

Changes in Plant Mitochondrial Electron Transport Alter Cellular Levels of Reactive Oxygen Species and Susceptibility to Cell Death Signaling Molecules

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Transgenic tobacco (*Nicotiana tabacum*) lacking mitochondrial alternative oxidase (AOX) have been compared with wild-type (Wt) tobacco using two different systems, either suspension cell cultures or leaves. In both systems, a lack of AOX was accompanied by an increase in some anti-oxidant defenses, consistent with the hypothesis that a lack of AOX increases the mitochondrial generation of reactive oxygen species (ROS). In most cases, this increase in anti-oxidant defenses could more than offset the presumed increased rate of ROS generation, resulting paradoxically in a lower steady-state level of ROS than was found in Wt leaves or suspension cells. We also found that the amount of cell death induced by salicylic acid or nitric oxide correlated strongly with the level of ROS (irrespective of the level of AOX), while death induced by azide was dependent upon the presence or absence of AOX. These results suggest that susceptibility to cell death by signaling molecules (salicylic acid and nitric oxide) is dependent upon the steady-state cellular level of ROS and that AOX levels clearly contribute to this steady state, perhaps by influencing the rate of mitochondrial-generated ROS and hence the cellular level of anti-oxidant defenses.

Keywords: Alternative oxidase — Anti-oxidant defenses — Cell death — Mitochondrial electron transport — Reactive oxygen species — Transgenic tobacco.

Abbreviations: AA, antimycin A; AOX, alternative oxidase; APx, ascorbate peroxidase; CAT, catalase; CuZnSOD, copper zinc superoxide dismutase; cyt, cytochrome; DAB, 3,3'-diaminobenzidine; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; ETC, electron transport chain; FeSOD, iron superoxide dismutase; GPx, glutathione peroxidase; H₂O₂, hydrogen peroxide; mROS, mitochondrial reactive oxygen species; MnSOD, manganese superoxide dismutase; NBT, nitrotriazolium blue chloride; N₃, azide; NO, nitric oxide; PCD, programmed cell death; PTOX, plastid terminal oxidase; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; SA, salicylic acid; SNP, sodium nitroprusside; Wt, wild-type.

Introduction

The plant mitochondrial electron transport chain (ETC) is branched, such that electrons in the ubiquinone pool can be passed to O₂ via the usual cytochrome (cyt) pathway (that includes complex III, cyt c and cyt oxidase) or they may pass to oxygen via a second terminal oxidase called alternative oxidase (AOX) (Finnegan et al. 2004). The AOX pathway is non-energy conserving (i.e. it is not coupled to proton translocation) and also bypasses the last two sites of energy conservation associated with the cyt pathway. The ubiquitous presence of AOX in the plant kingdom as well as its sporadic distribution in fungi and protists is well established. More recently, AOX genes have also been found in both the eubacterial and animal kingdoms (Stenmark and Nordlund 2003, McDonald and Vanlerberghe 2004).

Mitochondrial electron transport is associated with the generation of reactive oxygen species (ROS) such as superoxide and H₂O₂, which we refer to herein as mitochondrial ROS (mROS). Complexes I and III probably represent the primary sites of mROS generation. While the relative importance of these two sites for mROS generation and the factors influencing their rates of mROS production are largely unknown, an important generalization is that mROS formation increases as the ETC becomes more highly reduced (Møller 2001). mROS generation by isolated mitochondria is therefore increased under ADP-limiting conditions that increase mitochondrial transmembrane potential and decreased by uncouplers that dissipate transmembrane potential. 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 over-reduction of specific ETC components. However, most of the above conclusions are based upon

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studies of animal mitochondria, and the degree to which these generalizations might be extended to plant mitochondria remains unclear (Møller 2001).

Since ROS can damage macromolecules, their cellular levels are managed by both avoidance and scavenging mechanisms (Mittler et al. 2004). By accepting electrons from ubiquinone, AOX may prevent over-reduction at complex I and/or III. Hence, 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, since AOX respiration is less tightly coupled to ATP production, under conditions in which ADP availability is limiting. This hypothesis is supported by studies with isolated mitochondria from several plant (and other) species, as well as studies performed with transgenic tobacco suspension cells lacking AOX. Using isolated mitochondria, several groups have shown that when AOX is inactive (due to chemical inhibition of AOX and/or lack of biochemical activation of AOX), ETC activity generates more ROS (Popov et al. 1997, Purvis, 1997, Braidot et al. 1999, Casolo et al. 2000, Camacho et al. 2004, Czarna and Jarmuszkiewicz 2005). Further, it has been shown that transgenic tobacco suspension cells lacking AOX have more ROS emanating from the mitochondrion than do Wt cells (Maxwell et al. 1999) and that the exaggerated level of ROS in these cells is amplified further during phosphate-limited growth, a condition in which the Wt cells induce large amounts of AOX protein (Parsons et al. 1999, Sieger et al. 2005). Hence, while the physiological role of AOX in plants and other organisms is still a matter of considerable debate, a developing idea is that this pathway has a role in dampening the generation of mROS (Finnegan et al. 2004).

Recently, Umbach et al. (2005) reported the characterization of transgenic *Arabidopsis thaliana* plants lacking AOX. Based upon measures of leaf oxidative damage

(lipid peroxidation), microarray analyses of leaf gene transcript levels and imaging of root ROS levels (using a ROS-sensitive fluorescent probe), they found no evidence that the lack of AOX had impacted mROS generation under standard growth conditions. In a second study by the same group, *Arabidopsis* plants lacking AOX were found to grow more slowly than Wt plants at low temperature, but again there was no evidence that this was associated with increased mROS generation (Fiorani et al. 2005).

The results obtained with transgenic *Arabidopsis* plants lacking AOX differ from those obtained with transgenic tobacco suspension cells lacking AOX and could presumably be due to either species-dependent differences or differences in the response of plants vs. suspension cells to a lack of AOX. To distinguish between these possibilities and to examine further the physiological function of AOX respiration, the present study compared the response of both tobacco plants and tobacco suspension cells to a lack of AOX. In both systems, the results are consistent with the hypothesis that a lack of AOX increases the generation of mROS.

