

REVIEW

The role of the mitochondrion in plant responses to biotic stress

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Recent studies suggest that the plant mitochondrion may play a role during biotic stress responses, such as those occurring during incompatible plant–pathogen interactions. There are indications that signal molecules or pathways initiated by such interactions may directly or indirectly target mitochondrial components and that an important consequence of this targeting is an early disruption of mitochondrial homeostasis, resulting in an increased generation of mitochondrial reactive oxygen species (mROS). These mROS may then initiate further mitochondrial dysfunction and further mROS generation in a self-amplifying manner. The mROS, as well as the graded dysfunction of the mitochondrion may act as cellular signals that initiate graded cellular responses ranging from defense gene induction to initiation of programmed cell death. However, these events may be attenuated by the unique components of the plant electron transport chain that act to substitute for dysfunctional components, dampen mROS generation or facilitate in defining the cellular level of ROS and antioxidant defense systems.

Introduction

Upon recognition of a pathogen, plants mount a resistance response meant to cease pathogen growth and disease development (Dangl and Jones 2001, Greenberg and Yao 2004, Lam et al. 2001). The resistance response can include activation of local and systemic defenses (e.g. expression of pathogenesis-related proteins) and induction of a localized plant cell death at the site of infection called the hypersensitive response (HR). The HR is a form of programmed cell death (PCD) and shares some molecular and biochemical similarities with animal apoptosis.

Salicylic acid (SA), nitric oxide (NO) and reactive oxygen species (ROS) (particularly H_2O_2) increase in

abundance following pathogen recognition and each are important signaling molecules that promote and coordinate defense and HR responses (Alvarez 2000, Delledonne 2005, Laloï et al. 2004, Neill et al. 2002, Torres and Dangl 2005, Wendehenne et al. 2004). The increase in ROS (the so-called oxidative burst) involves activation of a plasma membrane-localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. During the HR, this is accompanied by an active down-regulation of ROS-scavenging systems to further promote ROS accumulation (Mittler et al. 1998, Vacca et al. 2004). There are also complex synergistic (and possibly antagonistic) interactions between SA, NO and ROS that define the responses to biotic stress (Delledonne 2005).

Abbreviations – AA, antimycin A; ANT, adenine nucleotide translocator; AOX, alternative oxidase; BA, bongkreik acid; CsA, cyclosporin A; cyt, cytochrome; $\Delta\Psi_m$, mitochondrial transmembrane potential; DPI, diphenylene iodonium; ETC, electron transport chain; GDC, glycine decarboxylase; HR, hypersensitive response; IMM, inner mitochondrial membrane; IMS, intermembrane space; mROS, mitochondrial reactive oxygen species; NO, nitric oxide; OMM, outer mitochondrial membrane; PCD, programmed cell death; PPIX, protoporphyrin IX; PTP, permeability transition pore; ROS, reactive oxygen species; SA, salicylic acid; VDAC, voltage-dependent anion channel.

It is hypothesized that plant mitochondria act in the perception of biotic stress and take part in initiating responses such as the HR (Jones 2000, Lam et al. 2001). In part, this hypothesis derives from studies of animal apoptosis, where mitochondria play an active role (see reviews by Bratton and Cohen 2001, Crompton 1999, Kuwana and Newmeyer 2003, Ly et al. 2003, Newmeyer and Ferguson-Miller 2003, van Loo et al. 2002). Animal apoptosis involves activation of an aspartate-specific cysteine protease (caspase) cascade. Activation is achieved by the release of mitochondrial intermembrane space (IMS) proteins, in particular the electron transport chain (ETC) component cytochrome (cyt) *c*, to the cytosol. Cyt *c* then combines with other cytosolic components to form a caspase-activating complex. The caspase cascade acts to amplify the original death-inducing signal and participates in the ordered disassembly of the cell. Cyt *c* release is tightly regulated: antiapoptotic Bcl-2 family members present on the outer mitochondrial membrane (OMM) act to prevent cyt *c* release, whereas proapoptotic Bcl-2 members can translocate from cytosol to the OMM and promote cyt *c* release.

The mechanism by which IMS proteins are released to the cytosol during animal apoptosis remains a topic of debate (Ly et al. 2003). Potential mechanisms are broadly divided into three types: (1) the inner mitochondrial membrane (IMM) experiences a large increase in permeability because of opening of the permeability transition pore (PTP). The PTP resides at contact sites between the inner and outer membranes and its core components include the IMM-localized adenine nucleotide translocator (ANT), the OMM-localized voltage-dependent anion channel (VDAC) and the matrix-localized cyclophilin-D. Pore opening results in a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), which is followed by an influx of water and solutes to the matrix. This causes matrix swelling and selective rupture of the OMM (because of its smaller surface area in comparison to the IMM), allowing the release of IMS proteins. Cyclosporin A (CsA) and bongkreikic acid (BA) are pharmacological inhibitors of PTP opening, acting by interaction with cyclophilin-D or ANT, respectively. A key requirement for pore opening is the accumulation of Ca^{2+} in the mitochondrial matrix and susceptibility to Ca^{2+} -induced opening is influenced by numerous other aspects of mitochondrial status (Crompton 1999). Also, the pro- and antiapoptotic proteins may act by promoting or inhibiting PTP opening; (2) proteins residing in and/or recruited to the OMM can produce a pore that allows release of IMS proteins to the cytosol. VDAC, as well as proapoptotic proteins (e.g. Bax) may be components of this pore, whereas antiapoptotic proteins (e.g. Bcl-2) may inhibit pore formation; (3) the VDAC

closes in response to death stimuli and because VDAC and ANT coordinately shuttle adenosine diphosphate (ADP) into the matrix in exchange for adenosine triphosphate (ATP), this closure depletes matrix ADP. This leads to an initial increase in $\Delta\Psi_m$ that promotes enhanced generation of ROS by the ETC (see below). These factors damage the IMM, leading to an influx of solutes and water, followed by swelling and rupture of the OMM.

