Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 137: 392–406. 2009

REVIEW

Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase?

Greg C. Vanlerberghe[a,b,*], Marina Cvetkovska[a,b] and Jia Wang[a,b]

[a]Department of Biological Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, Ontario, Canada M1C 1A4
[b]Department of Cell and Systems Biology, University of Toronto, 25 Harbord St., Toronto, Ontario, Canada M5S3G5

Correspondence
*Corresponding author,
e-mail: gregv@utsc.utoronto.ca

Received 19 March 2009; revised 28 April 2009

doi: 10.1111/j.1399-3054.2009.01254.x

All plants maintain a non-energy-conserving pathway of mitochondrial electron transport referred to as alternative oxidase (AOX) respiration. Here, we briefly review some of the most prevailing themes for the metabolic and physiological roles of this respiratory pathway. Many of these themes relate to the potential of AOX to provide *metabolic homeostasis* in response to fluctuating cellular conditions, such as is often seen during stress. We then review reverse genetic experiments that have been used to test these hypotheses. To date, such experiments have been limited to just two dicot species and have only targeted one member (a stress-induced member) of the AOX multigene family. Nonetheless, the experiments to date strongly reinforce the idea that AOX respiration is of particular importance during abiotic and biotic stress. Finally, we propose that another core role of AOX may be to modulate the strength of a stress-signaling pathway from the mitochondrion that controls cellular responses to stress. In this way, AOX could be acting to provide a degree of *signaling homeostasis* from the mitochondrion. This hypothesis may provide explanation for some of the disparate results seen in reverse genetic experiments regarding the impact of AOX on the reactive oxygen network and oxidative damage.

Introduction

Besides cytochrome (cyt) oxidase (Complex IV), all plants have an additional terminal respiratory oxidase called alternative oxidase (AOX) that catalyzes the oxidation of ubiquinol and reduction of O_2 to H_2O (Finnegan et al. 2004). AOX is non-proton pumping and since it bypasses proton-pumping Complexes III and IV, electron flow to AOX dramatically reduces the energy yield of respiration. In plants, AOX is encoded by a small nuclear gene family. It is an interfacial membrane protein on the matrix side of the inner mitochondrial membrane and exists as a homodimer. Many AOX gene family members contain a conserved Cys residue that confers tight biochemical control over the enzyme. In this case, the dimer may be either non-covalently linked (reduced form) or covalently linked by a regulatory disulfide bond between the conserved Cys residues of the two monomers (oxidized form). Reduction of this disulfide bond can be achieved by the oxidation of specific tricarboxylic acid (TCA) cycle substrates, and based upon the substrate specificity of this process, it is hypothesized that specifically matrix NADPH provides the reducing power for this regulatory reduction. Once reduced, AOX can be activated by specific α-keto acids, most notably pyruvate. Such activation is due to the ability of pyruvate to interact with the exposed sulfhydryls of the regulatory Cys to produce a thiohemiacetal. Our understanding of these biochemical controls remains

Abbreviations – AOX, alternative oxidase; cyt, cytochrome; ETC, electron transport chain; PCD, programmed cell death; ROS, reactive oxygen species; SA, salicylic acid; WT, wild-type.

incomplete, and it is certain that some AOX gene family members are subject to different biochemical controls. Nonetheless, our current knowledge does indicate that the partitioning of electrons between the non-energy-conserving AOX pathway and the energy-conserving cyt pathway is subject to tight biochemical control and hence likely very responsive to metabolic conditions within the mitochondrion (Finnegan et al. 2004).

Because of its non-energy-conserving nature, there has been considerable interest to understand the role of AOX respiration, in addition to its well-established role to generate heat in highly specialized thermogenic tissues such as the floral receptacle of the sacred lotus (Watling et al. 2006). One approach taken over the last decade has been to characterize transgenic or T-DNA knockout plants in which expression of an AOX gene family member has been altered. Here, we review some of the prevailing hypotheses for the role of AOX respiration and summarize specifically the work done using reverse genetics to test such ideas. We also introduce a new hypothesis for the role of this non-energy-conserving pathway and provide some suggestions toward testing the hypothesis.