Results

Transgenic tobacco plants and suspension cells with silenced AOX expression

Antisense and RNA interference constructs were used to generate a collection of transgenic tobacco plants and suspension cells with silenced AOX expression (see Materials and Methods). Western blot analyses indicated that leaf mitochondria isolated from three transgenic tobacco lines (RI9, RI29 and AS8) contained little to no detectable AOX protein in comparison with Wt leaves (Fig. 1A). These results are consistent with how the leaves responded to treatment with AA (see Materials

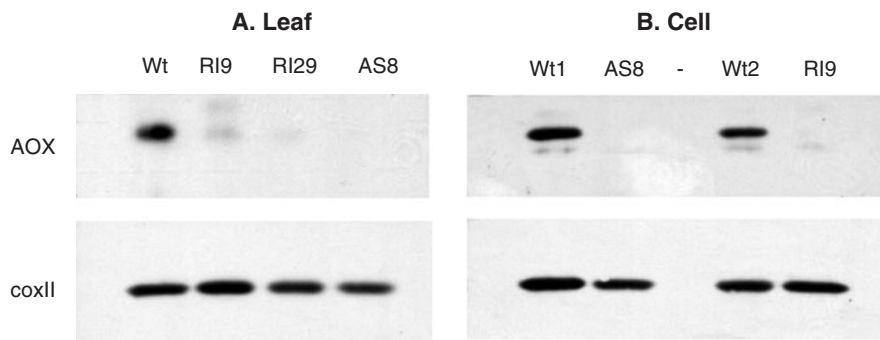


Fig. 1 Level of AOX and cyt oxidase subunit II (coxII) protein in mitochondria isolated from Wt and transgenic tobacco leaves (A) and tobacco suspension cells (B). Mitochondrial proteins (50 µg) were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies raised against AOX or coxII. See Materials and Methods for an explanation of the different transgenic lines. Representative results are shown.

and Methods). After AA treatment, Wt leaves remained viable while leaves of each of the transgenic lines displayed severe necrosis (data not shown).

Western blot analyses also indicated that mitochondria from the transgenic suspension cell lines (AS8 and RI9) contained little to no detectable AOX protein in comparison with their corresponding Wt cell line (Fig. 1B) (see Materials and Methods for an explanation of Wt1 and Wt2). Further, both AS8 and RI9 cells showed little to no AOX capacity (*n*-propyl gallate-sensitive oxygen uptake in the presence of CN) when measured in whole cells or isolated mitochondria in comparison with the Wt cell lines (Sieger et al. 2005; data not shown).

Genes encoding anti-oxidant defenses are up-regulated in leaves lacking AOX

Since AOX is hypothesized to dampen mROS generation (see Introduction), we examined transcript levels of genes that encode enzymes involved in anti-oxidant defense. We included in our analysis enzymes that localize to the mitochondrion [manganese superoxide dismutase (MnSOD)], cytosol [ascorbate peroxidase (APx), copper-zinc superoxide dismutase (CuZnSOD) and glutathione peroxidase (GPx)], peroxisome [catalase (CAT)] and chloroplast [iron superoxide dismutase (FeSOD)]. Consistently, we found that all of these transcripts (with the possible exception of GPx) were at higher levels in the leaves of the three transgenic lines lacking AOX than in the Wt leaves (Fig. 2).

The steady-state cellular level of ROS is decreased in leaves lacking AOX

It is well recognized that cellular levels of ROS are dependent upon both the rate at which ROS are generated by various cellular processes and the rate at which ROS are scavenged by the various anti-oxidant defenses (Mittler et al. 2004). We used in situ staining of Wt and transgenic leaves with nitrotetrazolium blue chloride (NBT) and 3,3'-diaminobenzidine (DAB) to compare their relative steady-state levels of superoxide and H₂O₂, respectively. Strikingly, we found that these ROS species (particularly H₂O₂) were lower in each of the three transgenic leaves lacking AOX than in the Wt (Fig. 3A, B).

Leaf AOX levels impact susceptibility to cell death induced by salicylic acid or nitric oxide

Salicylic acid (SA) and nitric oxide (NO) are well established to act as signaling molecules during programmed cell death (PCD) events such as the hypersensitive response (Alvarez 2000, Wendehenne et al. 2004, Delledonne 2005). We compared the susceptibility of Wt and transgenic plants to cell death induced by these signaling molecules. The transgenic leaves (that lack AOX,