A distinct feature of plant mitochondria is the presence of several unique ETC components beside those components associated with the usual cyt pathway (that consists of Complexes I–IV and cyt *c*). Besides Complex I (the rotenone-sensitive NADH dehydrogenase oxidizing matrix NADH), the IMM contains alternative rotenone-resistant NAD(P)H dehydrogenases (Finnegan et al. 2004, Rasmusson et al. 2004). These include both 'internal' enzymes oxidizing matrix NAD(P)H and 'external' enzymes that oxidize NAD(P)H on the external side of the IMM. The alternative dehydrogenases reduce the energy yield of respiration because they are non-proton pumping and bypass the proton-pumping Complex I. Several alternative NAD(P)H dehydrogenases possess EF-hand motifs for Ca^{2+} binding, consistent with the observation that their activity is modulated by Ca^{2+} . The IMM also contains an additional terminal oxidase (beside Complex IV or cyt oxidase) called alternative oxidase (AOX) that catalyzes the oxidation of ubiquinone and reduction of O_2 to H_2O (Finnegan et al. 2004). AOX also reduces the energy yield of respiration because it is non-proton pumping and bypasses proton-pumping Complexes III and IV.

Mitochondrial electron transport is associated with the generation of ROS such as superoxide and H_2O_2 , which are referred to in this review specifically as mitochondrial ROS (mROS). Because ROS can damage macromolecules, their cellular levels are managed through avoidance and scavenging mechanisms (Mittler et al. 2004). As in animals, Complexes I and III likely represent the primary sites of mROS generation (Møller 2001). The relative importance of these two sites of mROS generation and the factors influencing their rates of mROS production are largely unknown but an important generalization is that mROS formation increases as the ETC becomes more highly reduced. mROS generation by isolated mitochondria is therefore increased under ADP-limiting conditions that increase $\Delta\Psi_m$ and decreased by uncouplers that dissipate $\Delta\Psi_m$. mROS formation is also increased by inhibition of specific sites in the ETC such as inhibition of Complex III by antimycin A (AA) or inhibition of Complex I by rotenone. These inhibitors presumably promote mROS formation by promoting overreduction of specific ETC components (Møller 2001).

The alternative dehydrogenases and AOX may impact the rate of mROS production. By accepting electrons from ubiquinone, AOX may prevent overreduction at Complex I and/or III. This route of electron transport could be important in dampening mROS formation under conditions in which cyt pathway components have suffered stress-induced damage or, because AOX respiration is less tightly coupled to ATP production, under conditions in which ADP availability is limiting. Such a role for AOX is supported by the finding that transgenic cells lacking AOX have more ROS emanating from the mitochondrion (Maxwell et al. 1999). How the alternative NAD(P)H dehydrogenases impact ROS generation is unknown. On the one hand, they may themselves represent sites of ROS generation. Alternatively, they may act to dampen ROS generation because (1) their activity will bypass Complex I, a known ROS producer and (2) unlike Complex I, their activity will not contribute to $\Delta\Psi_m$.

Below, we review recent literature investigating the potential role of plant mitochondria in biotic stress responses. Fig. 1 is a summary of the main questions being addressed. We propose some working models to aid further research in this area.

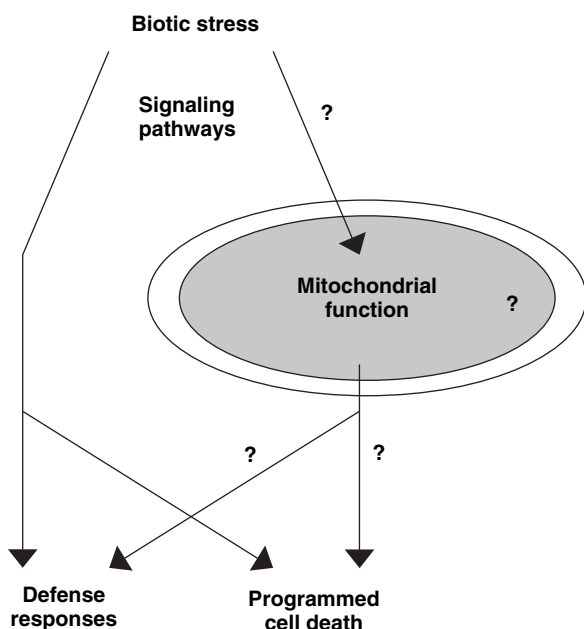


Fig. 1. A framework for investigating the role of plant mitochondria in biotic stress. The following are the key questions being addressed in this review and illustrated in this figure: (1) Do any signaling molecules or pathways initiated by biotic stress impact mitochondrial function? (2) What changes occur in mitochondrial function? (3) Does the mitochondrion play an active role in programmed cell death events such as the hypersensitive response? (4) Is the induction of any defense responses to biotic stress dependent upon mitochondrial events?

Recent studies suggest that plant mitochondria may be a target of biotic stress

Beside other well-studied signaling roles for SA during biotic stress (see Introduction), it has recently been suggested that SA may directly impact mitochondria. It was shown that SA disrupts mitochondrial function in a concentration-dependent manner in tobacco suspension cells (Norman et al. 2004). At low concentrations, it acted as an uncoupler, whereas at higher concentrations it strongly inhibited electron flow. These effects were seen in both whole cells and isolated mitochondria and provide a rationale for studies showing that SA could dramatically inhibit ATP synthesis by tobacco cells (Xie and Chen 1999). It may also provide a rationale for why SA is able to induce AOX because AOX expression appears to increase in response to disruptions in respiratory homeostasis induced by diverse means (Finnegan et al. 2004). Norman et al. (2004) found that SA inhibited electron flow upstream of the ubiquinone pool, perhaps by acting as a quinone analog interacting with Complex I or II. Significantly, the concentrations of SA required to induce these dramatic effects are within the range often used by investigators when examining effects of externally supplied SA. A key unresolved question is whether endogenous localized concentrations of SA that accompany pathogen infection are sufficient to impact mitochondrial function. If they are, it opens up the possibility that some “signaling functions” of SA act via effects on the mitochondrion.

Norman et al. (2004) also found that AOX expression correlated with the ability of SA to disrupt mitochondrial function. Low concentrations of SA caused only transitory increases in cellular SA and this correlated well with both transitory mitochondrial dysfunction and transitory increases in AOX expression. Hence, AOX may represent an excellent ‘reporter gene’ to evaluate whether mitochondrial dysfunction is occurring during biotic stress. Several studies suggest that this is the case (see later). For example, AOX was amongst the early response genes induced in *Arabidopsis* during bacterial infection (Lacomme and Roby 1999). AOX induction was transient (as expected for the increase in SA) and specific to an avirulent interaction (as are increases in SA).

Interestingly, recent work with animal mitochondria shows that SA interacts directly with Complex I, causing an increase in Complex I-generated ROS, which then contributes to a permeability transition, cyt c release and apoptosis (Battaglia et al. 2005). If SA targets plant mitochondria in a similar fashion, it could play a role in the early generation of mROS noted in recent studies (see below).

