Mitochondrial Reactive Oxygen Species. Contribution to Oxidative Stress and Interorganellar Signaling

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The inner membrane of a plant mitochondrion contains the mitochondrial electron transport chain (mtETC), consisting of protein complexes that use an energy source-derived reductant to form a proton gradient across the membrane. This proton gradient drives ATP synthesis, a primary mitochondrial function. Ultimately, electrons in the mtETC are transferred through one of two pathways from ubiquinone to a terminal oxidase, where oxygen is reduced to water. Specifically, electrons pass through either the “standard” cytochrome pathway, which includes two sites of proton pumping downstream of the ubiquinone pool, to cytochrome c oxidase (COX), or the alternative pathway, which consists of the cyanide-insensitive alternative oxidase (AOX) and has no proton-pumping sites after the ubiquinone pool (Finnegan et al., 2004). Because the mtETC harbors electrons with sufficient free energy to directly reduce molecular oxygen, it is considered the unavoidable primary source of mitochondrial reactive oxygen species (mtROS) production, a necessary accompaniment to aerobic respiration.

Formation of mtROS takes place under normal respiratory conditions but can be enhanced in response to a range of abnormal conditions, including exposure to biotic and abiotic stresses. The marked reactivity of ROS toward biological molecules, including lipids, proteins, and nucleic acids, requires multiple mechanisms for keeping mtROS levels under control, including pathways that attenuate mtROS formation in response to imposed stresses and protective, antioxidant enzyme systems. However, when mtROS formation exceeds normal levels despite the operation of these protective mechanisms, there are downstream consequences for the cell, including altered gene expression and even programmed cell death (PCD). Not simply agents of damage, mtROS also play roles in the signaling required to bring about these changes. The cell ultimately must strike a balance between the level of ROS required to elicit an appropriate response to a changing condition while at the same time keeping ROS levels sufficiently low to prevent large-scale cellular damage. On the other hand, cells must be able to determine a mtROS-initiated, extreme measure at the cellular level is appropriate for the good of the plant as a whole (e.g. the hypersensitive response to pathogen attack). This article provides an overview of our current understanding of plant mtROS. While much remains to be established, mtROS clearly play important roles in the responses of plants to the variety of environmental conditions they experience on a regular basis, as well as more extreme environmental stresses.

GENERATION of mtROS

The known sites of mtROS production in the mtETC are complexes I and III, where superoxide anion (\(O_2^-\)) is formed and in turn is reduced by dismutation to \(H_2O_2\) (Raha and Robinson, 2000; Møller, 2001; Sweetlove and Foyer, 2004). \(H_2O_2\), a compound of relatively low toxicity, can react with reduced \(Fe^{2+}\) and \(Cu^{+}\) to produce highly toxic hydroxyl radicals and, beinguncharged, can also penetrate membranes and leave the mitochondrion (Grene, 2002; Sweetlove and Foyer, 2004). The ubisemiquinone intermediate formed at complexes I and III is the principal electron donor to oxygen, although other complex I sites are also potential donors (Raha and Robinson, 2000; Sweetlove and Foyer, 2004). Thus, the overall reduction level of the mitochondrial ubiquinone pool will be the primary determinant of mtROS output (Sweetlove and Foyer, 2004).

The amount of ROS produced by mitochondria and the fraction of total cellular ROS that come from mitochondria are difficult to determine, in part because ROS levels in general are difficult to measure accurately (Veljovic-Jovanovic et al., 2002; Halliwell and Whitteman, 2004). For isolated mitochondria and sub mitochondrial particles, ROS evolution varies with conditions but falls within the wide range of 0.2 to 30.0 nmol min \(^{-1}\) mg protein \(^{-1}\) for either \(H_2O_2\) or superoxide (studies compiled in Møller, 2001; Popov et al., 2003). While we are unaware of any quantitative measurements of ROS production by mitochondria in vivo, estimates (Foyer and Noctor, 2003) indicate that the in situ level of mtROS evolution normally will be considerably less than that of chloroplasts or peroxisomes in the light due to the operation of photosynthesis and...
photorespiration. However, in the dark or in non-green tissues, mitochondria will be a major source of ROS (e.g., Puntarulo et al., 1988). Production of mtROS will increase if the rate of electrons leaving the mtETC through the terminal oxidases is slowed and/or the rate of electron input increases in excess of the ability of the two respiratory pathways to process the electrons, leading to an overreduced ubiquinone pool. This principle has been demonstrated in isolated mitochondria, where rates of H$_2$O$_2$ and superoxide generation show a substrate-dependent increase upon the addition of specific inhibitors of either the cytochrome pathway or AOX (Popov et al., 1997; Møller, 2001). Mitochondria in situ show the same effect. Studies using whole cells have shown increased ROS production specifically from mitochondria in the presence of the cytochrome pathway inhibitor antimycin A (AA; Maxwell et al., 1999; Yao et al., 2002).