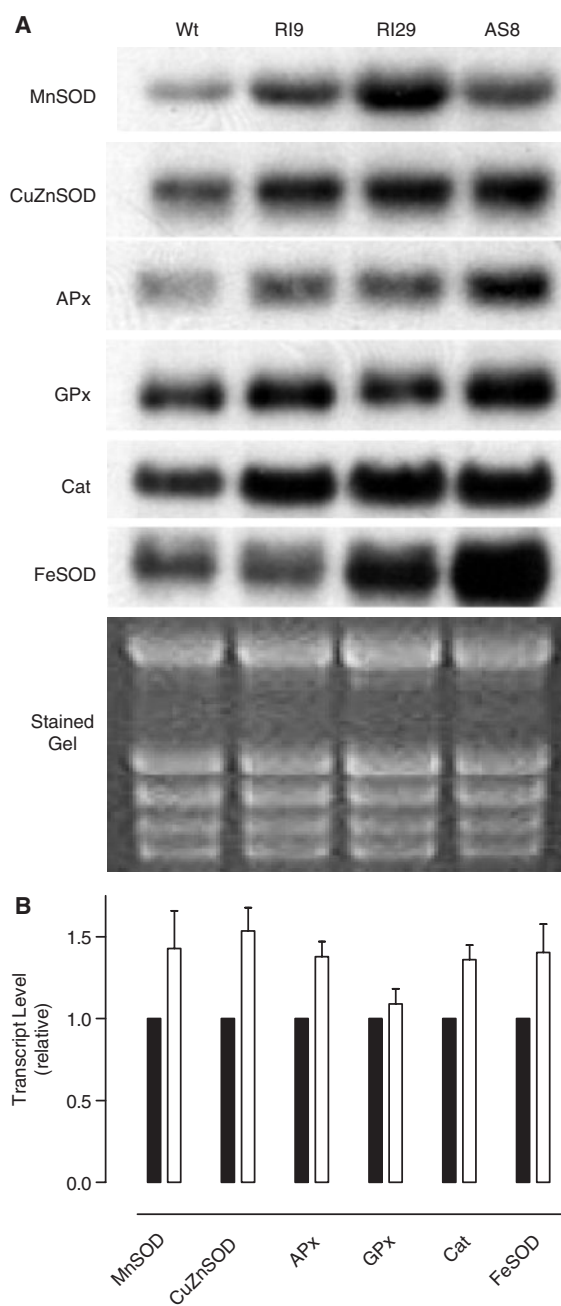


Fig. 2 Leaf transcript levels in Wt and three transgenic lines (RI9, RI29 and AS8) with silenced leaf AOX expression. The genes analyzed encode enzymes involved in anti-oxidant defense. (A) Representative Northern blots for each gene transcript analyzed. Also shown is a representative ethidium bromide-stained gel. (B) Relative transcript levels determined by densitometer analyses of Northern blots. Two to three separate experiments were done for each transcript and each plant line, with similar results in each experiment. Since all three transgenic plant lines showed the same transcript level patterns (relative to Wt), their densitometer data (open bars) were combined. Hence, these data represent the average \pm SE from 6–9 separate Northern blots for each transcript. Wt densitometer values (solid bars) were arbitrarily set to 1.

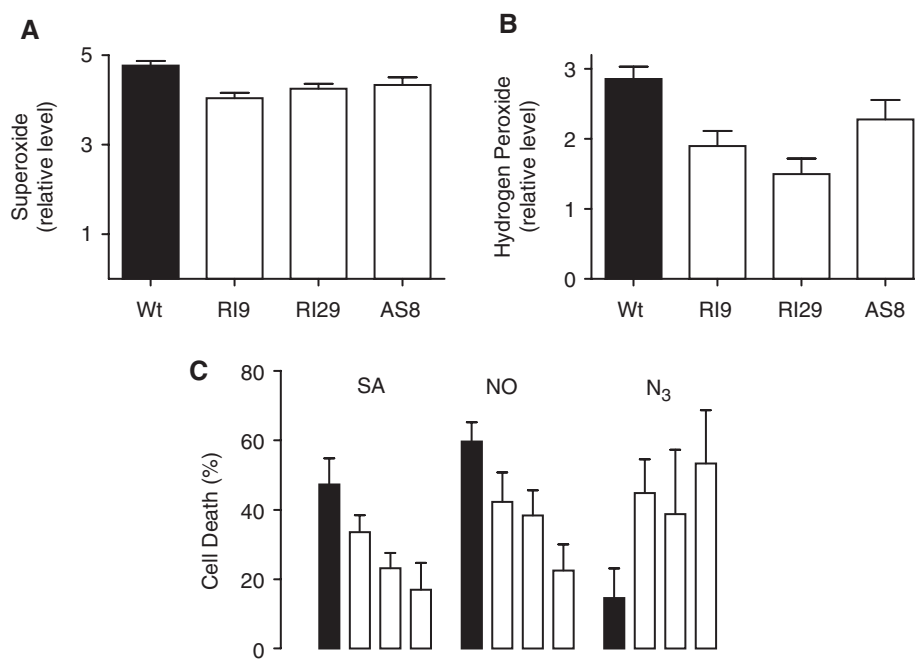


Fig. 3 Levels of ROS and susceptibility to cell death in Wt and transgenic tobacco leaves. Relative steady-state levels of superoxide (A) and hydrogen peroxide (B) were assessed by in situ staining with NBT or DAB as described in Materials and Methods. Leaf cell death (C) was assessed 48 h after treatment with SA, SNP (NO donor) or N₃ as described in Materials and Methods. In (C), the black bar represents the Wt and the three open bars represent (from left to right) RI9, RI29 and AS8. All results represent the mean \pm SE and are based upon multiple (3–16) independent experiments.

maintain lower steady-state cellular levels of ROS and exhibit up-regulated anti-oxidant defenses in comparison with the Wt) were found to be less susceptible than the Wt to cell death induced by treatment with SA or NO (generated from sodium nitroprusside; SNP) (Fig. 3C). On the other hand, the transgenic lines were clearly more susceptible to the complex IV inhibitor azide (N₃).

Suspension cells lacking AOX have up-regulated anti-oxidant defenses but with a variable impact on steady-state ROS level

The same anti-oxidant defense gene transcripts analyzed in leaves (see above) were also analyzed in the suspension cell lines (Fig. 4). AS8 cells lacking AOX displayed large increases in GPx and CAT transcripts, along with smaller increases in CuZnSOD and FeSOD, in comparison with Wt1 (Fig. 4A, B). However, the APx transcript was unchanged and the MnSOD transcript was found to be consistently lower in AS8 than in Wt1. On the other hand, RI9 cells lacking AOX displayed increased GPx (and perhaps CuZnSOD) but had little change or even small decreases in other transcript levels in comparison with Wt2 (Fig. 4A, C).

The steady-state cellular level of ROS in the suspension cell lines was evaluated using the ROS-sensitive

fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Similar to leaves, RI9 suspension cells maintained lower levels of steady-state ROS than Wt2 cells (see below). However, similar to previous results (Maxwell et al. 1999), AS8 cells contained much higher ROS levels than Wt1 (see below).