Another signal molecule during biotic stress is NO, which along with SA and ROS, has been shown to promote the HR (see Introduction). In animals, NO is a modulator of mitochondrial-mediated apoptosis, in part because it causes a strong reversible inhibition of cytochrome oxidase (Vieira and Kroemer 2003). Plant cytochrome oxidase is similarly sensitive to NO but whether the physiological NO concentrations generated during plant–pathogen interactions are sufficient to inhibit cytochrome oxidase and whether such inhibition contributes to defense responses or the HR remains unknown. An important factor in this regard may be the cellular source of NO. Animals have a mitochondrial-localized NO synthase. The situation in plants has been less clear but a recent publication has identified a NO synthase localizing to mitochondria (Guo and Crawford 2005). Under some conditions, the plant ETC may also generate NO from nitrite (Planchet et al. 2005). These studies provide potential means by which NO could be generated in close proximity to cytochrome oxidase, hence perturbing mitochondrial function.

An important set of virulence factors in pathogenic fungi is the so-called host-selective toxins that interact with host molecules to cause plant cell death and contribute to disease development. One such toxin, victorin, was shown to bind to and inhibit mitochondrial glycine decarboxylase (GDC), suggesting that GDC inhibition acted to promote cell death (Curtis and Wolpert 2002). Victorin treatment of oat leaves resulted in a loss of $\Delta\Psi_m$, followed by an ability of victorin to gain access to the mitochondrial matrix. This was interpreted to indicate that a permeability transition had occurred and that victorin used the PTP to gain access to matrix GDC. However, more recent results suggest that cell death precedes access of victorin to the cell interior and that victorin likely interacts with a cell surface protein to initiate defense responses and cell death (Curtis and Wolpert 2004, Tada et al. 2005). In this respect, the virulence of the toxin may reside in its ability to elicit a plant PCD pathway. These results shed doubt on the importance of victorin-induced GDC inhibition in promoting cell death, but they do not preclude a role for the mitochondrion in this cell death. In particular, Yao et al. (2002) have shown that victorin induces a burst of mROS preceding death (see below).

Ceramides are lipids that act as important second messengers in animals, where the balance between ceramides and their phosphorylated derivatives may regulate apoptosis. Animal studies indicate that ceramide can cause a direct inhibition of Complex III, which, by promoting mROS generation, initiates apoptosis (Gudz et al. 1997, Quillet-Mary et al. 1997). Interestingly, an *Arabidopsis* mutant defective in ceramide kinase (and hence accumulating ceramide) shows excessive PCD in

response to bacterial infection (Liang et al. 2006). It will be interesting to examine whether this enhanced cell death is because of ceramide targeting of the ETC.

In summary, a number of molecules commonly associated with biotic stress may have a direct impact on ETC components such as Complexes I, III and IV. As discussed next, a common consequence of this targeting may be an increase in mROS.

Recent studies suggest that an increase in mROS formation is an early consequence of biotic stress

Earlier studies showed that intracellular sources of ROS might contribute to the pathogen-induced oxidative burst (e.g. Allan and Fluhr 1997, Naton et al. 1996) and a review by Bolwell and Wojtaszek (1997) suggested a need to investigate whether the mitochondrion represented such a source. A few recent studies have now directly addressed this question by using ROS-sensitive fluorescent dyes and other imaging techniques to localize ROS generation in vivo and in response to pathogens or their elicitors.

Harpins are virulence factors produced by bacterial pathogens such as *Pseudomonas syringae*. Application of purified harpin to plant tissue can elicit a rapid HR-like cell death and some studies have examined the impact of such harpin treatments on mitochondria. By double staining *Arabidopsis* cell cultures with both a mitochondrial-specific dye and a ROS-indicating dye, it was shown that a large and early ROS burst associated with harpin treatment emanated specifically from the mitochondrion, suggesting the ETC as the likely source of ROS (Krause and Durner 2004). This burst of mROS was associated with a decline in $\Delta\Psi_m$ and cellular ATP levels and the appearance of cytosol-localized cytochrome *c*. All these events preceded PCD by several hours. The results are consistent with those of another study in which harpin was shown to dramatically inhibit ATP synthesis in tobacco cell cultures (Xie and Chen 2000). That study found that the early harpin-induced burst of ROS could be completely inhibited by diphenylene iodonium (DPI), a finding usually interpreted to indicate that ROS production is occurring via the DPI-sensitive NADPH oxidase. However, DPI is also a potent inhibitor of Complex I (Møller 2001). Hence, another interpretation of the DPI result could be that ROS is being generated by the mitochondrion in response to harpin and that this ROS generation can be dampened by DPI inhibition of Complex I. The study of Xie and Chen (2000) also found that harpin treatment dramatically reduced the in vivo capacity for cytochrome *c* pathway electron transport downstream of ubiquinone. This would be consistent with a loss of cytochrome *c* from the mitochondrion, although this was not examined.

The above studies show that harpin has a rapid and dramatic impact on mitochondria, an interesting observation in light of the recent proposal that most *P. syringae* virulence factors likely function by targeting the plasma membrane, chloroplast or mitochondrion of host cells (Greenberg and Vinatzer 2003).

Greenberg and colleagues have studied mitochondrial events associated with HR induction by *P. syringae* as well as PCD induced by protoporphyrin IX (PPIX) or by light treatment of the *Arabidopsis* accelerated cell death 2 (ACD2) mutant. ACD2 encodes a protein that attenuates PCD, probably by sequestering or metabolizing porphyrin-related molecules (such as PPIX) that can be photo-activated, leading to the production of ROS. Interestingly, the localization of ACD2 shifts from being largely chloroplastic to including the mitochondrion during PCD-inducing treatments. Yao and Greenberg (2006) reported that a very early event (1.5 h) associated with death-inducing treatment of wild-type or ACD2 plants was a burst of mROS, localized using ROS-sensitive fluorescent dyes. This was followed slightly later by a loss of $\Delta\Psi_m$ (quantified using flow cytometry) that, if blocked by CsA or ROS scavengers, was able to attenuate the PCD (Yao and Greenberg 2006, Yao et al. 2004). These elegant studies provide the most convincing data to date that mitochondrial events precede and contribute toward plant PCD.