Hypotheses regarding the metabolic and physiological roles of AOX in plants

The role of a non-energy-conserving AOX pathway of respiration has intrigued plant biologists for several decades, and many influential reviews have discussed the hypotheses regarding its role. Examples of such reviews include Lambers (1982), Purvis and Shewfelt (1993), Theodorou and Plaxton (1993), Millar and Day (1997), Vanlerberghe and McIntosh (1997), Sakano (1998), Simons and Lambers (1999), Hansen et al. (2002), Moore et al. (2002), Netting (2002), Raghavendra and Padmasree (2003) and Arnholdt-Schmitt et al. (2006). Below are brief descriptions of what we consider to be some prevailing themes regarding the role of AOX in plant metabolism and physiology. We emphasize that, in most cases, these roles are not mutually exclusive and would likely to act in a complementary fashion. Also, the importance of these different potential roles for AOX is likely to depend upon the developmental, metabolic or physiological status of the plant. Hence, the key consideration may not be so much whether these are roles for AOX, but rather: what are the specific developmental, metabolic or physiological conditions under which these roles are manifest?

Optimization of respiratory metabolism

In general, respiration is a process that couples carbon metabolism to electron transport and ATP synthesis. Since the AOX pathway of electron transport is not associated with energy conservation, it provides flexibility within this otherwise tightly coupled system. This should broaden the metabolic conditions under which respiration can function effectively, increase the metabolic tasks that respiration can achieve, and allow for transitions between catabolic and anabolic modes of respiration. For example, in some cases, AOX activity may be necessary to provide adequate respiratory carbon intermediates for biosynthesis (Lambers 1982, Simons and Lambers 1999), to burn excess carbohydrate (Lambers 1982, Simons and Lambers 1999), to overcome the inherent limitations of ADP and/or P_i seen during P-limited growth (Theodorou and Plaxton 1993), to regulate cellular levels of pyruvate (Vanlerberghe and McIntosh 1997, Zabalza et al. 2009) or to allow for the oxidation of carbon by pathways subject to different levels of control by ATP turnover (e.g. glycolysis vs oxidative pentose phosphate pathway)(Simons and Lambers 1999). Each of these processes can be aided by an AOX pathway able to adjust the degree of coupling between carbon metabolism and electron transport.

Integration of respiratory metabolism with other metabolic and cellular processes

Respiration does not function in isolation but rather must integrate with other processes. These include other major metabolic pathways that interface with respiration (e.g. nitrogen assimilation and photosynthesis) (Gardeström et al. 2002) as well as cellular activities that are directly supported by respiration such as growth and ion transport. These processes external to respiration can dramatically alter the supply of or demand for carbon skeletons, reducing power and ATP, and hence AOX could provide a ready means by which respiration could adjust to the new needs. For example, AOX activity could provide a means to adjust metabolism in response to the large differences in demand for reducing power associated with the assimilation of NH_4^+ vs the assimilation of NO_3^- (Escobar et al. 2006). AOX activity may also oxidize reducing power associated with malate metabolism during Crassulacean acid metabolism (CAM) (Robinson et al. 1992), reducing power generated by photorespiration (Gardeström et al. 2002) or other excess reductant associated with photosynthesis (Raghavendra and Padmasree 2003, Yoshida et al. 2007). By modulating energy yield, AOX may also play a role in optimizing the rate of highly energy-consuming processes such as growth (Hansen et al. 2002, Moore et al. 2002), perhaps to match growth rate with the availability of key resources such as mineral nutrients. Finally, it has been suggested that AOX activity may provide a means to

regulate cytosolic pH, particularly during periods of stress (Netting 2002, Sakano 1998).