Susceptibility to cell death by SA or NO is dependent upon steady-state levels of ROS, irrespective of AOX levels

The opposing results of the two transgenic suspension cell lines lacking AOX (in terms of steady-state ROS level) afforded us an opportunity to examine whether susceptibility of suspension cells to cell death induced by SA or NO correlates with steady-state ROS level or AOX level. For this, we determined the steady-state ROS level for each of the four suspension cell lines just prior to treatment, along with quantifying the extent of cell death 48 h after treatment with SA or NO. For both SA (Fig. 5A) and NO (Fig. 5B), we found an excellent correlation across cell lines between susceptibility and steady-state ROS level, irrespective of the presence or absence of AOX. On the other hand, susceptibility to cell death by N₃ did not correlate with ROS level but rather was clearly dependent upon the presence or absence of AOX (Fig. 5C). These results are consistent with

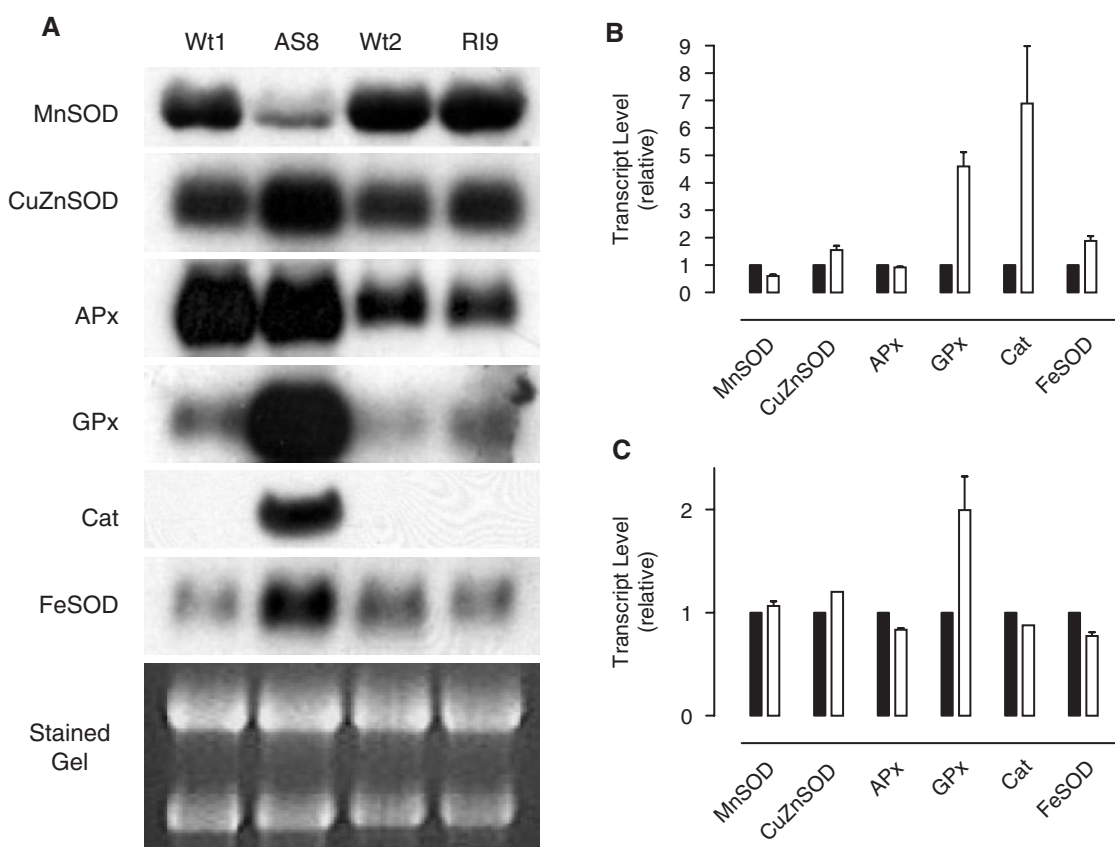


Fig. 4 Suspension cell transcript level for genes encoding enzymes involved in anti-oxidant defense. (A) Representative Northern blots for each gene transcript analyzed, illustrating some of the differences seen between AS8 and RI9. Also shown is a representative ethidium bromide-stained gel. (B) Relative transcript levels in transgenic AS8 cells that lack AOX in comparison with its corresponding Wt culture (Wt1). (C) Relative transcript levels in transgenic RI9 cells that lack AOX in comparison with its corresponding Wt culture (Wt2). For both (B) and (C), relative transcript levels are based upon densitometric analyses of Northern blots. Two separate experiments were done for each transcript and cell line, with similar results in each experiment. Wt densitometer values (solid bars) were arbitrarily set to 1.

the leaf data, in which susceptibility to SA and NO correlated with the ROS level while susceptibility to N_3 was dependent upon the AOX level.

Leaves and suspension cells lacking AOX display increased expression of a structurally related but plastid-localized oxidase

Plant plastids contain a protein that oxidizes plastoquinol and reduces oxygen to water (Peltier and Cournac 2002, Aluru et al. 2006). This plastid terminal oxidase (PTOX) is structurally related to AOX and, since it might have a functionally analogous role to AOX (but within the plastid), we examined whether PTOX expression was impacted by a lack of AOX. We found that, in both leaves and suspension cells lacking AOX, the expression of PTOX was consistently increased (Fig. 6).

Discussion

Our 'in planta' results suggest that the mitochondrial ETC generates more ROS when AOX is absent and that these additional mROS result in an increase of anti-oxidant defenses both within and outside the mitochondrion. Such results have already been seen using transgenic (AS8) tobacco suspension cells (Maxwell et al. 1999, Parsons et al. 1999), but our data suggest that this is similarly the case in tobacco leaves. As previously discussed (Dutilleul et al. 2003), the actual rate of ROS generation within tissues cannot be experimentally determined and the levels of ROS measured in tissues are a function of both the rate of ROS generation and the rate at which the ROS are scavenged by anti-oxidant defenses. Given these limitations, the best indicator that the rate of mROS generation has increased in the transgenics lacking AOX is the consistent