In another interesting study, $\Delta\Psi_m$ and mROS generation of camptothecin-treated and digitonin-permeabilized protoplasts were monitored by flow cytometry (Weir et al. 2003). This study also found an early (1.5 h) burst in mROS and this corresponded closely with an increase of $\Delta\Psi_m$. This was then followed slightly later by a decrease in both these parameters. The initial increase in $\Delta\Psi_m$ (similar to that reported in an early study by Naton et al. 1996) is of particular interest. It is in keeping with animal models in which impaired ATP/ADP exchange between the cytosol and matrix (perhaps because of VDAC closure) promotes an initial increase in $\Delta\Psi_m$ that, by promoting overreduction of the ETC, promotes mROS generation and mitochondrial dysfunction. The decreased expression of ANT during heat shock or senescence associated PCD of *Arabidopsis* cells provides another hint that impaired ATP/ADP exchange may be an early event in PCD (Swidzinski et al. 2002). In another study, victorin was shown to elicit a very rapid (30 min) increase in mROS (Yao et al. 2002). In this case, localization of the ROS was based on a cytochemical assay that showed H_2O_2 eruptions at specific sites on the OMM.

The above studies indicate that increased mROS is an early event that clearly precedes PCD and likely also precedes other documented mitochondrial events such as loss of $\Delta\Psi_m$ and cyt *c* release (see later). As well, the

results suggest that the mROS released is obligatory to PCD in that, in some cases, it was shown that scavenging of the ROS attenuated PCD. We suggest that the early burst of mROS being noted in these studies is because of a disruption of metabolic homeostasis in the mitochondrion, possibly because of molecules (such as those described in the previous section) that target the ETC. Also, we suggest that an important consequence of this mROS burst will be a self-amplifying cycle in which the increased mROS leads to mitochondrial damage, resulting in further increases in mROS and further damage. The culmination of these events will be the catastrophic mitochondrial dysfunction associated with changes in the permeability or integrity of the mitochondrial membranes (see later). This hypothesis is outlined in Fig. 2.

Several studies have documented the sensitivity of mitochondria (particularly components of energy metabolism) to oxidative stress, suggesting that ROS accumulation can promote damage and dysfunction (Bartoli et al. 2004, Kristensen et al. 2004, Sweetlove et al. 2002, Taylor et al. 2002). Some of the identified components that appear particularly susceptible to oxidative stress include aconitase, GDC, ATP synthase, cyt *c* and VDAC. As outlined more later, the self-amplifying cycle of mROS generation and mitochondrial dysfunction may be an important feature promoting PCD.

There is also evidence that the ROS-scavenging capacity of the mitochondrion is modulated in response to pathogen infection. In particular, increases in mitochondrial superoxide-scavenging capacity combined with decreases in the H_2O_2 -scavenging components of the organelle were seen during *Botrytis cinerea* infection of tomato leaves and it was hypothesized that this could promote accumulation of mitochondrial H_2O_2 (Kuźniak and Skłodowska 2004). Such results imply an active mechanism to ensure accumulation of specific ROS species at the mitochondrion.

Recent studies suggest that mitochondria do play an active role in plant PCD

A possible role of plant mitochondria in PCD was indicated by studies showing that when pro- or antiapoptotic animal proteins such as Bax or Bcl-2 were expressed in plants, they were able to, respectively, promote or inhibit PCD (Lam et al. 2001). Plants lack clear homologs of these proteins and so the functional relevance of these observations remains speculative. However, the studies did emphasize that manipulation of components at the OMM impacted PCD, implying that plant mitochondria could play an active role in the process.

Table 1 summarizes some recent literature in which mitochondrial events were examined during PCD and the

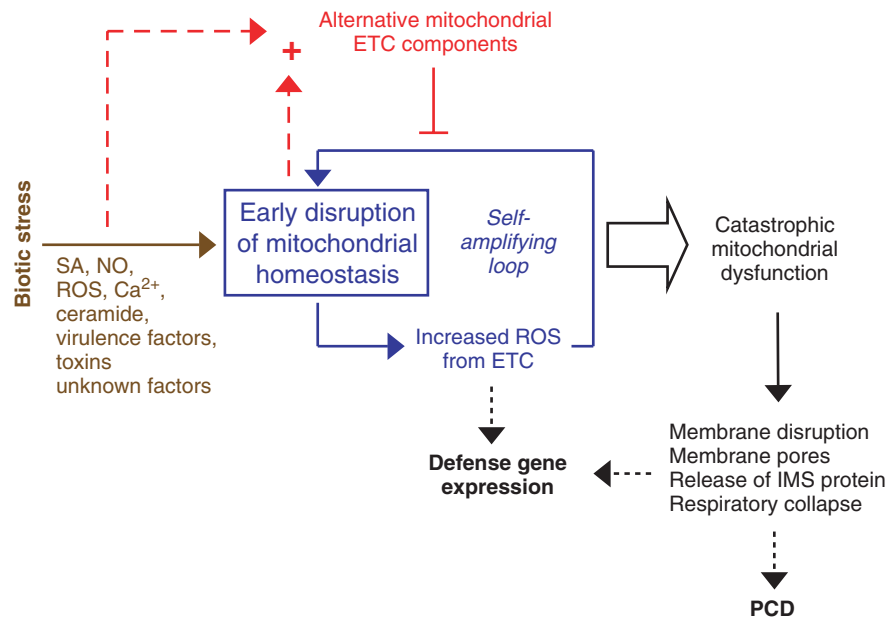


Fig. 2. A working model for the role of mitochondria in biotic stress responses such as the hypersensitive response. The model suggests that biotic stress-induced factors such as salicylic acid (SA), nitric oxide (NO), H_2O_2 , Ca^{2+} , ceramide, virulence factors or other unknown agents promote defense gene expression and programmed cell death by inhibiting the function of cytochrome (cyt) pathway electron transport chain (ETC) components such as Complex I, III or IV. This dysfunction promotes increased reactive oxygen species (ROS) generation by the ETC (mitochondrial ROS [mROS]), thus initiating further damage and dysfunction in a self-amplifying manner, and ultimately leading to the catastrophic dysfunction associated with permeability transition, loss of outer membrane integrity and release of intermembrane space proteins (including cyt c) to the cytosol. However, the unique alternative components of the ETC (the rotenone-resistant NAD(P)H dehydrogenases and alternative oxidase) can attenuate these events by functionally replacing cyt pathway components and attenuating mROS generation. Further, the activity and/or expression of these alternative components may be enhanced by some of the same factors (e.g. SA, NO, Ca^{2+}) responsible for inducing cyt pathway dysfunction. See text for further details.