A reactive oxygen species-avoidance mechanism

The mitochondrial electron transport chain (ETC) is a source of reactive oxygen species (ROS), and studies have shown that the rate of ETC-generated ROS is strongly dependent upon membrane potential, due to the close relationship between membrane potential and the reduction state of ETC components (Møller 2001). Increased membrane potential correlates with more highly reduced ETC components and this increases the probability for single electron transfer to O_2, generating superoxide. The magnitude of membrane potential is dependent upon the activity of the energy-dissipating systems, particularly oxidative phosphorylation. For example, when ADP is being actively phosphorylated (state 3), membrane potential is lower than when ADP is limiting (state 4). As a result, ROS generation increases in state 4 compared with state 3 conditions. Similarly, increased energy dissipation (resulting in reduced ROS generation) can be achieved by artificial uncouplers as well as by the action of uncoupling proteins. Given that electron flow from ubiquinone to O_2through AOX is not coupled to proton translocation (hence dissipating energy as heat) and given that this activity could also reduce the rate of electron flow from ubiquinone to O_2 through the energy-conserving cyt pathway, an important hypothesis has been that AOX may act to dampen ROS generation by the mitochondrial ETC (Purvis and Shewfelt 1993). AOX may also reduce ROS generation by other means. For example, if AOX represents a means to consume reducing equivalents associated with photosynthetic metabolism (see above), then its activity might indirectly dampen ROS generation by the photosynthetic ETC.

A cell 'survival protein'

The expression of some AOX gene family members is strongly induced by inhibition of the cyt pathway at Complex III or Complex IV and, under such conditions, high AOX protein and activity can support respiration (Vanlerberghe and McIntosh 1994). Hence, one role of AOX may be to support metabolism under conditions in which the capacity of the cyt pathway has been strongly suppressed. Sulfide, cyanide and nitric oxide are all strong inhibitors of cyt oxidase (while AOX is insensitive to these compounds), and each of these metabolites can be a by-product of metabolism occurring within the mitochondrion. Since AOX is also induced by TCA cycle inhibitors, inhibitors of oxidative phosphorylation and artificial uncouplers, it is possible that induction is also a general response to disruptions in energy metabolism (Saisho et al. 2001, Vanlerberghe and McIntosh 1996). AOX is also strongly induced by salicylic acid (SA), a cell signaling molecule that can accumulate to high levels during biotic stress and which appears to disrupt mitochondrial function by a poorly understood mechanism (Norman et al. 2004; van der Merwe and Dubery 2006). Since AOX represents a heme-independent respiratory pathway, it could also be used following conditions that prevent heme accumulation, such as anoxia (Millar et al. 2004). Other abiotic stress such as acute ozone exposure may also negatively impact the cyt pathway function and require an upregulation of AOX to support metabolism (Ederli et al. 2006). Hence, AOX may act as a 'survival protein' during disruptions in energy metabolism.

Reverse genetics has been used to manipulate AOX respiration in plants

Over the last decade, reverse genetics has been used to test hypotheses regarding the role of the AOX pathway. The majority of these studies are summarized in Table 1. We begin here with a few general observations about this body of work:

1. To date, studies involving the manipulation of AOX gene expression to investigate its physiological role have been limited to just two plant species, *Arabidopsis thaliana* and *Nicotiana tabacum* (Table 1). Transgenic *Solanum tuberosum* that over-express AOX have also been reported and shown to have an increased maximal capacity for AOX respiration, but otherwise these plants have not been further characterized (Hiser et al. 1996). In future, studies should be expanded to other species such as the monocot rice.
2. All studies to date have manipulated the expression of just a single AOX gene family member, the *AOX1a* gene (Table 1). In *N. tabacum* and *A. thaliana*, this gene family member has been shown to be highly stress-responsive as well as highly responsive to inhibition of the cyt pathway, such as by antimycin A (Clifton et al. 2005, 2006, Mittler et al. 2004, Saisho et al. 1997, Vanlerberghe and McIntosh 1992, 1994). Other gene family members in *A. thaliana* (and likely *N. tabacum* as well) are generally not responsive to these treatments and appear rather to have strict tissue and developmental stage-specific expression (Considine et al. 2002; Wang and Vanlerberghe, unpublished). In one study (Kitashiba et al. 1999), an antisense

Table 1. A summary of publications that have used transgenic or mutant plants with altered expression of specific AOX genes to investigate the role of this respiratory pathway in cell metabolism and physiology, particularly under stress conditions..