Of primary interest in mitochondrial research is the likelihood that naturally occurring physiological and environmental conditions encountered by plants can give rise to an overreduced ubiquinone pool and concomitant increased mtROS production. For example, the endogenous signaling molecule nitric oxide (NO) is an inhibitor of COX, but not AOX (Millar and Day, 1996), at concentrations achieved in vivo during normal seedling development (Caro and Puntarulo, 1999). Therefore, NO production could lead to increased mtROS formation. Many stresses cause oxidative damage in plant tissues, and increases in mtROS resulting from mtETC perturbations have been implicated as at least partly responsible for the damage and plant responses observed in several of these cases, including chilling (Prasad et al., 1994a, 1994b; Purvis et al., 1995), salt stress (Hernández et al., 1993; Mittova et al., 2003), and phosphate deficiency (Juszczuk et al., 2001; Parsons et al., 1999; Malusa’ et al., 2002).

The preceding examples together with other studies strongly suggest that mtROS are involved in the responses of plants to stresses, as well as other plant processes. This means that plants are able to detect changes in mtROS output against a background of ROS production from other sources. Because mtROS output is predicted to be relatively constant during the course of a day/night cycle (Foyer and Noctor, 2003; Sweetlove and Foyer, 2004), changes in mtROS levels, even if low in magnitude, could be distinguished and registered by local detection mechanisms. The concept of local detection is consistent with accumulating evidence that the specific source of ROS is important in determining appropriate cellular responses (Dutilleul et al., 2003; Laloi et al., 2004; Clifton et al., 2005). A change in mtROS level is likely to have a consequence, including direct damage if levels increase (in which case damaged molecules could be part of signaling) or as early participants themselves in a signaling pathway(s). Therefore, as discussed below, mitochondria must be capable of controlling their ROS levels under normal conditions, detoxifying excess ROS, repairing oxidative damage arising from ROS formation, and modulating mtROS production appropriately for signaling.

CONSEQUENCES OF mtROS IN MITOCHONDRIA

Oxidative Damage to Mitochondrial Lipids

Peroxidation of mitochondrial membrane polyunsaturated fatty acids is initiated by the abstraction of a hydrogen atom by ROS, especially by hydroxyl radicals. This leads to the formation of cytotoxic lipid aldehydes, alkenals, and hydroxyalkenals (HAEs), such as the much-studied 4-hydroxy-2-nonenal (HNE) and malondialdehyde. Inhibition of the mtETC with AA can generate mitochondrial HAEs to levels similar to those generated by general oxidative stress through chemical treatments such as H$_2$O$_2$ or menadione (a compound that causes superoxide production; Sweetlove et al., 2002; Winger et al., 2005). Once formed, lipid peroxidation products can cause cellular damage by reacting with proteins, other lipids, and nucleic acids. Key oxylipins and smaller, lipid-derived reactive electrophile species may also be produced from lipid peroxidation (Almerás et al., 2003), but, to our knowledge, there is no direct evidence of these compounds being produced in plant mitochondria from oxidative stress.

Effects of ROS on Mitochondrial Proteins

Proteins can be damaged and/or inhibited by oxidative conditions in several ways, including: (1) direct oxidation of amino acids by ROS, such as the oxidation of Cys residues to form disulfide bonds, oxidation of Met residues to form Met sulfoxide, and oxidation of Arg, Lys, Pro, and Thr residues, which creates carbonyl groups in the side chains (Berlett and Stadtman, 1997; Dean et al., 1997); (2) oxidation that breaks the peptide backbone (Dean et al., 1997); (3) reactions with lipid peroxidation products (such as HNE); (4) reactions with reactive nitrogen species that are formed by reaction of NO with ROS (Sakamoto et al., 2003); and (5) direct ROS interaction with metal cofactors, illustrated by the TCA cycle iron-sulfur enzyme aconitase, which is sensitive to H$_2$O$_2$ (Verniquet et al., 1991) and superoxide (Flint et al., 1993).