reader is referred to this literature for a more in-depth analysis of this topic. An understanding of how mitochondria contribute to PCD will depend upon elucidating the timing of mitochondrial events, of which we still have only a rudimentary knowledge. As summarized in Table 1, numerous studies have documented decreases in $\Delta\Psi_m$ that precede PCD. In some cases (but not all), this decrease (and in some cases PCD itself) can be attenuated by CsA, consistent with the drop in $\Delta\Psi_m$ representing a permeability transition. Often closely associated with the loss of $\Delta\Psi_m$ is a loss of cyt c to the cytosol. This might also be consistent with a permeability transition because many animal models of cyt c release are dependent upon the permeability transition (see Introduction). However, interpretations of such data remain difficult because the mechanism of cyt c release in plants has not been investigated. As noted in the previous section, a breakthrough in our understanding may reside with studies that have shown a very early and localized increase in mROS. If, as we suggest, this mROS promotes a self-amplifying cycle of mitochondrial dysfunction, then this could lead to the often-documented (and often slightly later) events of declining $\Delta\Psi_m$ and cyt c release. Interestingly, a recent

article shows that cyt c release can be blocked by antioxidants, perhaps evidence that cyt c release is dependent upon mROS generation (Vacca et al. 2006).

A central feature of many models of mitochondrial dysfunction and release of IMS proteins during animal apoptosis is opening of the PTP (see Introduction). Plant mitochondria are known to contain the key components (VDAC, ANT, cyclophilin-D) that constitute the animal PTP. Hence, a key question is whether a similar permeability transition occurs in plants. The elegant study of Arpagaus et al. (2002) strongly suggests that such a permeability transition can indeed occur in plants and that conditions promoting PTP opening are similar to those described in animals. Under PTP-inducing conditions, swelling of purified potato mitochondria proceeded with kinetics similar to that in animals and this resulted in selective rupture of the OMM and release of IMS proteins, including cyt c. Similar to animals, these events were absolutely dependent upon the presence of Ca^{2+} (other cations such as Mg^{2+} were not effective) and were potentially inhibited by CsA. Similar to animals, the ability of Ca^{2+} to induce pore opening was modulated by other key factors. For example, the presence of P_i was

Table 1. A summary of some recent studies linking the plant mitochondrion to programmed cell death (PCD). Only a subset of these represents biotic stress-induced PCD, enforcing the idea that the mitochondrion may be a common component amongst diverse PCD pathways. In the majority of these studies, PCD was confirmed by markers such as nuclear condensation, cytoplasmic shrinkage or oligonucleosomal cleavage of DNA. $\Delta\Psi_m$, mitochondrial transmembrane potential; CsA, cyclosporin A; ROS, reactive oxygen species; OMM, outer mitochondrial membrane; AOX, alternative oxidase; ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel; IMS, intermembrane space; PPIX, protoporphyrin IX; NO, nitric oxide; cyt, cytochrome.

Experimental system	Mitochondrial events	Reference
<i>Petroselinum crispum</i> ; suspension cells infected with <i>Phytophthora infestans</i>	Increased $\Delta\Psi_m$ and ROS accumulation in individual fungus-infected cells precedes PCD	Naton et al. 1996
<i>Helianthus annuus</i> ; PCD of tapetal cells in cytoplasmic male sterile plants	Cyt c release precedes loss of OMM integrity	Balk and Leaver 2001
<i>Arabidopsis thaliana</i> ; PCD of synergid cells	Mutant defective in the mitochondrial protein GFA2 is defective in PCD	Christensen et al. 2002
<i>Nicotiana tabacum</i> ; SA and H ₂ O ₂ -induced PCD of suspension cells	Cells lacking AOX show increased susceptibility to PCD; cyt c release	Robson and Vanlerberghe 2002
<i>Citrus sinensis</i> ; NO-treated suspension cells	Decrease in $\Delta\Psi_m$ and PCD blocked by CsA	Saviani et al. 2002
<i>A. thaliana</i> ; heat shock or senescence-associated PCD of suspension cells	Decreased expression of ANT during PCD	Swidzinski et al. 2002
<i>A. thaliana</i> ; oxidative stress-induced PCD of suspension cells	Mitochondria from cells given oxidative stress generate increased ROS	Tiwari et al. 2002
<i>Triticum aestivum</i> ; root mitochondria under anoxia	Anoxia plus Ca ²⁺ induces mitochondrial swelling and cyt c release in CsA-insensitive manner	Violainen et al. 2002
<i>Avena sativa</i> ; leaves treated with the host-selective toxin victorin	A burst of mROS clearly precedes a later decrease in $\Delta\Psi_m$	Yao et al. 2002
<i>Zinnia elegans</i> ; tracheary element differentiation	Decrease in $\Delta\Psi_m$ and CsA-independent cyt c release prior to PCD	Yu et al. 2002
<i>A. thaliana</i> ; isolated nuclear, cytosolic and mitochondrial fractions from heat-shocked suspension cells	An IMS-localized nuclease activity promotes high molecular weight DNA cleavage and chromatin condensation.	Balk et al. 2003
<i>Nicotiana benthamiana</i> ; leaf PCD activation following virus-induced silencing of proteasome subunits	High ROS production, decreased $\Delta\Psi_m$; cyt c release	Kim et al. 2003
<i>N. tabacum</i> ; ozone-induced leaf PCD	Cyt c release	Pasqualini et al. 2003
<i>Oryza sativa</i> ; lesion mimic mutant	Hyperphosphorylation of the mitochondrial protein prohibitin	Takahashi et al. 2003
<i>Sugarbeet</i> ; camptothecin-induced PCD, digitonin-permeabilized protoplasts	Early increase, followed by later decrease in mROS and $\Delta\Psi_m$	Weir et al. 2003
<i>A. sativa</i> ; leaves treated with the host-selective toxin victorin	A subpopulation of mitochondria lose $\Delta\Psi_m$ prior to PCD, whereas others in the same cell retain $\Delta\Psi_m$	Curtis and Wolpert 2004
<i>A. thaliana</i> ; harpin-treated suspension cells	Rapid increase in mROS and decrease in $\Delta\Psi_m$	Krause and Durner 2004
<i>A. thaliana</i> ; heat shock or senescence associated PCD of suspension cells	Preferential maintenance of specific mitochondrial proteins (e.g. manganese superoxide dismutase; VDAC) during PCD	Swidzinski et al. 2004
<i>Papaver rhoeas</i> ; pollen PCD during self-incompatibility response	Very rapid cyt c release	Thomas and Franklin-Tong 2004
<i>A. thaliana</i> ; ceramide, PPIX and elicitor-induced PCD in protoplasts	Decrease in $\Delta\Psi_m$ is a early marker of PCD; CsA can partially block the decrease in $\Delta\Psi_m$ and PCD but not cyt c release	Yao et al. 2004
<i>Glycine max</i> ; NO and H ₂ O ₂ -induced PCD of suspension cells	Changes in mitochondrial K ⁺ -channel activity	Casolo et al. 2005
<i>A. thaliana</i> ; dark-induced senescence of attached leaves	Increased oxidative damage and accelerated senescence in mutant lacking mitochondrial NO synthase	Guo and Crawford 2005