Condition (species/tissue)	Gene manipulation	Major findings	Reference
Biotic stress-related			
(*N. tabacum*/suspension cells)	Knockdown of *AOX1a*	Knockdown of *AOX1a* increased susceptibility to PCD induced by cyt pathway dysfunction or by treatment with SA, H_2O_2 or a protein phosphatase inhibitor	Robson and Vanlerberghe (2002) and Vanlerberghe et al. (2002)
(*N. tabacum*/leaves)	Knockdown and over-expression of *AOX1a*	Knockdown of *AOX1a* did not compromise SA-induced or N-gene-mediated resistance to tobacco mosaic virus (TMV). Over-expression of AOX did result in smaller hypersensitive response lesions, suggesting a link with this PCD event	Ordog et al. (2002)
(*N. tabacum*/leaves)	Knockdown and over-expression of *AOX1a*	Manipulation of *AOX1a* gene expression had no effect on basal susceptibility to tobacco mosaic virus (TMV) or SA-induced resistance to systemic viral disease	Gilliland et al. (2003)
(*N. tabacum*/leaves and suspension cells)	Knockdown of *AOX1a*	In both leaves and suspension cells, susceptibility to SA- and NO-induced PCD was dependent upon the steady-state cellular level of ROS and AOX levels clearly contributed to this steady-state	Amirsadeghi et al. (2006) and Robson et al. (2008)
Low temperature stress			
(*A. thaliana*/leaves)	Knockdown and over-expression of *AOX1a*	At low temperature (12°C), knockdown of *AOX1a* reduced early shoot growth, whereas over-expression of *AOX1a* enhanced early shoot growth	Fiorani et al. (2005)
(*A. thaliana*/leaves)	Over-expression of wheat *AOX1a*	Over-expression of wheat *AOX1a* in *A. thaliana* delayed expression of the endogenous *A. thaliana AOX1a* gene following shift to low temperature (4°C)	Sugie et al. (2006)
(*A. thaliana*/leaves)	T-DNA knockout of *AOX1a*	At low temperature (4–10°C), knockout of *AOX1a* enhanced expression of ROS scavengers, increased C:N ratios and lowered lipid peroxidation levels	Watanabe et al. (2008)
Light and drought stress			
(*A. thaliana*/leaves)	Knockdown and over-expression of *AOX1a*	In plants over-expressing *AOX1a*, the mitochondria had an increased capacity to synthesize ascorbic acid and such plants displayed increased ascorbic acid levels	Bartoli et al. (2006)
(*A. thaliana*/leaves and roots)	T-DNA knockout of *AOX1a*	Transfer of knockout plants from low to medium irradiance during drought induced a severe stress that included anthocyanin accumulation and increases in stress-responsive transcripts, particularly those encoding chloroplast-localized ROS scavengers	Giraud et al. (2008)
Nutrient stress			
(*N. tabacum*/suspension cells)	Knockdown of *AOX1a*	Knockdown of *AOX1a* magnified ROS generation and restricted carbon metabolism during P-limited growth	Parsons et al. (1999) and Yip and Vanlerberghe (2001)

Table 1. Continued.