Proteomics approaches have been undertaken to determine the damaging effects of oxidative stress on mitochondrial proteins (Sweetlove et al., 2002; Kristensen et al., 2004; Taylor et al., 2005). These studies primarily used chemical or environmental stresses to impose general, non-mitochondria-specific oxidative conditions, though some studies also employed AA (see below), which causes mtROS production specifically. Proteins that accumulate but that are degraded (as determined by identification of fragments) as a result of high ROS levels imposed by addition of H$_2$O$_2$ or menadione were identified (Sweetlove et al., 2002). Oxidatively damaged mitochondrial proteins include subunits of the pyruvate dehydrogenase complex, subunits of ATP synthase, and enzymes of the TCA cycle.
Further, mitochondrial proteins oxidized by treatment with H$_2$O$_2$ were “tagged” with dinitrophenylhydrazine, which forms covalent bonds with carbonyl groups resulting from oxidation of amino acids (Kristensen et al., 2004). Thirty-eight labeled, oxidized mitochondrial proteins were identified. Several of these proteins were among those previously observed to be damaged by oxidative stresses (Sweetlove et al., 2002; Taylor et al., 2002, 2005). Although such proteomics experiments do not survey the full mitochondrial proteome, it is clear that several important mitochondrial proteins are damaged by general oxidative stresses and therefore could be damaged by mtROS. Most significantly for this discussion, many proteins degraded during more general oxidative treatments were also degraded following AA treatment (Sweetlove et al., 2002), demonstrating that mtROS specifically can damage key mitochondrial proteins.

Treatment of mitochondria with HNE or paraquat (which causes superoxide formation in chloroplasts and mitochondria) or cold or drought treatment of plants leads to formation of a covalent HNE-derived adduct of the lipoic acid moiety of several mitochondrial enzymes, including Gly decarboxylase (an enzyme in the photorespiratory pathway), 2-oxoglutarate dehydrogenase (a TCA cycle enzyme), and pyruvate decarboxylase (Millar and Leaver, 2000; Taylor et al., 2002, 2005). Although it has not been proven that the damage is from mtROS specifically or mitochondrial lipid peroxidation products produced during these stresses, inhibition of the mtETC does result in elevated malondialdehyde levels (Taylor et al., 2005; Winger et al., 2005). These results indicate that oxidative conditions due to production of mtROS could cause damage to proteins through formation of HAEs that react with the lipoic acid moiety. HNE can also form adducts with Cys, His, and Lys residues, causing altered enzyme function (Schaur, 2003). One of these reactions is likely the cause of HNE inhibition of AOX (Winger et al., 2005). While this seems counterintuitive since one role of AOX is to help prevent mtROS formation, it may allow cells to sense an extreme stress that dramatically enhances the accumulation of mtROS and initiation of a more extreme response, such as PCD (see below).

While oxidative damage to proteins occurs under stressful conditions, it has also been shown to be a normal part of Arabidopsis (Arabidopsis thaliana) leaf maturation (Johansson et al., 2004). In addition, selective protein oxidation has been suggested to have a role in controlling the course of metabolic activity during seed germination where the mitochondrial ATP synthase was one of the targeted proteins (Job et al., 2005). If this is true, then mtROS can also impact cell physiology through this oxidative mechanism.

Oxidative Damage to mtDNA

Hydroxyl radicals are highly reactive and can damage nuclear and mitochondrial DNA, which cells try to repair (Roldan-Arjona et al., 2000; Doudican et al., 2005). Accumulated damage to mtDNA, which can be caused by mtROS over the course of the lives of animals, causes decreased mitochondrial function and can contribute to aging and diseases (Allen, 1996; Raha and Robinson, 2000; Trifunovic et al., 2004; Wallace, 2005). For mtDNA, damage from oxidative stress (including mtETC inhibition by AA) and effects on mitochondrial function have been studied in other organisms (Raha and Robinson, 2000; Doudican et al., 2005; Wallace, 2005). However, there is a paucity of such studies using plant models. In the long term, plants could avoid the consequences of DNA damage by replacing organs that contain damaged genomic or mtDNA. On the other hand, there could be an accumulation of mutations in meristematic tissues that are maintained in cells of organs that develop from these tissues, though we are not aware of any studies addressing this issue. One factor helping prevent the adverse effects of mtDNA damage in plant cells is the redundancy of genomic sequences in each mitochondrion (e.g. Mackenzie et al., 1994; Backert et al., 1997). The copy number of individual genomic regions can help determine levels of gene expression (Mause and Hauswirth, 1995; Hedtke et al., 1999) and, therefore, could help dictate the extent of the effects of mutation caused by ROS. In addition, the number of mitochondria in plant cells ranges from hundreds to, perhaps, thousands (Douce, 1985). Heteroplasmy and recombination help to clear deleterious mutations from damaged mitochondrial genomes and maintain their functional integrity (Barr et al., 2005). Although a low rate of recombination in mitochondria seems to cause an accumulation of nonsynonymous mutations in mitochondrial genomes at an accelerated rate relative to nuclear genomes, the rate of accumulation is slow enough that severe fitness losses can only occur on a time scale of tens of millions of years (Lynch and Blanchard, 1998). Nevertheless, these “long-term” strategies would not alleviate consequences of potential mtDNA damage in cells by severe oxidative stress.