Table 1. Continued

Experimental system	Mitochondrial events	Reference
<i>N. tabacum</i> ; protoplasts subjected to salt stress	Decrease in $\Delta\Psi_m$ and initiation of PCD is dependent upon increases in cytosolic Ca^{2+} and is delayed by CsA	Lin et al. 2005
<i>A. thaliana</i> ; ovule abortion during in response to salt stress	Early ROS accumulation; early decrease in $\Delta\Psi_m$	Hauser et al. 2006
<i>N. tabacum</i> ; heat-shock-induced PCD of suspension cells	Cyt <i>c</i> release blocked by ROS scavengers; cytosolic cyt <i>c</i> degraded by caspase-like activity	Vacca et al. 2006
<i>A. thaliana</i> ; PPIX and <i>P. syringae</i> -induced PCD in protoplasts	Early (1.5 h) increase in mROS; translocation of PCD modulator ACD2 to mitochondrion	Yao and Greenberg 2006

necessary for Ca^{2+} -induced opening, the threshold $[Ca^{2+}]$ needed for opening was lower at reduced $\Delta\Psi_m$ and opening was promoted by compounds capable of thiol oxidation. In animals, oxidation of critical thiols of the ANT promotes pore opening and this may explain why ROS are often reported to enhance PTP opening (Kanno et al. 2004). Arpagaus et al. (2002) did not examine whether ROS could promote pore opening but did demonstrate that pore opening could occur under anoxia, thus precluding ROS as an absolute requirement for permeability transition.

An important area of future study will be to determine whether the release of any IMS proteins from mitochondrion to cytosol plays an active role in plant PCD, analogous to the situation in animals. For example, although the release of cyt *c* to the cytosol does appear to be an event often coinciding with plant PCD, there is at present little compelling data to indicate that this relocalization is an obligatory event for PCD induction and no indication that cyt *c* has interacting partners in the cytosol, similar to that seen in animals. One possibility is that cyt *c* release in plants simply represents a more passive (primitive?) mechanism promoting PCD than is documented in animals. For example, a progressive loss of cyt *c* could amplify mROS generation.

Although an active role for cytosolic cyt *c* in PCD remains speculative, one study has provided compelling evidence for another IMS protein promoting PCD. Using a cell-free system, Balk et al. (2003) have shown that an IMS DNase activity could mediate the generation of 30-kb DNA fragments as well as DNA condensation. Such activity is reminiscent of apoptosis-inducing factor, an animal protein that once released from the IMS moves to the nucleus and brings about cleavage of DNA into large fragments and chromatin condensation. Several apoptosis-inducing factor homologs are present in the *Arabidopsis* genome. Finally, activation of caspases is a central feature of the mitochondria-dependent pathway of animal apoptosis (see Introduction). Accumulating evidence suggests that caspase-like activities are also

activated during plant PCD but this activation has not yet been strongly linked to the mitochondrion (Sanmartín et al. 2005).

Recent studies suggest that the expression of some plant defense genes may be modulated by mitochondrial function

Beside a role in the HR, mitochondria may represent an important intermediate between the perception of biotic stress and downstream responses such as the induction of defense gene expression (Jones 2000, Lam et al. 2001). Studies that have investigated this hypothesis are outlined below.

Polyamines such as spermine are proposed to play a role during biotic stress responses. Spermine accumulates dramatically and in an N-gene-specific manner in the apoplast of tobacco mosaic virus (TMV)-infected tobacco because of upregulation of genes involved in spermine biosynthesis (Yamakawa et al. 1998, Yoda et al. 2003). Accumulated polyamines are subsequently degraded in the apoplast by polyamine oxidase, generating H_2O_2 that may contribute to plant responses (Yoda et al. 2003). A series of recent publications have investigated the series of events that may link this apoplastic degradation of spermine to downstream events that include the mitochondrion. The findings of these studies are summarized in Fig. 3. Exogenous application of spermine to tobacco leaves could induce defense responses and cell death, mediated by a pathway involving activation of mitogen activated protein (MAP) kinases and resulting in increased expression of HR marker genes and transcription factors (Takahashi et al. 2003, 2004, Uehara et al. 2005). Interestingly, activation of the MAP kinase cascade and downstream changes in gene expression could be blocked by BA, the inhibitor of animal PTP opening (see Introduction). This suggests that mitochondrial events (dysfunction leading to PTP?) are required for MAP kinase activation by spermine. Spermine also induced AOX, perhaps indicative of mitochondrial dysfunction. It was also shown that AOX induction and