Condition (species/tissue)	Gene manipulation	Major findings	Reference
(*N. tabacum*/suspension cells)	Knockdown of *AOX1a*	During P- or N-limited growth, knockdown of *AOX1a* enhanced biomass accumulation and caused other nutrient specific effects on cellular redox and carbon balance	Sieger et al. (2005)
Ozone stress			
(*N. tabacum*/leaves)	Knockdown and over-expression of *AOX1a*	Over-expression of *AOX1a* increased the level of ozone-induced damage	Pasqualini et al. (2007)
Other			
(*N. tabacum*/leaves)	Knockdown and over-expression of *AOX1a*	When cyt pathway disabled, knockdown of AOX results in ethanol accumulation	Vanlerberghe et al. (1995)
(*S. tuberosum*/leaf and tuber)	Over-expression of *AOX1*	Over-expression increased AOX capacity	Hiser et al. (1996)
(*N. tabacum*/suspension cells)	Knockdown and over-expression of *AOX1a*	Use confocal microscopy to show that knockdown of *AOX1a* increased the level of ROS emanating from the mitochondrion	Maxwell et al. (1999)
(*N. tabacum*/pollen)	Antisense *AOX1a* gene fragment from *A. thaliana* under the control of tapetum-specific promoter	Knockdown of tapetal cell AOX[a] in *N. tabacum* caused partial male sterility	Kitashiba et al. (1999)
(*N. tabacum*/leaves)	Knockdown and over-expression of *AOX1a*	Under a range of tested conditions, there was little, if any, effect of AOX protein level on the actual partitioning of electrons to the AOX pathway, indicating that such partitioning is likely dependent upon post-translational mechanisms	Guy and Vanlerberghe (2005)
(*A. thaliana*/leaves and roots)	Knockdown and over-expression of *AOX1a*	Under normal growth conditions, knockdown of *AOX1a* had little impact on transcript level of oxidative stress-related proteins but did impact the transcript level of chloroplast-localized proteins	Umbach et al. (2005)
(*A. thaliana*/leaves)	T-DNA knockout of *AOX1a*	When cyt pathway artificially disabled in the light, knockdown of AOX results in increased glycine to serine ratio	Strodtkötter et al. (2009)

[a]It is unclear which *N. tabacum* AOX gene family member may have been targeted by the *A. thaliana* antisense gene fragment.

AOX1a gene fragment from *A. thaliana* was used to knockdown AOX expression in *N. tabacum* tapetal cells and this was shown to reduce pollen viability. Unfortunately, it is not clear which *N. tabacum* AOX gene family member may have been targeted in these experiments. In future, reverse genetic experiments should also be applied to the tissue and developmental stage-specific members of AOX gene families.

3. To date, studies indicate that manipulation of *AOX1a* gene expression is not compensated for by changes in expression of other AOX genes. For example, suppression or knockout of *AOX1a* did not impact expression of the other four AOX gene family members found in *A. thaliana* (Giraud et al. 2008, Umbach et al. 2005). Similarly, although wild-type (WT) tissues can readily survive chemical inhibition of the cyt pathway by high level expression of AOX (Vanlerberghe and McIntosh 1994), transgenic *N. tabacum* or *A. thaliana* with knockdown of *AOX1a* cannot survive such treatments (Umbach et al. 2005, Vanlerberghe et al. 1994), confirming that, at least under these conditions, none of the other AOX genes compensate for the lack of *AOX1a*.
4. Several studies suggest that AOX levels are co-ordinated with the levels of other mitochondrial ETC components, particularly other energy-dissipating systems such as the alternate (rotenone-resistant) NAD(P)H dehydrogenases and uncoupling proteins (Clifton et al. 2005, Elhafez et al. 2006, Yoshida et al. 2008). Therefore, several of

the AOX reverse genetics studies have examined expression of other ETC components to evaluate whether global changes take place in the ETC that adjust or compensate for the altered AOX level. In some cases, little change in ETC components (including the alternate dehydrogenases and uncoupling proteins) has been seen (Umbach et al. 2005), whereas in other cases at least a subset of genes encoding other energy-dissipating systems was found to increase (Giraud et al. 2008, Watanabe et al. 2008). Although more work is necessary to firmly establish the point, it seems that composition of the ETC is not dramatically reprogrammed when *AOX1a* levels are altered.