ANTIOXIDANT COMPOUNDS AND PROTECTIVE ENZYMES IN MITOCHONDRIA

Plant mitochondria can modulate superoxide production from the mtETC by two mechanisms that act to keep the ubiquinone pool reduction level low. The first, AOX, is not inhibited by the proton gradient across the inner membrane and can function when the cytochrome pathway is impaired (Finnegan et al., 2004). That AOX might act to maintain a basal ubiquinone pool reduction state was initially proposed by Purvis and Shewfelt (1993) and is supported by studies with roots treated with a cytochrome pathway inhibitor (Millenaar et al., 1998). Further, operation of AOX diminishes mtROS production (Purvis, 1997, Popov et al., 1997; Maxwell et al., 1999). Increased expression of alternative mitochondrial NAD(P)H dehydrogenases, which do not translocate protons, often
accompanies increased AOX expression (Clifton et al., 2005). Use of these enzymes serves as an alternative to complex I and could further help decrease mtROS production. The second, uncoupling protein (UCP), also is found in the inner mitochondrial membrane. UCP uncouples by facilitating a proton leak across the membrane and consequently reduces inhibition of the mtETC (Sluse and Jarmuszkiwicz, 2004; Hourtong-Cabassa et al., 2004). Like AOX, UCP function in isolated mitochondria decreases ROS formation (Kowaltowski et al., 1998; Casolo et al., 2000). On the other hand, ROS are actually required for UCP activity (Pastore et al., 2000; Considine et al., 2003), a direct activator being HNE (Smith et al., 2004). This ROS requirement by UCP is in contrast to the effect of ROS on AOX. Exposure of AOX to experimental oxidative stress can drive the protein into the inactive, disulfide-linked form (Vanlerberghe et al., 1999; A.L. Umbach, unpublished data), and HNE inhibits AOX (Winger et al., 2005). Therefore, while both AOX and UCP may act to forestall mtROS production, only UCP may be able to operate when ROS levels become increased.

Once superoxide has been generated from the mtETC, the first step in detoxification, dismutating superoxide to H$_2$O$_2$, is catalyzed by mitochondrial manganese superoxide dismutase (MnSOD; Klieberstein et al., 1998; Möller, 2001). The presence of catalase, though reported in maize (Zea mays) mitochondria, does not appear to be typical (Sweetlove and Foyer, 2004). Consequently, H$_2$O$_2$ is removed by other mechanisms in plant mitochondria. One of these is the mitochondrial glutathione-ascorbate cycle (Jiménez et al., 1997; Chew et al., 2003). Ascorbate peroxidase reduces H$_2$O$_2$ to H$_2$O using ascorbate, which is reduced by the sequential action of monodehydroascorbate reductase and dehydroascorbate reductase, which uses reduced glutathione in the final reduction step. Lastly, glutathione reductase regenerates reduced glutathione. Peroxiredoxins also could reduce mitochondrial H$_2$O$_2$. These enzymes use reduced thioredoxin or glutathione sequentially with glutathione as reductant sources, which in turn are reduced by thioredoxin reductase and glutathione reductase (Sweetlove and Foyer, 2004). An Arabidopsis mitochondrial type II peroxiredoxin (PrxH F) has been identified by proteomics and characterized (Heazlewood et al., 2004; Finkemeier et al., 2005), and plant mitochondria contain thioredoxins (Sweetlove and Foyer, 2004). Ultimately, the electrons for all these systems are derived from mitochondrial NADPH, and a high level of employment of these systems could affect overall mitochondrial and even cellular redox status.