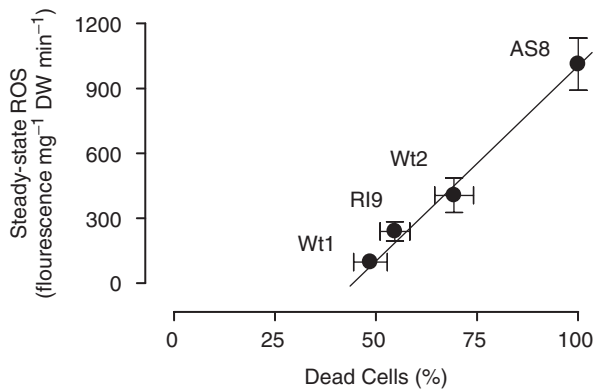
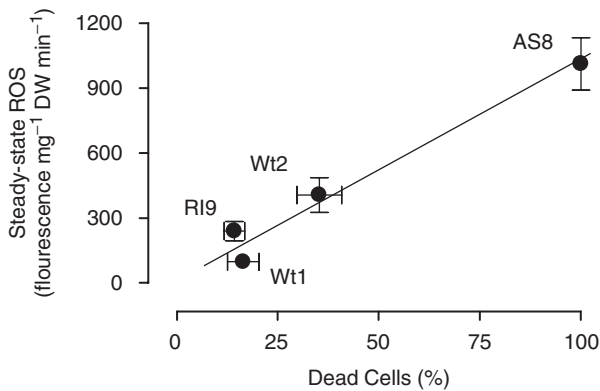
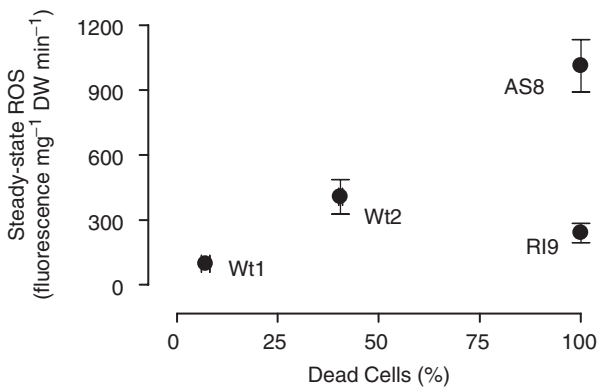
A. Salicylic acid**B. Nitric oxide****C. Azide**

Fig. 5 Levels of ROS and susceptibility to cell death in Wt and transgenic tobacco suspension cells. Steady-state ROS levels were assessed using the ROS-sensitive probe DCFH-DA as described in Materials and Methods. Cell death was assessed (using Evan's blue) 24 h after addition of SA (A), SNP (NO donor) (B) or N₃ (C) to cell cultures, as described in Materials and Methods. Results represent the mean \pm SE and are based upon multiple (3–10) independent experiments. In some cases, error bars are smaller than the data symbols.

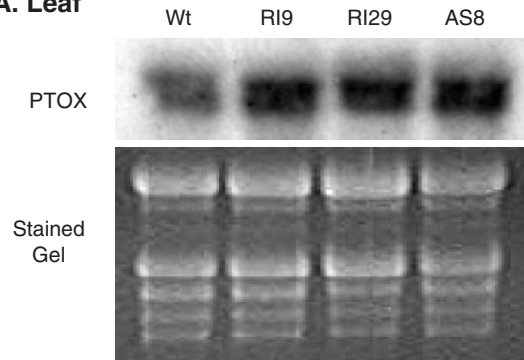
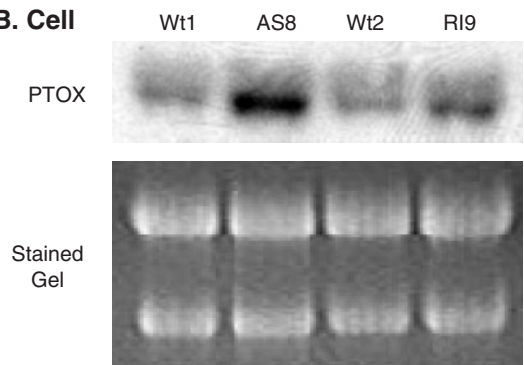
A. Leaf**B. Cell**

Fig. 6 Northern blots showing transcript levels of PTOX in Wt and transgenic leaf (A) and Wt and transgenic suspension cells (B). Also shown are representative ethidium bromide-stained gels. Representative results from two independent experiments with all lines are shown.

up-regulation of several anti-oxidant defenses in all lines of both suspension cells and plants examined. Presumably, up-regulation of these anti-oxidant defenses represents a ROS-induced stress response. This interpretation of our 'in planta' results is further supported by several 'in organello' studies showing that when AOX is inactive, rates of mROS generation increase (see Introduction). Nonetheless, another interpretation is that, while rates of ROS generation have increased (and induced anti-oxidant defenses), this additional ROS is being generated elsewhere (see later).

The lack of AOX in AS8 suspension cells is associated with a large increase in CAT and GPx transcript levels, as well as a large increase in the steady-state level of ROS. Both these results confirm previous reports (Maxwell et al. 1999, Parsons et al. 1999, Sieger et al. 2005). This suggests that up-regulation of antioxidant defenses in these transgenic suspension cells is not enough to compensate fully for the enhanced rate of mROS generation and hence mROS can actually be visualized emanating from the mitochondrion and accumulating in the cell

(Maxwell et al. 1999). It was initially surprising then that the second transgenic suspension cell line (RI9) as well as all three of the transgenic plant lines actually displayed lower steady-state ROS levels than their corresponding Wt cell culture or plant. These results suggest that the up-regulation of anti-oxidant defenses in these transgenic cells and leaves has 'overcompensated' for the lack of AOX, resulting in lower than normal ROS levels within the cell.