5. Since AOX gene expression is often shown to be highly stress-responsive, most reverse genetic studies have subjected the transgenic and mutant plants to various stress conditions (both biotic and abiotic) (Table 1). In many cases, the manipulation of AOX level appears to have relatively minor consequences under non-stress conditions, but more severe consequences are seen under the stress condition. Such results are consistent with the idea that AOX respiration has an important role(s) under stress conditions (Simons and Lambers 1999).

Reverse genetics has provided some insight into the role of AOX respiration in plants

The following are some of the major findings of studies using reverse genetics to test hypotheses about the role of AOX respiration in plants. These studies are also summarized in Table 1.

Impacts on carbon and nitrogen metabolism

Studies have begun to examine the degree to which manipulated levels of AOX impacts the primary pathways of carbon and nitrogen metabolism. Interestingly, the general picture emerging is that there are impacts on metabolism, but this is primarily seen only under stress conditions. For example, Watanabe et al. (2008) found an increase of C:N ratio in *A. thaliana* leaves lacking AOX, but only after transfer to low temperature. Similarly, the C:N ratio of tobacco suspension cells lacking AOX was similar to WT under an optimal nutrient regime but higher than WT during P- or N-limited growth (Sieger et al. 2005). Such cells also had higher carbohydrate pools. Giraud et al. (2008) also reported that *A. thaliana* plants lacking AOX accumulated higher levels of numerous carbohydrates, but only under stress conditions, in this case a combination of moderate light and drought. These studies suggest that AOX respiration aids carbon metabolism under stress and that the cyt pathway alone is unable to compensate for a lack of AOX, resulting in an accumulation of carbohydrate substrate. Several studies suggest that the lack of AOX under stress can also lead to redirections in carbon metabolism, perhaps due to particular bottlenecks in metabolism. For example, Parsons et al. (1999) found that the pool size of amino acids derived from downstream carbon intermediates (such as 2-oxoglutarate) was declined in cells lacking AOX and growing under P limitation. This study also found altered levels of amino acids derived from phosphoenolpyruvate and pyruvate, indicating some disruption of normal metabolism at this regulatory hub in respiration. Umbach et al. (2005) noted an increased transcript level of oxidative pentose phosphate pathway enzymes in *A. thaliana* lacking AOX, also potentially indicative of changes in respiratory carbon flow. Finally, Giraud et al. (2008) found many changes in the metabolite profile of *A. thaliana* plants lacking AOX, and these changes were most pronounced when the plants were under stress.

Impacts on photosynthetic metabolism

Umbach et al. (2005) used microarray analyses to compare the transcriptome of WT *A. thaliana* with that of plants lacking AOX. The analysis indicated that transcripts encoding chloroplast proteins (particularly proteins associated with the light reactions of photosynthesis and stress-related proteins) were among the most perturbed sets of genes. These results are in keeping with the hypothesis that AOX may aid photosynthetic metabolism, most probably due to its ability to oxidize excess reducing equivalents from the chloroplast or associated with photorespiration. Direct evidence that a lack of AOX impedes photosynthesis was reported by Giraud et al. (2008) who showed that during a combined light and drought stress, plants lacking AOX displayed lower levels of photochemical efficiency and dissipated more excitation energy by non-photochemical quenching. Changes in transcript and metabolite profile of these plants were more similar to other studies in which chloroplast metabolism had been perturbed than to studies in which mitochondrial metabolism had been perturbed. Amirsadeghi et al. (2006) found that the expression of plastid terminal oxidase was increased in plants lacking AOX. Plastid terminal oxidase is a plastoquinol oxidase in chloroplasts that could act as a means to dispose excess electrons associated with the photosynthetic ETC. A major challenge for the future will be to elucidate the means by which AOX respiration is interacting with and aiding photosynthesis.