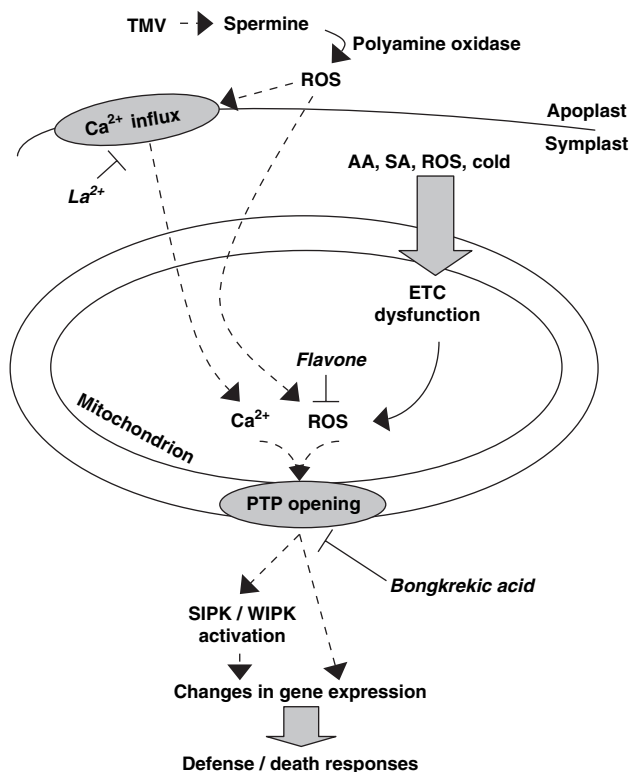


Fig. 3. A working model for how mitochondria may act as a focal point for the perception of and response to biotic stress. Several studies have shown that stress-induced changes in gene expression can be blocked by bongkreikic acid, suggesting that opening of a mitochondrial permeability transition pore (PTP) is a necessary step for gene induction. Opening of the PTP may be promoted by stress-induced changes in Ca^{2+} , reactive oxygen species or mitochondrial function. The results outlined here are primarily based upon the work of Lee et al. (2002), Maxwell et al. (2002), Takahashi et al. (2003b, 2004) and Uehara et al. (2005). See text for further details.

activation of the MAP kinases could be blocked by the antioxidant flavone and by the Ca^{2+} channel blocker La^{2+} suggesting that increases in ROS and influx of Ca^{2+} are cellular events upstream of the mitochondrial events. Critically, increased ROS and especially increased mitochondrial Ca^{2+} are thought to be critical factors promoting PTP opening (Arpagaus et al. 2002, Crompton 1999). The requirement of spermine and mitochondrial events for changes in defense gene induction should now be evaluated using recently isolated spermine-deficient mutants of *Arabidopsis* (Imai et al. 2004).

As noted above, one source of the ROS generated during biotic stress could derive from the metabolism of amines by cell wall-localized amine oxidases. Interestingly, animals have an amine oxidase that localizes to the OMM and which is able to induce the mitochondrial pathway of PCD via H_2O_2 generation (Maccarrone et al. 2001). To our knowledge, there has been no report of

a similar activity in plant mitochondria but this might represent a fruitful area for study.

A similar BA-sensitive pathway to that outlined above was described by Maxwell et al. (2002) (Fig. 3). They found that treatment of tobacco cells with AA resulted in the rapid expression of eight different genes, as identified using differential display. Only one of these genes encoded an ETC component (AOX), whereas the others encoded proteins more generally implicated in either senescence or (biotic) stress responses. All eight genes were also induced by treatment of cells with SA or H_2O_2 . Each of the gene-inducing treatments was associated with increased cellular levels of ROS and gene induction could be partially blocked by antioxidants that lowered ROS levels, suggesting ROS as an important intermediary in gene induction. The authors also showed that pretreatment of cells with BA blocked induction of all eight genes regardless of whether AA, SA or H_2O_2 was used as the inducing agent (Maxwell et al. 2002).

Interpretation of the above studies is still somewhat speculative because it is not well established that the plant PTP is BA sensitive. Nonetheless, the most parsimonious explanation of results to date is that a plant BA-sensitive PTP exists, that this pore opens in response to signaling molecules commonly associated with biotic stress (spermine, SA, H_2O_2) or mitochondrial dysfunction (AA) and that the state of this pore affects defense gene expression (Fig. 3). If this is the case, then plant mitochondria may indeed act as a focal point for the perception of and response to biotic stress.

Further evidence that mitochondria may act in the perception of and response to stress (albeit abiotic stress in this case) comes from studies of the *fro1* mutant of *Arabidopsis* (Lee et al. 2002). Mutant plants are unable to induce a set of cold-responsive genes, thus reducing their capacity to cold acclimate. The defect is specific to cold stress in that expression of the genes in response to other treatments (abscisic acid, NaCl) is normal. Interestingly, *FRO1* encodes a protein similar to the 18-kDa Fe-S subunit of complex I from diverse organisms and was shown to localize to *Arabidopsis* mitochondria. *Fro1* plants also displayed constitutively higher levels of ROS, even in the absence of stress. Because Complex I is considered a major site of mROS production, one potential explanation is that mutation of the 18-kDa subunit has increased the ROS-generating activity of Complex I. The authors hypothesize that the constitutive generation of ROS makes mutant plants less responsive to what would normally be a cold-induced increase in ROS generation. Presumably, this cold-induced ROS generation would also involve Complex I, thus representing part of a cold-stress-activated signal path from

mitochondrion to nucleus, with mROS as a key intermediate (Fig. 3).

Other mutations of Complex I or Complex IV (but in these case mutations that dramatically compromise their activity) have been shown to increase stress gene expression and/or stress tolerance (Dutilleul et al. 2003, Kuzmin et al. 2004). These mutations illustrate that the consequence of a major mitochondrial deficiency is not limited to PCD (perhaps because of compensatory activities; see below) but can also be linked to protective (defense) responses.

Several studies investigated a possible role for the mitochondrion in the SA-mediated development of local and systemic resistance to viruses. Interest in this area stemmed from studies suggesting that AOX played some active role in the induction of resistance. Results from the use of transgenic plants with increased and decreased levels of AOX have largely negated any direct role for AOX in the development of resistance (Gilliland et al. 2003, Ordog et al. 2002).

Recent studies suggest that the unique components of the plant mitochondrial ETC play a complex role in biotic stress responses

Although there is now a large body of circumstantial evidence that plant mitochondria play a regulatory role in PCD (Table 1), analogous to the relatively well-defined active role of mitochondria in animal apoptosis, it is also evident that the regulation in plants must differ from that in animals. This is best exemplified by the lack of plant homologs of many of the key pro- and antiapoptotic proteins described in animals (Lam et al. 2001). Hence, an important next step will be to define the pro- and antiapoptotic players in a plant mitochondria-dependent PCD pathway.

As discussed earlier, the generation of mROS and mitochondrial dysfunction are early events associated with biotic stress and preceding PCD. Dysfunction may be the result of key mitochondrial components (e.g. Complexes I, III, IV) being targeted by biotic stress signals, leading to the self-amplifying cycle of increased mROS and increased dysfunction described earlier (Fig. 2). However, a striking feature of plant mitochondria in comparison to their animal counterparts is the existence of additional ETC components that increase the points of entry and exit of electrons in the respiratory chain as well as providing a high degree of flexibility in terms of the coupling of electron transport to oxidative phosphorylation (see Introduction). This is significant because these components (the rotenone-resistant alternative dehydrogenases and AOX) represent a potential means to modulate a mitochondria-dependent PCD because they

could compensate for dysfunctional ETC components, as well as providing a means to dampen the mROS generation associated with escalating dysfunction (Fig. 2). In other words, they may represent antiapoptotic components of plant mitochondria, the levels of which could define cell fate (e.g. defense vs death). It is certainly clear, for example, that AOX can prevent the PCD initiated by a loss of cyt pathway function downstream of ubiquinone (Vanlerberghe et al. 2002).