At present, we cannot explain the different response (in terms of steady-state ROS level) of AS8 vs. RI9 suspension cells. One possible explanation is that the degree of suppression of AOX gene expression differs in the two lines and that this impacts the outcome in terms of how much additional mROS is produced, how the anti-oxidant defenses respond and, ultimately, what steady-state level of ROS is attained. However, as the level of AOX protein and capacity is essentially at or below detection limits in both cell lines, we cannot establish experimentally any difference in residual AOX between the two transgenic lines. A second possible explanation relates to culture age. The AS8 cells have been in culture continuously for ~10 years longer than the RI9 cells. One intriguing possibility is that cumulative oxidative damage to mitochondria as a result of lacking AOX is, in a self-amplifying manner, resulting in even greater amounts of mROS generation. It is this sort of cumulative oxidative damage of mitochondrial DNA, for example, which is thought to be responsible for the increased mROS generation associated with aging, the so-called 'free radical theory' of aging (Balaban et al. 2005). If this is the case, it could provide an explanation as to why AS8 cells have much higher ROS levels (and more highly induced anti-oxidant defenses) than do RI9 cells. It should be noted, however, that important components of the free radical theory of aging (such as the relationship between mitochondrial DNA mutations and rates of mROS generation) remain vigorously debated (Loeb et al. 2005, Trifunovic et al. 2005).

Another striking difference between the two transgenic suspension cell lines was the massive induction of CAT in AS8 but absolutely no induction in RI9. In this regard, it is interesting that CAT is a ROS scavenger that, due to its low affinity for H₂O₂, might only be useful in the removal of excessive amounts of ROS (Mittler 2002).

Our results show that a lack of leaf AOX impacts anti-oxidant defenses well beyond those localized to the mitochondrion. For example, significant changes were seen in the level of gene transcripts encoding peroxisome- and chloroplast-localized ROS scavengers (i.e. CAT and FeSOD). In photosynthetic tissue, it is expected that the ROS-scavenging capacity of these compartments would already be high to deal with the large fluxes of ROS associated with photosynthesis and photorespiration

(Foyer and Noctor 2003). Therefore, it is significant that these transcripts were seen to increase even further in the absence of AOX. These pervasive changes throughout the leaf cell may provide an explanation for why the ROS-scavenging network would appear to have 'overcompensated' for the new rate of ROS generation, hence drawing levels of ROS to below that seen in the Wt leaf. Other disparate studies have also shown the capacity of mitochondrial alterations to impact the ROS-scavenging network of leaves (Dutilleul et al. 2003).

We envisage at least two possibilities (that are not mutually exclusive) as to why the lack of AOX is able to impact in such a pervasive way the ROS-scavenging network in leaves. As elaborated upon earlier, the most straightforward possibility is that, in the absence of AOX, the rate of mROS generation increases and this additional mROS is able to intensify signaling pathway(s) that control the expression of anti-oxidant defense genes from multiple compartments. However, another possibility is that the lack of AOX perturbs cellular metabolism in such a way that other sources of ROS are generated, which then initiate such signaling pathways. For example, one of the hypotheses about AOX function in leaves is that it supports photosynthetic metabolism (Gardeström et al. 2002). AOX could act as a means to dispose of reductant that is generated by photosynthetic electron transport but which is in excess of that needed for chloroplast metabolism. Also, AOX could play a role in oxidizing the large amount of mitochondrial reductant generated by photorespiratory glycine oxidation (Gardeström et al. 2002). If a lack of AOX compromises these processes, one possibility is that over-reduction of the photosynthetic ETC could occur and that a resulting increased ROS generation from this ETC might signal up-regulation of some anti-oxidant defenses.

PTOX is a plastoquinol oxidase embedded in the thylakoid membrane of chloroplasts and could act as a means to dispose of excess electrons associated with photosynthetic electron transport (Peltier and Cournac 2002). We found that PTOX transcript levels were increased in leaves lacking AOX, suggesting that these two structurally related oxidases (Berthold and Stenmark 2003) might be acting functionally in a coordinated manner. In the absence of AOX to oxidize excess reductant from the chloroplast, PTOX might represent a means to transfer excess electrons back to oxygen. In any case, the results are consistent with the idea that chloroplast metabolism is being perturbed by the lack of AOX. In keeping with this result, microarray analyses of Arabidopsis plants lacking AOX suggested that transcripts encoding metabolic components of the chloroplast were one of the most perturbed sets of genes (Umbach et al. 2005). A recent study in the green alga *Chlamydomonas reinhardtii* showed that both AOX and PTOX were strongly induced during

phosphate-limited growth and that neither was induced in mutant cells defective in acclimation to phosphate limitation (Moseley et al. 2006). This is further evidence that these two oxidases may act in a coordinated manner. Interestingly, we found that PTOX expression was also increased in the non-photosynthetic suspension cells lacking AOX. PTOX is known to be present in different plastid types and, together with the plastid NADH dehydrogenase, could also act to pass electrons from non-photosynthetically generated reductant pools to O₂ (Peltier and Cournac 2002). That AOX and PTOX may act in a coordinated manner is a hypothesis that will now require further investigation.

Some cellular processes are hypothesized to be strongly dependent upon cellular levels of ROS (Apel and Hirt 2004). An example is PCD, such as that exemplified by the hypersensitive response, a feature of plant disease resistance that involves the formation of a necrotic lesion at pathogen infection sites (Lam et al. 2001, Greenberg and Yao 2004). Extensive research has established that both SA and NO are key signaling molecules involved in this plant PCD (Alvarez 2000, Wendehenne et al. 2004, Delledonne 2005). Importantly, the action of these molecules is hypothesized to be either dependent upon or greatly enhanced by levels of ROS in the cell.

We felt that our collection of plants and suspension cells with altered cellular levels of ROS afforded an opportunity to test hypotheses about the interplay of cellular ROS with cell death signaling molecules. In both leaves and suspension cells, we found that higher cellular levels of ROS correlated well with increased susceptibility to SA and NO. This result is consistent with hypotheses that ROS can act synergistically with SA and NO to induce cell death (Delledonne et al. 2005). The results also show that susceptibility to these signaling molecules does not directly correlate with the presence or absence of AOX in that Wt leaves are more susceptible than transgenic leaves lacking AOX while Wt1 suspension cells are more resistant than AS8 cells lacking AOX. Hence, the level of AOX determines susceptibility only in so far as it can impact the rate of mROS generation and the cellular level of antioxidant defenses. In this way, AOX can define the cellular level of ROS. In turn, the ROS level then determines susceptibility, since these ROS interact with the signaling molecules (SA and NO) to initiate death pathways.

