acid and gibberellic acid) are likely to be involved in cell death regulation [89,90]. Continued work with these powerful systems should result in the further molecular definition of these pathways and poses the challenge to produce an integrated map that connects these pathways at the molecular level.

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References

1 Kroemer, G. *et al.* (1995) The biochemistry of programmed cell death. *FASEB J.* 9, 1277–1287
2 Morel, J-B. and d Dangl, J.L. (1997) The hypersensitive response and the induction of cell death in plants. *Cell Death Differ.* 4, 671–683
3 Hoeberichts, F.A. and Woltering, E.J. (2002) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *BioEssays* 25, 47–57
4 Neill, S.J. *et al.* (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot.* 53, 1237–1247
5 Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251–275
6 Kangasjärvi, J. *et al.* (1994) Plant defence systems induced by ozone. *Plant Cell Environ.* 17, 783–794
7 Rao, M.V. and Davis, K.R. (2001) The physiology of ozone-induced cell death. *Planta* 213, 682–690
8 Pellinen, R.I. *et al.* (2002) H_2O_2 activates cell death and defense gene-expression in birch (*Betula pendula*). *Plant Physiol.* 130, 549–560
9 Langebartels, C. *et al.* (2002) Oxidative stress and defense reactions in plants exposed to air pollutants and UV-B radiation. *Oxidative Stress in Plants* (Inzé, D., Van Montagu, M., *et al.* eds), pp. 105–135, Taylor & Francis
10 Langebartels, C. *et al.* (2002) Oxidative burst and cell death in ozone-exposed plants. *Plant Physiol. Biochem.* 40, 567–575
11 Dangl, J.L. *et al.* (1996) Death don't have no mercy: cell death programs in plant–microbe interactions. *Plant Cell* 8, 1793–1807
12 Jabs, T. *et al.* (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* 273, 1853–1856
13 Overmyer, K. *et al.* (2000) The ozone-sensitive *Arabidopsis rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. *Plant Cell* 12, 1849–1862
14 Berger, S. (2001) Jasmonate-related mutants of *Arabidopsis* as tools for studying stress signals. *Planta* 214, 497–504
15 Beers, E.P. and McDowell, J.M. (2001) Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Curr. Opin. Plant Biol.* 4, 561–567
16 Vranová, E. *et al.* (2002) Signal transduction during oxidative stress. *J. Exp. Bot.* 53, 1227–1236
17 Schraudner, M. *et al.* (1998) Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. *Plant J.* 16, 235–245
18 Rao, M.V. and Davis, K.R. (1999) Ozone-induced cell death occurs via

two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. *Plant J.* 17, 603–614
19 Wohlgemuth, H. *et al.* (2002) Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. *Plant Cell Environ.* 25, 717–726
20 Pellinen, R. *et al.* (1999) Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *Plant J.* 20, 349–356
21 Orozco-Cárdenas, M. *et al.* (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* 13, 179–191
22 Dat, J. *et al.* (2003) Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. *Plant J.* 33, 621–632
23 Bolwell, G.P. *et al.* (2002) The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *J. Exp. Bot.* 53, 1367–1376
24 Dwyer, S.C. *et al.* (1996) Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. *Biochem. Biophys. Acta* 1289, 231–237
25 Keller, T. *et al.* (1998) A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. *Plant Cell* 10, 255–266
26 Torres, M.A. *et al.* (1998) Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*gp91^{phox}*). *Plant J.* 14, 365–370
27 Torres, M.A. *et al.* (2002) Arabidopsis gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 517–522
28 Simon-Plas, F. *et al.* (2002) The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells. *Plant J.* 31, 137–147
29 Yoshioka, H. *et al.* (2003) *Nicotiana benthamiana* gp91^{phox} homologs *NbrbohA* and *NbdbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. *Plant Cell* 15, 706–718
30 Wendehenne, D. *et al.* (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci.* 6, 177–183
31 Delledonne, M. *et al.* (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13454–13459
32 Alamillo, J.M. and Garcia-Olmeedo, F. (2001) Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. *Plant J.* 25, 529–540
33 Cazalé, A.-C. *et al.* (1998) Oxidative burst and hypo-osmotic stress in tobacco cell suspensions. *Plant Physiol.* 116, 659–669