Impacts on the reactive oxygen network

AOX activity is an energy-dissipating mechanism and could act directly to reduce mitochondrial ETC-generated ROS and hence the immediate mitochondrial levels of ROS. To date, the most convincing evidence in support of this hypothesis is that of Maxwell et al. (1999), who used ROS-sensitive fluorescent dyes and confocal microscopy to show that knockdown of AOX in *N. tabacum* suspension cells increased ROS levels emanating specifically from the mitochondrion. It remains to be determined if similar results will be seen in planta, where such experiments are no doubt being hindered by the technical challenges. Nonetheless, numerous studies have documented changes in ROS levels, expression or activity of ROS-scavenging enzymes or changes in oxidative damage in plants after the alteration of AOX levels. Amirsadeghi et al. (2006) found that in *N. tabacum* leaves lacking AOX there was increased expression of ROS-scavenging enzymes which resulted in a lower steady-state level of ROS than found in the WT, suggesting some over-compensation by the ROS-scavenging network. Similarly, *A. thaliana* plants lacking AOX had higher expression of some ROS-scavenging enzymes when given a low temperature stress and, again, some over-compensation of the ROS-scavenging network is implied because the levels of lipid peroxidation in these plants were consistently lower than those in the WT (Watanabe et al. 2008). However, other studies have noted increased ROS levels in *A. thaliana* plants lacking AOX and under stress (Giraud et al. 2008). Some studies have shown that the greater induction of some ROS-scavenging enzymes in plants lacking AOX (compared with WT) is strongly dependent upon the imposition of stress (Giraud et al. 2008, Sieger et al. 2005). Conversely, it has been shown that over-expression of AOX can reduce the expression of ROS-scavenging enzymes (Maxwell et al. 1999, Pasqualini et al. 2007) as well as the levels of cellular ROS under cold stress (Sugie et al. 2006). Although the above studies reinforce the idea that AOX levels impact the reactive oxygen network, it still needs to be confirmed (in planta and in vivo) that this is due to changes in the rate of mitochondrial-generated ROS. For instance, it seems equally plausible at this point that the level of AOX is influencing chloroplast-generated ROS and that this factor is driving the changes in the reactive oxygen network (Amirsadeghi et al. 2006, Giraud et al. 2008). Also, the above studies indicate a variable relationship between AOX level and the level of cellular ROS (or oxidative damage) that may be due to differences in the response of the ROS-scavenging network. Hence, more clarity still needs to be brought to the functional relationship between AOX and components of the reactive oxygen network.

Impacts on growth and development

Most studies report no obvious change in plant growth and development due to alteration of AOX levels. However, there are a few notable exceptions. When a tapetum-specific promoter was used to drive expression of an antisense AOX construct in tobacco, it reduced AOX protein levels in the tapetal cells and resulted in significant losses of pollen viability (Kitashiba et al. 1999). In another study, loss of AOX protein in *A. thaliana* was reported to reduce root growth at the early stage of seedling development by approximately10% (Giraud et al. 2008). Fiorani et al. (2005) saw a strong correlation between AOX level and shoot growth of *A. thaliana* plants at low temperature. Plants lacking AOX showed reduced leaf area, whereas plants over-expressing AOX showed increased leaf area in comparison to the WT. These differences disappeared at normal growth temperature. Finally, Sieger et al. (2005) showed that when *N. tabacum* suspension cells lacking AOX were grown under macronutrient deficiency, they accumulated significantly more biomass than WT cells, which induce large amounts of AOX protein under these conditions. It was suggested that, in the suspension cell system at least, the large induction of AOX in the WT is important to uncouple carbon metabolism from ATP generation and growth. The cells lacking AOX are not able to uncouple these processes, resulting in greater accumulation of biomass and hence a more severe reduction in tissue levels of the limiting macronutrient (Sieger et al. 2005).