We have previously shown that AOX is able to maintain respiratory metabolism, growth and viability if the cyt pathway is inhibited downstream of ubiquinone (Vanlerberghe et al. 2002). As expected then, susceptibility to cell death by the complex IV inhibitor N₃ was directly dependent upon the presence or absence of AOX. Leaves and suspension cells lacking AOX were susceptible to N₃ while those containing AOX (Wt) were resistant. Beside the

ability of NO to act as a signaling molecule, it is also a well-known potent inhibitor of complex IV, while AOX is resistant to NO (Millar and Day 1996). Hence, an important question regarding the ability of NO to induce plant cell death (such as during the hypersensitive response) is whether NO is primarily acting as a signal molecule or as a respiratory inhibitor. Our results suggest the former. If cell death induced by NO in our leaf experiments was occurring primarily due to its ability to act as a complex IV inhibitor, we would have expected that the transgenic lines lacking AOX would have been the most susceptible (similar to the N₃ result). What we found, in fact, is that the leaves lacking AOX were nonetheless the more resistant to NO. This provides strong evidence that the ability of NO to induce cell death in these experiments is due to its ability to act (along with cellular ROS) as a signaling molecule rather than targeting complex IV.

Conclusion

Our results indicate that the mitochondrion, and AOX specifically, can have a significant impact on the cellular balance between ROS generation and scavenging in both tobacco suspension cells and tobacco leaves. This is of particular interest in leaves given that another leaf organelle (the chloroplast) is often regarded as playing a dominant role in terms of both ROS generation and scavenging. Our results suggest an unexpectedly prominent role for the mitochondrion in these processes as well.

Materials and Methods

Transgenic plants and suspension cells

For silencing of AOX, a T-DNA construct encoding an intron-spliced hairpin RNA was built into the pKANNIBAL vector described by Wesley et al. (2001). This construct contained two copies of an AOX cDNA in an inverted repeat orientation (sense and antisense arms) separated by a pyruvate orthophosphate dikinase intron. For the sense arm, a 1.4 kb AOX cDNA (Vanlerberghe et al. 1994) was excised with a single *EcoRI* digest and cloned into pKANNIBAL. Orientation of the sense arm was confirmed by a *BglII* digest resulting in four expected fragments of 4.5, 1.5, 0.74 and 0.69 kb. To obtain the antisense arm, a similar AOX cDNA was first cloned into the *EcoRI* site of pBluescript SK (+/−) (Stratagene). The orientation of this AOX cDNA was confirmed by a *NotI*–*BglII* digest resulting in a 0.52 kb diagnostic fragment and then excised with *BamHI*–*Clal* and subcloned in antisense orientation into pKANNIBAL. The resulting construct, driven by the cauliflower mosaic virus (CaMV) 35S promoter and with the octopine synthase transcription termination sequence was cut by a single *NotI* digestion and cloned into the binary plant transformation vector pART27 (Gleave 1992). The resulting plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 and used to transform tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) by the leaf disc method of Horsch et al. (1986). Thirty primary transformants were selected, and the progeny from 22 of these lines showed

a Mendelian segregation ratio of 3:1 for resistance to kanamycin, indicative of insertion of the T-DNA at a single locus. Homozygous progeny from the second generation of these 22 lines were screened by injection of leaves with the complex III inhibitor AA. It is expected that tissue with effective silencing of AOX will die in response to this treatment due to a complete inhibition of respiration (Vanlerberghe et al. 1995). Two transgenic lines (termed RI9 and RI29) that showed extensive death in response to 5 μ M AA were identified in this manner. Fresh leaf tissue from Wt (non-transformed) and RI9 (homozygous progeny from the second generation) were also used to generate suspension cell cultures using the method described previously (Vanlerberghe et al. 1994). We also utilized a previously generated transgenic tobacco line termed AS8. This line contains an antisense construct of AOX and displays no detectable AOX protein or activity in leaves or as a suspension cell culture (Vanlerberghe et al. 1994, Vanlerberghe et al. 1995, Sieger et al. 2005). Hence, this study has utilized two Wt suspension cell cultures, one that was originally generated and maintained alongside the AS8 suspension cell culture (referred to here as Wt1) and one that was more recently generated and maintained alongside the RI9 suspension cell culture (referred to here as Wt2). This allows the silenced suspension cells to be compared with Wt cells that have been cultured for a comparable period of time (i.e. compare Wt1 with AS8 and Wt2 with RI9).

Growth conditions

Plants were raised in controlled-environment growth chambers (Model PGR-15, Conviron, Winnipeg, Canada) with a 16 h photoperiod, a temperature of 28°C/22°C (light/dark), a growth irradiance of approximately 400 μ mol m⁻² s⁻¹ and a relative humidity of 60%. Plants were grown in 130 mm plastic pots containing a general purpose growing medium (Pro-mix BX, Premier Horticulture Ltd, Rivière-du-Loup, Quebec, Canada) and were irrigated with water or a 10 \times diluted Hoagland's solution as necessary. For all experiments, plants were used at 6–8 weeks after initiating germination in vermiculite. At this stage, plants had five mature or fully emerged leaves, and no obvious differences were seen in the growth or development of the transgenic plants compared with the Wt.

Suspension cells were grown in axenic batch culture in a previously described medium (Linsmaier and Skoog 1965) that contains 88 mM sucrose as carbon source. The cultures (200 ml culture in a 500 ml Erlenmeyer flask) were maintained in the dark on a rotary shaker (140 r.p.m.) at 28°C and were subcultured every 7 d by dilution in fresh growth medium. Cells were used at 2 d after subculture (early exponential growth phase) for all experiments.

Mitochondrial isolation and analysis

Previously described methods were used for the isolation of mitochondria from tobacco leaves (Vanlerberghe et al. 1995) or suspension cells (Robson and Vanlerberghe 2002) and for immuno-blot analysis of mitochondrial proteins (Robson and Vanlerberghe 2002).