Interestingly, numerous studies have shown increases in AOX expression in response to pathogen infection, signaling molecules (SA, NO, H₂O₂) or elicitors (Bruggman et al. 2005, Huang et al. 2002, Krause and Durner 2004, Maxwell et al. 2002, Vanlerberghe and McIntosh 1996, Mizuno et al. 2005, Murphy et al. 2001, Lacomme and Roby 1999, Ordog et al. 2002, Takahashi et al. 2003, Simons et al. 1999, Zottini et al. 2002). As discussed earlier, one interpretation is that increased AOX expression simply represents an all-purpose response to disruptions of respiratory homeostasis. However, another interpretation is that it represents a defensive response against the initiation of PCD, much like increases in expression of ROS-scavenging systems. Such a response could be important in limiting cell death progression and in this regard it is interesting that overexpression of AOX has been shown to reduce the size of HR lesions induced by TMV (Ordog et al. 2002). In the case of ROS-scavenging systems, however, it has also been shown that an active decline in their capacity may be an important means to commit a cell to PCD (see Introduction). It would be interesting to examine whether capacity of the alternative ETC components might also be declining in such instances. It is also possible that once a threshold level of ROS is reached in the mitochondrion, AOX might be inactivated by oxidation of critical sulfhydryl residues involved in α -keto acid activation. This has been demonstrated to occur when cells are treated exogenously with H₂O₂ (Vanlerberghe et al. 1999). Such inactivation could again act to amplify mROS levels.

Chloroplasts are a large source of ROS and so it is often assumed that the steady-state level of cellular ROS as well as the capacity of cellular ROS-scavenging systems is largely determined by this organelle. However, as reviewed by Foyer and Noctor (2003), recent studies indicate an unexpectedly influential role for mitochondria in determining the cellular level of ROS and capacity of both intra- and extramitochondrial antioxidant defenses. Hence, another role for the alternative components of the mitochondrial ETC during biotic stress (beside a role in compensating for dysfunctional ETC components and/or controlling mROS production after imposition of the stress) is that they may play a key role in *constitutively* defining the cellular level of ROS and

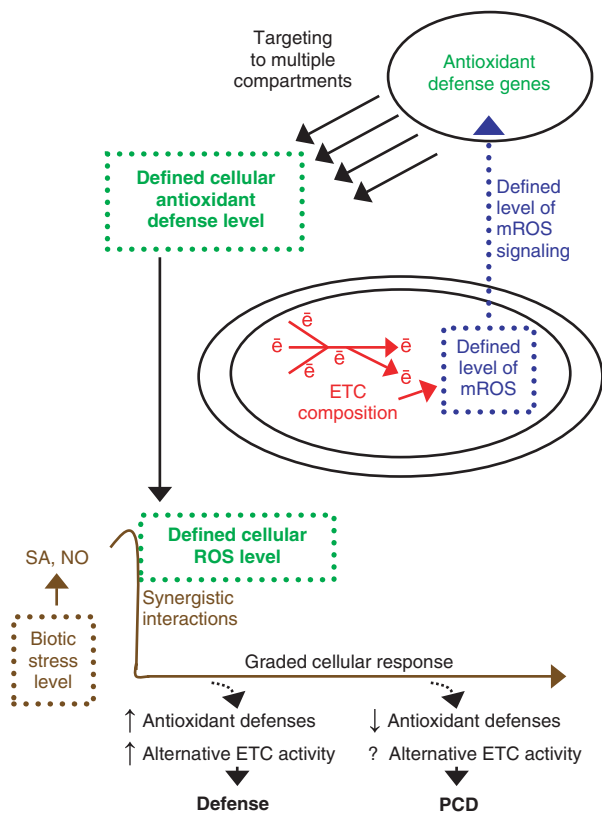


Fig. 4. A working model for how mitochondria may impact biotic stress responses by defining cellular levels of reactive oxygen species (ROS) and antioxidant defense capacity. Recent studies suggest that the rate of electron transport chain (ETC)-generated ROS (mitochondrial ROS [mROS]) is dependent upon the composition of the ETC (such as the level of alternative oxidase [AOX]) and that these mROS influence the antioxidant defense capacity of multiple cellular compartments by influencing nuclear gene expression. This antioxidant defense capacity, in turn, defines the steady-state cellular level of ROS. ROS level is critical during biotic stress, possibly because of its synergistic (and potentially antagonistic) interactions with key biotic stress signaling molecules such as salicylic acid and nitric oxide. Cellular responses to these signal interactions can range from defense gene expression to programmed cell death. Progression toward the later may depend upon active decreases in antioxidant defenses and perhaps antiapoptotic components of the ETC (such as AOX). See text for further details.

capacity of antioxidant defenses in the plant. This could dramatically impact the plant response to biotic stress (e.g. defense vs death) if in fact this response is modulated by the cellular level of ROS and capacity of antioxidant defenses (see Introduction). These ideas are summarized in Fig. 4. Recently, we have investigated these points by making use of a collection of transgenic plants and suspension cells with altered levels of AOX (Amirsadeghi, Robson and Vanlerberghe, unpublished). In both plants and suspension cells, we found that genetic manipulation of AOX levels altered both the steady-state level of ROS

and the capacity of cellular antioxidant defenses. We found that susceptibility of these plants or cells to death-inducing stimuli that may act synergistically with ROS (i.e. SA, NO) correlated well with the steady-state level of ROS. These results suggest that susceptibility to cell death initiated by these signaling molecules is strongly dependent upon the steady-state cellular level of ROS and that AOX levels clearly contribute to this steady-state by influencing the rate of mROS generation and the cellular level of antioxidant defenses. It remains to be seen how these plants will respond to various biotic stress.

The potential regulatory role of the rotenone-resistant dehydrogenases in plant PCD has not yet been investigated. However, we hypothesize that both the increases in cytosolic Ca^{2+} associated with biotic stress as well as the potential targeting of Complex I by SA could act to engage these components of the ETC.

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