In addition to directly detoxifying ROS, these and other enzyme systems, together with their electron donors, may be involved in repairing lipid peroxidation damage and some forms of protein oxidation. For example, glutathione S-transferases (GSTs) can inactivate HNE by converting it to the glutathione conjugate GS-HNE, and there is evidence for at least one GST in plant mitochondria (Heazlewood et al., 2004). The type II peroxiredoxin/thioredoxin system mentioned above could also reduce lipid peroxides (Rouhier et al., 2004). Also, although most oxidative damage to proteins is irreversible and results in protein degradation, Arabidopsis mitochondria contain a protein disulfide isomerase that, functioning together with thioredoxin and NADPH, can reduce disulfides resulting from protein oxidation (Sweetlove et al., 2002). It remains to be determined whether these mechanisms function in plant mitochondria to decrease oxidative damage.

Mitochondria can control their oxidative state indirectly through metabolism as well. One example is the γ-aminobutyrate shunt for succinate synthesis, which bypasses two enzymes of the TCA cycle that are known to become inactivated by oxidative stress. Plants that lack this bypass show increased tissue damage and H$_2$O$_2$ levels, perhaps because less NAD(P)H is generated in the mitochondria than is needed to maintain reducing conditions (Bouché et al., 2003; Bouché and Fromm, 2004). Another example is the biosynthesis of ascorbate, the last step of which involves the mtETC (Siendones et al., 1999; Bartoli et al., 2000; Millar et al., 2003). Stresses on the mtETC could perturb ascorbate synthesis with drastic effects on mitochondrial and cellular redox balance (Millar et al., 2003), as exemplified by ascorbate-deficient Arabidopsis mutants (Pavet et al., 2005).

The antioxidant systems of plant mitochondria could connect mtROS to other cellular processes through their effects on the overall availability and reduction state of glutathione, ascorbate, thioredoxin, and NADPH in the cell. For example, glutathione and ascorbate induce expression of plant defense genes and are proposed signals of cellular redox status and PCD (Foyer et al., 1997; Noctor and Foyer, 1998; de Pinto et al., 2002). Glutathione also is a potential component of cold stress signaling (Foyer et al., 1997), and thioredoxin is involved in redox activation of numerous enzyme systems (Gelhaye et al., 2005). Mitochondrial events can also modify rates of ROS evolution elsewhere in the cell. The mtETC is recognized as needed for processing excess reductant produced by the light reactions of photosynthesis. Should reductant levels originating from the chloroplast exceed the processing ability of the mtETC, not only would mtROS production likely increase, but chloroplast ROS production could also increase due to an overly reduced photosynthetic reaction system (Fernie et al., 2004; Finnegan et al., 2004). Therefore, mitochondrial antioxidant systems are positioned to affect ROS and redox status and modulate many cellular functions (Foyer et al., 1997; Noctor and Foyer, 1998).

mtROS AS SIGNALS AFFECTING NUCLEAR GENE EXPRESSION AND CELL FATE

Altered Nuclear Gene Expression

Altered mitochondrial function can cause altered nuclear gene expression through mitochondria-to-nucleus...
signaling, which is referred to as mitochondrial retrograde regulation (MRR). This phenomenon has been studied in yeast, animal cell cultures, and plants (for review, see Butow and Avadhani, 2004; Rhoads and Vanlerberghe, 2004). There are several interesting examples in which nuclear gene expression was altered in plants by mitochondrial dysfunction caused by nuclear mutation or transgenic expression of mitochondrial proteins (Kushnir et al., 2001; Gómez-Casati et al., 2002; Rhoads et al., 2005). Although not directly demonstrated in these examples, there is evidence for the importance of mtROS in plant MRR. Inhibition of the cytochrome pathway by AA results in production of mtROS (Maxwell et al., 1999; Yao et al., 2002) and altered nuclear gene expression (Saisho et al., 1997; Yu et al., 2001; Karpova et al., 2002; Maxwell et al., 2002). The direct involvement of mtROS in MRR in plants is strongly suggested by the dramatic reduction of AA-initiated gene induction observed when antioxidants are added prior to AA (Maxwell et al., 1999).