Northern blot analyses

RNA extraction and Northern blot analyses were performed essentially as described by Sieger et al. (2005). All RNA gels contained 20 μ g of total RNA per lane. To generate hybridization probes, partial cDNAs were amplified from tobacco leaf RNA using a reverse transcription-PCR (RT-PCR) kit (Access RT-PCR, Promega, Madison, WI, USA) and cloned into pGEM-T Easy (Promega). The cDNAs were then excised

from these plasmids and gel purified. The amplified cDNAs included CAT (U03473; Chen et al. 1993), GPx (X60219; Criqui et al. 1992), APx (U15933; Orvar and Ellis 1995), MnSOD (X14482; Bowler et al. 1989), CuZnSOD (X55974; Tsang et al. 1991), FeSOD (M55909; Van Camp et al. 1990) and PTOX (see below). The primer sequences used for RT-PCR were: CAT fwd 5'-CTTACCTGTGCTGATTTTCTCC-3' and CAT rev 5'-TGTCCTCCGAATAGTAAAGACC-3'; GPx fwd 5'-CCAGTCAATCCAGCAAGC-3' and GPx rev 5'-TTCTTGATATCC TTCTCCATG-3'; APx fwd 5'-TGCGCTCCTCTTATGCTCC-3' and APx rev 5'-GACAAAAACCAACAATTCCACC-3'; MnSOD fwd 5'-TGCAGACCTTTTCGCTCC-3' and MnSOD rev 5'-CATGTCACCTCAGCCTTATTTCC-3'; CuZnSOD fwd 5'-AAAA TGGTGAAGGCCGTC/TGC-3' and CuZnSOD rev 5'-GAGGCCC/TATC/TATACCACAAGC-3'; and FeSOD fwd 5'-CTCCAGCTCCTCCTTATCC-3' and FeSOD rev 5'-TCGTGCCTGCTAGATTTGC-3'.

To isolate a tobacco PTOX cDNA, degenerate primers were designed based upon the known dicot PTOX sequences of *Arabidopsis thaliana* (Q56X52), *Capsicum annuum* (AAG02288) and *Lycopersicon esculentum* (AAG02287). The primer sequences used were: Dicot PTOX fwd 5'-AAGATWCTTGAYACTTT-3' and Dicot PTOX rev 5'-MCCYCCVGTTRTAGTA-3'. Using RT-PCR, these primers amplified the expected size product (~432 bp), which was then ligated into pGEM-T Easy and sequenced. The identity of the tobacco PTOX was confirmed by alignment of the translated protein sequence with known PTOX proteins. Based upon the partial tobacco PTOX cDNA isolated and sequenced, gene-specific primers (tobacco PTOX fwd 5'-CTTGATACTTTGTATCACGACC-3' and tobacco PTOX rev 5'-AATTCCTCTCCTTGCTCC-3') were then used in an RT-PCR to generate a 377 bp product that was cloned into pGEM-T Easy, cut out of the plasmid using *EcoRI* and gel purified for use as hybridization probe.

After X-ray film development, Northern blots were quantified by densitometry using an imaging system (Alpha Innotech Corporation, San Leandro, CA, USA) and associated software (AlphaEaseFc).

Analysis of reactive oxygen species

For leaves, NBT and DAB were used for in situ visualization of superoxide and H₂O₂, respectively, similar to as described in Dutilleul et al. (2003). Leaves were cut from plants and immediately vacuum infiltrated with either 0.5 mg ml⁻¹ NBT in 10 mM potassium phosphate buffer (pH 7.8) or 1 mg ml⁻¹ DAB in dH₂O (pH 3.8). The leaves were then incubated in the dark at room temperature for 1 h (NBT) or 14 h (DAB). Chlorophyll was subsequently extracted using 95% ethanol at 70°C for several hours. Once chlorophyll was completely removed, the ethanol was replaced with 25% glycerol and the leaves were visually scored for color intensity.

For suspension cells, the ROS-sensitive fluorescent probe DCFH-DA was used to estimate cellular levels of H₂O₂. The method was similar to that described by Parsons et al. (1999). Briefly, 20 μ M DCFH-DA was added to the cell culture flask and an aliquot of cells was taken periodically for 30 min and immediately mixed with cold 5 mM KCN (final concentration). These samples were then immediately frozen and stored in liquid nitrogen. For each flask, a single aliquot of cells was also washed twice with water, frozen and lyophilized to determine culture density (g DW l⁻¹). The following day, samples were thawed on ice and centrifuged (2 min, 16,000 \times g, 4°C). The supernatant was then diluted 10-fold with water and the fluorescence of the sample was

measured with a Turner Quantech digital filter fluorometer (Model FM109535, Barnstead/ThermoLyne) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. In each experiment, duplicate samples were taken at each time point. In most cases, fluorescence yield increased linearly with time after the addition of DCFH-DA. Linear regression analysis of the data was used to determine the rate of fluorescence increase over time, and this was normalized to culture density.

Chemical treatments

Chemicals to be injected into leaves were made up in concentrated stocks and then diluted in 10 mM MES buffer (pH 6.5) to final concentrations of 3.5 mM SA, 2 mM SNP and 2 mM NaN_3 . Plants were taken from the growth chambers, and defined leaf panels (outlined by the mid-vein and secondary veins) were completely infiltrated from the abaxial side using a 25G needle. Care was taken to inject equivalent leaves (middle and upper leaves) between different individual plants. After injection, plants were returned to the growth chambers. Chemical treatment of suspension cells involved addition of filter-sterilized concentrated stock solutions directly into cultures to yield the following final concentrations: 500 μM SA, 600 μM SNP and 100 μM NaN_3 .

Analysis of cell death

The extent of leaf cell death was determined at 48 h after chemical treatments by visual estimation of the percentage of infiltrated area that had browned and dried. Viability of suspension cells at 24 h after chemical treatments was determined using Evans blue, as previously described (Robson and Vanlerberghe 2002).

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