34 Wendehenne, D. *et al.* (2002) Nitrate efflux is an essential component of the cryprogein signaling pathway leading to defense responses and hypersensitive cell death in tobacco. *Plant Cell* 14, 1937–1951
35 Ono, E. *et al.* (2001) Essential role of the small GTPase Rac in disease resistance of rice. *Proc. Natl. Acad. Sci. U.S.A.* 98, 759–764
36 Baxter-Burrell, A. *et al.* (2002) RopGAP4-dependant Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. *Science* 269, 2026–2028
37 Tiwari, B.S. *et al.* (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant Physiol.* 128, 1271–1281
38 Lam, E. *et al.* (2001) Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* 411, 848–853
39 Yu, X.H. *et al.* (2002) Mitochondrial involvement in tracheary element programmed cell death. *Cell Death Differ.* 9, 189–198
40 Maxwell, D.P. *et al.* (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8271–8276
41 Robson, C.A. and Vanlerberghe, G.C. (2002) Transgenic plants lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. *Plant Physiol.* 129, 1908–1920
42 Ordog, S.H. *et al.* (2002) Mitochondrial alternative oxidase is not a critical component of plant viral resistance but may play a role in the hypersensitive response. *Plant Physiol.* 129, 1858–1865
43 Sweetlove, L.J. *et al.* (2002) The impact of oxidative stress on *Arabidopsis* mitochondria. *Plant J.* 32, 891–904
44 Devadas, S.K. *et al.* (2002) The *Arabidopsis hrl1* mutation reveals novel overlapping roles for salicylic acid, jasmonic acid, and ethylene signaling in cell death and defense against pathogens. *Plant J.* 30, 467–480
45 Ghassemian, M. *et al.* (2000) Regulation of abscisic acid signalling by the ethylene response pathway in *Arabidopsis*. *Plant Cell* 12, 1117–1126
46 Van Camp, W. *et al.* (1998) H_2O_2 and NO: redox signals in disease resistance. *Trends Plant Sci.* 3, 330–334
47 Crowell, D.N. *et al.* (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. *Plant Mol. Biol.* 18, 459–466
48 Rao, M.V. *et al.* (2002) Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. *Plant J.* 32, 447–456
49 Draper, J. (1997) Salicylate, superoxide synthesis and cell suicide in plant defence. *Trends Plant Sci.* 2, 162–165
50 Aviv, D.H. *et al.* (2001) Runaway cell death, but not basal disease resistance, in *lsd1* is SA- and *NIM1/NPR1*-dependent. *Plant J.* 29, 381–391
51 Durner, J. *et al.* (1997) Salicylic acid and disease resistance in plants. *Trends Plant Sci.* 2, 266–274
52 van Wees, S. and Glazebrook, J. (2003) Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. *Plant J.* 33, 733–742
53 Rao, M.V. *et al.* (2000) Jasmonic acid signalling modulates ozone-induced hypersensitive cell death. *Plant Cell* 12, 1633–1646
54 Örvar, B.L. *et al.* (1997) Pre-activating wounding response in tobacco prior to high-level ozone exposure prevents necrotic injury. *Plant J.* 11, 203–212
55 Mazel, A. and Levine, A. (2001) Induction of cell death in *Arabidopsis* by superoxide in combination with salicylic acid or with protein synthesis inhibitors. *Free Radic. Biol. Med.* 30, 98–106
56 Leslie, C.A. and Romani, R.J. (1988) Inhibition of ethylene biosynthesis by salicylic acid. *Plant Physiol.* 88, 833–837
57 O'Donnel, P.J. *et al.* (2003) Susceptible to intolerance – a range of hormone actions in a susceptible *Arabidopsis* pathogen response. *Plant J.* 33, 245–257
58 Wang, K.L.C. *et al.* (2002) Ethylene biosynthesis and signaling networks mediating responses to stress. *Plant Cell* 14, S131–S151
59 Hadfield, K.A. and Bennett, A.B. (1997) Programmed senescence of plant organs. *Cell Death Differ.* 4, 662–670
60 Drew, M.C. *et al.* (2000) Programmed cell death and aerenchyma formation in roots. *Trends Plant Sci.* 5, 123–127
61 Lund, S. *et al.* (1998) Ethylene regulates the susceptible response to pathogen infection in tomato. *Plant Cell* 10, 371–382
62 Vahala, J. *et al.* (1998) Induction of an ACC synthase cDNA by ozone in light-grown *Arabidopsis thaliana* leaves. *Physiol. Plant.* 103, 45–50
63 Tuomainen, J. *et al.* (1997) Ozone induction of ethylene emission in tomato plants: regulation by differential transcript accumulation for the biosynthetic enzymes. *Plant J.* 12, 1151–1162
64 Nakajima, N. *et al.* (2002) Improvement in ozone tolerance of tobacco plants with an antisense DNA for 1-aminocyclopropane-1-carboxylate synthase. *Plant Cell Environ.* 25, 727–736
65 Moeder, W. *et al.* (2002) Ethylene synthesis regulated by bi-phasic induction of ACC synthase and ACC oxidase genes is required for H_2O_2 accumulation and cell death in ozone-exposed tomato. *Plant Physiol.* 130, 1918–1926