Although inhibition by antioxidants of gene induction from cytochrome pathway disruption shows the importance of ROS in this process, it does not address whether mtROS leave the mitochondria and initiate gene expression changes. One of the two ubisemiquinone binding sites of complex III is close to the mitochondrial intermembrane space, providing a mechanism for superoxide production accessible to the cytoplasm (Møller, 2001). Observations of $\text{H}_2\text{O}_2$ apparently escaping from mitochondria at specific sites (Yao et al., 2002) in response to AA treatment also suggest that direct effects of mtROS outside the mitochondria, such as initiation of gene expression changes, are possible and that release may be through specific channels or pores. Although clearly not definitively in plants, related observations support the hypothesis that plant mtROS are released at specific sites. Gene induction from AA treatment of suspension-cultured tobacco (Nicotiana tabacum) cells can be inhibited by inhibitors of the permeability transition pore (Maxwell et al., 2002), and superoxide has been measured directly leaving respiring isolated rat mitochondria by an apparently channel-mediated mechanism (Han et al., 2003). However, changes in the mitochondria initiated by increased mtROS may produce secondary signals from local (in or close to mitochondria) ROS detection mechanisms that are transmitted to the nucleus. As has been discussed elsewhere (Rhoads and Vanlerberghe, 2004), any of the known signaling pathways or pathways yet to be discovered could be involved in transmitting a signal to the nucleus. Attractive candidates are those signaling components that are likely involved in oxidative stress sensing and/or redox signaling. Several potential signaling proteins were localized to mitochondria (Heazlewood et al., 2004) and could be involved as local detection mechanisms for increased mtROS and initiation of signaling to the nucleus.

Among the common themes in plant MRR following mtETC inhibition are induction of genes encoding (1) proteins involved in mitochondrial respiration via reactions other than those in the cytochrome pathway, including AOX and alternative NAD(P)H dehydrogenases; (2) antioxidant enzymes, such as GSTs and a monodehydroascorbate reductase; and (3) proteins for plant defenses against biotic and abiotic stresses (Saisho et al., 1997; Yu et al., 2001; Maxwell et al., 2002; Clifton et al., 2005). At least for the Arabidopsis AOX1a gene, induction is most likely the result of increased transcription controlled at a promoter region (Dojcinovic et al., 2005; Zarkovic et al., 2005).

In plants that lack a fully functioning mtETC due to mutations in the mitochondrial genome, a new cellular homeostasis must be attained. Altered nuclear gene expression is part of this new homeostasis. Mutants exhibit altered expression of genes encoding AOXs, heat shock proteins, and antioxidant enzymes (Karpova et al., 2002; Dutilleul et al., 2003; Kuzmin et al., 2004). Thus, permanent alteration of mtETC function results in constitutively altered nuclear gene expression. Interestingly, tobacco plants impaired in complex I function exhibit increased respiration (Gutierres et al., 1997) but lower cellular $\text{H}_2\text{O}_2$ levels, likely due to the observed increased antioxidant enzyme expression and activity. These plants also exhibit increased levels of NAD, NADH, and NADPH (Dutilleul et al., 2005), but no change in redox components glutathione and ascorbate (Dutilleul et al., 2003) Overall, they have greatly altered metabolism to adjust to severely altered mitochondrial function but are still dramatically altered phenotypically (Gutierres et al., 1997; Dutilleul et al., 2005). Because these plants have already attained an interesting new homeostasis, it is not clear what signaling from the mitochondria is responsible for changes in nuclear gene expression. Nevertheless, it is possible that increased mtROS production and/or shifted redox status directed by mitochondria contribute to signaling (Dutilleul et al., 2003, 2005).

Finally, mtROS could diffuse from mitochondria and contribute to other forms of signaling by increasing ROS at other locations in the cell (such as the chloroplast; see e.g. op den Camp et al., 2003, and refs. therein), which could result in altered nuclear gene expression.

**PCD and Response to Pathogens**

PCD is an important part of certain plant responses to stresses and includes the hypersensitive response to pathogens. An early cellular signal for this process is frequently an increase in tissue ROS production due to plasma membrane NADPH oxidase activity (Overmyer et al., 2003). Plant mitochondrial responses to PCD signals are similar to those of animal mitochondria and include undergoing a permeability transition, release of cytochrome $c$, and a decrease in ATP production (Xie and Chen, 2000; Arpagaus et al., 2002; Tiwari et al., 2002; Krause and Durner, 2004). Increases in mtROS production are coincident with these processes, probably
a result of the large perturbations experienced by the mtETC (Pel·lín et al., 1999; Yao et al., 2002; Krause and Durner, 2004).

Importantly, while an extramitochondrial trigger may initiate disruptions in mitochondrial function, it appears that loss of mitochondrial function and the concomitant mtROS signal that is generated are necessary for subsequent PCD. Evidence for this comes from work using toxins of plant pathogens that alter mitochondrial function and induce mtROS formation. Harpin is a bacterial protein elicitor of the hypersensitive response. Harpin addition to suspension-cultured Arabidopsis cells results in increased mtROS and cytochrome c release (Krause and Durner, 2004), consistent with the proposed role of mtROS in PCD signaling. Mitochondria are the first cellular compartments to show PCD responses, including opening of the mitochondrial permeability transition pore, when exposed to the fungal toxin victorin. The mitochondrial response occurs well before other cellular PCD-related responses are observed (Curtis and Wolpert, 2004). Most significantly, during the course of victorin action on oat (Avena sativa) cells, mtROS were produced and, when their production was reduced by ROS scavengers, subsequent PCD-related events were delayed (Yao et al., 2002). Other evidence for a direct role of mtROS in signaling PCD comes from work with tobacco suspension-cultured cells with greatly reduced levels of AOX. These cells, lacking this protective pathway, were much more susceptible to PCD-inducing substances (Robson and Vanlerbergh, 2002; Vanlerbergh et al., 2002) but were partly protected when treated with an antioxidant (Robson and Vanlerbergh, 2002). Conversely, tobacco plants overexpressing AOX produced smaller leaf lesions upon pathogen challenge (Ordog et al., 2002). Thus, mtROS appear to be an important factor in PCD signaling.

Chemical disruption of mitochondrial function can also bring about changes leading to PCD, similar to those described above for pathogen toxins. The cytochrome pathway inhibitor AA induced PCD in oat leaf mesophyll cells, with the attendant loss of mitochondrial membrane potential, apparent bursts of mitochondrial H$_2$O$_2$ from specific sites, and subsequent chromatin condensation. All these effects were prevented by ROS scavengers (Yao et al., 2002), suggesting that mtROS in this case, as with victorin, were the PCD signal. AA treatment induced gene expression in suspension-cultured tobacco cells, which were inhibited by bongkrekic acid, an inhibitor of the mammalian mitochondrial permeability transition pore (Maxwell et al., 2002), further demonstrating connections between mtROS, altered gene expression, and PCD. In addition to sharing downstream, PCD-related mitochondrial events, inhibition of the mtETC by chemicals or by mutation and inhibition by pathogen challenge share similar gene induction profiles, evidence of similar MRR signaling occurring in each of these cases. Nuclear genes induced by harpin overlap with nuclear genes induced by disruption of the mtETC by mitochondrial mutants (Karpova et al., 2002; Krause and Durner, 2004; Kuzmin et al., 2004). In addition, the profile of the whole transcriptome response to AA is most similar to the transcriptome responses to pathogen attack (Yu et al., 2001). Thus, pathogen challenge and mtETC disruption have similar cellular consequences, which suggests that overlapping MRR pathways are utilized. It may be that MRR signaling is a common component of the responses of plants to a variety of stresses that affect mitochondrial function.

**AOX, NO, and Oxygen Sensing**

ROS may act as signals in sensing oxygen itself, particularly the lack of it (Bailey-Serres and Chang, 2005). Although induction of ROS under oxygen deprivation may seem paradoxical, there is increasing evidence for the accumulation of ROS even under severe oxygen deficiency and a role for ROS in sensing the stress (e.g. Blokhina et al., 2001; Baxter-Burrell et al., 2002). Mitochondrial Ca$^{2+}$ ([Ca$^{2+}$]$_{mt}$) is released within minutes of oxygen limitation, critically contributing to the rise in anoxic cytosolic Ca$^{2+}$ ([Ca$^{2+}$]$_{cyt}$) that signals changes in nuclear gene expression (Subbaiah et al., 1998, and refs. therein; Tsuji et al., 2000; Kuzmin et al., 2004). Modulation of mtROS levels by AOX is likely to be an important early step preceding the above sequence of events. AOX, owing to its much lower affinity for oxygen ($K_m$ 10–25 $\mu$m; Ribas-Carbo et al., 1994) than that of COX ($K_m$ 140 $\mu$m; Millar et al., 1994), is likely to be inhibited by even a mild decrease in oxygen concentration and has been shown to be abolished under prolonged hypoxia (Szal et al., 2003). AOX activity may rapidly be abolished under hypoxic conditions (e.g. flooding) with a consequent increase in mtROS levels (Popov, 2003), ROS accumulation in mitochondria can stimulate (Ca$^{2+}$)$_{mt}$ efflux (e.g. Sandri et al., 1990) and is a prerequisite for anoxic rise in (Ca$^{2+}$)$_{cyt}$ (Waypa et al., 2002). In addition, many “anaerobic genes” are inducible by even a small decrease in oxygen concentration (e.g. Paul and Ferl, 1991). Thus, an elevation of mtROS due to the inhibition of AOX activity may be the trigger for the release of (Ca$^{2+}$)$_{mt}$ and changes in gene expression under oxygen deprivation.