66 Bent, A.F. *et al.* (1992) Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. *Mol. Plant–Microbe Interact.* 5, 372–378
67 Ohtsubo, N. *et al.* (1999) Ethylene promotes the necrotic lesion formation and basic PR gene expression in TMV-infected tobacco. *Plant Cell Physiol.* 40, 808–817
68 Ciardi, J.A. *et al.* (2001) Reduced expression of the tomato ethylene receptor gene *LeETR4* enhances the hypersensitive response to *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Plant–Microbe Interact.* 14, 487–495
69 Vahala, J. *et al.* (2003) Ethylene insensitivity modulates ozone-induced cell death in birch. *Plant Physiol.* 132, 185–195

70 Alvarez, M.E. *et al.* (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92, 773–784
71 Ciardi, J.A. *et al.* (2000) Response to *Xanthomonas campestris* pv. *vesicatoria* in tomato involves regulation of ethylene receptor gene expression. *Plant Physiol.* 123, 81–92
72 Bleecker, A.B. and Kende, H. (2000) Ethylene: a gaseous signal molecule in plants. *Annu. Rev. Cell Dev. Biol.* 16, 1–18
73 Ciardi, J. and Klee, H. (2001) Regulation of ethylene-mediated responses at the level of receptor. *Ann. Bot.* 88, 813–822
74 Simons, B.H. *et al.* (1999) Enhanced expression and activation of the alternative oxidase during infection of *Arabidopsis* with *Pseudomonas syringae* pv tomato. *Plant Physiol.* 120, 529–538
75 Turner, J.G. *et al.* (2002) The jasmonate signal pathway. *Plant Cell* 14, S153–S164
76 Weber, H. (2002) Fatty acid derived signals in plants. *Trends Plant Sci.* 7, 217–224
77 Stintzi, A. and Browse, J. (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lacks the 12-oxophyodienoic acid reductase required for jasmonate biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 10625–10630
78 Kachroo, P. *et al.* (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9448–9453
79 Seo, H.K. *et al.* (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4788–4793
80 Blée, E. (2002) Impact of phyto-oxylipins in plant defense. *Trends Plant Sci.* 7, 315–321
81 Schenk, P.M. *et al.* (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11655–11660
82 Stenzel, I. *et al.* (2003) Jasmonate biosynthesis and the allene oxide cyclase family of *Arabidopsis thaliana*. *Plant Mol. Biol.* 51, 895–911
83 Seo, S. *et al.* (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. *Plant Cell* 11, 289–298
84 Petersen, M. *et al.* (2000) *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell* 103, 1111–1120
85 Jonak, C. *et al.* (2002) Complexity, cross talk and integration of plant MAP kinase signalling. *Curr. Opin. Plant Biol.* 5, 415–424
86 Nibbe, M. *et al.* (2002) Cell death and calicylate- and jasmonate-dependent stress responses in *Arabidopsis* are controlled by single *cet* genes. *Planta* 216, 120–128
87 Asai, T. *et al.* (2000) Fumonisin BI-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. *Plant Cell* 12, 1823–1835
88 Creelman, R.A. and Mullet, J.E. (1997) Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 355–381
89 Klusener, B. *et al.* (2002) Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in *Arabidopsis* guard cells. *Plant Physiol.* 130, 2152–2163
90 Bethke, P.C. *et al.* (1999) Hormonally regulated programmed cell death in barley aleurone cells. *Plant Cell* 11, 1033–1045
91 O'Donnell, P.J. *et al.* (2001) Ethylene-dependent salicylic acid regulates an expanded cell death response to a plant pathogen. *Plant J.* 25, 315–323
92 Asai, T. *et al.* (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 415, 977–983
93 Ren, D. *et al.* (2001) Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*. *J. Biol. Chem.* 277, 559–565
94 Kovtun, Y. *et al.* (2000) Functional analysis of oxidative stress activated mitogen-activated protein kinase cascades in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2940–2945
95 Samuel, M.A. *et al.* (2000) Ozone treatment rapidly activates MAP kinase signalling in plants. *Plant J.* 22, 367–376
96 Moon, H. *et al.* (2003) NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proc. Natl. Acad. Sci. U.S.A.* 100, 358–363
97 Samuel, M.A. and Ellis, B.E. (2002) Double jeopardy: both over-expression and suppression of a redox-activated plant mitogen-activated protein kinase render tobacco plants ozone sensitive. *Plant Cell* 14, 2059–2069
98 Frye, C.A. *et al.* (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 373–378