NO seems to be intricately involved in this pathway. Both the Arg-dependent and the NO$_3$-dependent pathways of NO production are located mainly in mitochondria (for review, see Crawford and Guo, 2005). The NO$_3$-dependent pathway is stimulated under oxygen deprivation (Gupta et al., 2005), leading to NO accumulation (e.g. Dordas et al., 2003). NO inhibits COX, facilitating an increase in mtROS production even under mild hypoxia (Cooper and Davies, 2000). NO induces a rise in (Ca$^{2+}$)$_{cyt}$ (Lamotte et al., 2004), either directly or via its promotive effect on mtROS levels. Thus, NO, by stimulating the production of mtROS as well as increase in (Ca$^{2+}$)$_{cyt}$ may act as a parallel transducer of oxygen deprivation signals from mitochondria to the nucleus. In addition to activating adaptive signaling pathways, the pro-oxidant activity...
of NO can trigger mitochondrial damage and cell death (Casolo et al., 2005). Further, besides acting as a pro-oxidant, NO can act as an antioxidant under certain conditions, particularly during hormone-mediated cell death processes (e.g. Beligni et al., 2002; Guo and Crawford, 2005).

Accumulation of NO and superoxide can also lead to the generation of peroxynitrite under prolonged hypoxia. Peroxynitrite is a strong oxidizing and nitrating agent that crosses membranes readily (approximately 400-fold faster than superoxide; Marla et al., 1997). It can inhibit several enzymes, compromise mitochondrial integrity, and, ultimately, trigger cell death in intact plants (Alamillo and García-Olmedo, 2001; Turrens, 2003); although cell death does not appear to result in cultured cells (e.g. Lamotte et al., 2004).

**SUMMARY**

ROS are known to be important signaling molecules in plants (Van Breusegem et al., 2001; Neill et al., 2002; Laloì et al., 2004). ROS levels, antioxidant levels, and redox status are balanced by plant cells, including within mitochondria. Each is important for plant health. Perturbations of this balance are likely detected and the plant responds accordingly. Responses to changes in ROS levels include alteration of expression of nuclear genes encoding enzymes that function to regain homeostasis by producing antioxidant compounds and protective and repair enzymes. Currently, little is known about how increased mtROS production is conveyed to the nucleus. Because ROS are involved in many distinct aspects of plant responses to stresses, there must be distinct detection mechanisms that differentiate identity, levels, and perhaps even sources of ROS. Mitochondrial ROS must fit into this scheme. Evaluation of the current literature leads us to conclude that the outcome of various mitochondrial perturbations may not be easily predicted due to the variety of potential mitochondrial responses, including mtROS production, as well as other signals and changes that may be arising elsewhere in the cell simultaneously. These responses may occur sequentially under some stresses and lead to a predictable outcome. For example, a stress may disrupt the mitochondrial mtETC causing production of a sufficient level to initiate permeability transition pore opening, cytochrome c release, and PCD. On the other hand, slightly different treatments or the same treatments in different plant species may cause mitochondria to respond with parallel events, such as simultaneously signaling for changes in nuclear gene expression to regain homeostasis and initiation of PCD, with the actual outcome decided by yet other inputs. Identification of components of the various signaling pathways will likely help clarify this complex picture. In the most extreme case of high mtROS production, when cells sense a severe threat, mitochondria likely initiate PCD. Although plants employ this important option to “cut their losses” in situations such as pathogen attack, such an extreme outcome should be under tight control. Increased mtROS can cause critical shifts in cell redox status because antioxidant systems ultimately require electrons from redox pairs such as NADP/NADPH, which connect many aspects of metabolism. Major challenges will be to determine the roles of mtROS and the potential consequences of altered mtROS levels, such as production of other reactive molecules and shifts in redox balance, in plant developmental processes and stress responses.

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**LITERATURE CITED**


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