Impacts on stress tolerance and cell survival

Studies have used reverse genetics to evaluate the impact of AOX level on tolerance to various biotic and abiotic stress. Pasqualini et al. (2007) investigated the impact of AOX level on tolerance toward an acute ozone fumigation. Interestingly, although WT plants and plants with slightly suppressed levels of AOX showed no visible phenotype, plants over-expressing AOX displayed necrotic lesions over approximately 20% of their leaf area. This increased sensitivity correlated with reduced induction of ROS-scavenging enzymes in the over-expressors in response to ozone. As a result, while ROS levels increased only transiently after ozone exposure in the WT plants, ROS levels increased and persisted in the AOX over-expressing plants. Amirsadeghi et al. (2006) and Robson et al. (2008) studied the response of *N. tabacum* plants and suspension cells to SA and nitric oxide, signaling molecules that are often implicated

in biotic stress responses such as the hypersensitive response (a form of programmed cell death [PCD]). It was found that susceptibility to cell death by these signal molecules was dependent upon the steady-state cellular level of ROS and that AOX level was important toward establishing this steady-state level, likely due to its ability to influence the rates of ROS generation and/or scavenging. These studies are of interest since both SA and nitric oxide can impact mitochondrial function and both induce AOX expression (Huang et al. 2002, Maxwell et al. 2002, Norman et al. 2004). Hence, AOX may play a role in PCD and/or other biotic stress responses, as suggested by studies investigating the impact of AOX level on responses to infection with tobacco mosaic virus (Gilliland et al. 2003, Ordog et al. 2002). Perhaps, the most dramatic impact of AOX level on stress tolerance is that recently reported by Giraud et al. (2008). They showed that AOX knockout lines of *A. thaliana* were dramatically more susceptible to the combined stress of moderate light and drought than WT plants. The knockout lines showed decreases in photosynthetic performance, increases in anthocyanin and superoxide, and increased perturbation of their metabolite and transcript profiles. Further, combining these stresses with a high temperature stress resulted in death of the knockout lines while WT plants survived this treatment. Transgenic plants have also been used to establish a link between AOX level and the capacity of mitochondria to synthesize ascorbic acid. The oxidation of L-galactone-1,4-lactone to ascorbic acid is coupled to the reduction of cyt c in a reaction catalyzed by the inner mitochondrial membrane-localized enzyme L-galactone-1,4-lactone dehydrogenase (Millar et al. 2003). Bartoli et al. (2006) showed that higher levels of AOX increased the capacity for ascorbic acid synthesis, potentially due to the ability of AOX activity to keep the cyt c pool in a relatively more oxidized state. Given the well-established importance of ascorbic acid for plant stress tolerance [acting as both an antioxidant and signaling molecule; Foyer (2004)], this study provides another intriguing link between stress tolerance and AOX.

Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for AOX?

We suggest another hypothesis for the role of plant AOX. The hypothesis is founded on the premise that plant mitochondria are able to act as 'signaling organelles', particularly in response to cellular stress. That is, beside the well-studied role of these organelles in carbon and energy metabolism, mitochondria also initiate 'stress-signaling pathway(s)' that co-ordinate appropriate cellular response(s) to the stress. Excellent examples of this are the central role of animal mitochondria in apoptosis (Kroemer et al. 2007) and the retrograde response of yeast mitochondria to respiratory chain deficiency (Butow and Avadhani 2004). We further suggest that *AOX activity is able to define the strength of this stress-signaling pathway*. In this way, AOX level could control the scale of a stress response (e.g. weak or strong induction of stress-related genes) and/or the type of response (e.g. the extreme response of inducing PCD) (Fig. 1). Implicit in our hypothesis is that changes in AOX expression and activity represent a programmed response by the cell to modulate these stress-signaling pathway(s). The potential for control of mitochondrial signaling by AOX has been suggested previously (Clifton et al. 2006, Gilliland et al. 2003, Pasqualini et al. 2007) and is perhaps one means by which AOX could be involved in 'cell reprogramming' under stress, as elaborated by Arnholdt-Schmitt et al. (2006).

Three key questions regarding the potential role of AOX in defining the strength of a stress-signaling pathway are: (1) What is the nature of this signal path being initiated in the mitochondrion? (2) Does AOX act as a positive or negative regulator of this signal path? (3) What aspect(s) of cellular function does this signaling pathway control? The answers to these questions are unknown but we favor a working hypothesis in which AOX negatively regulates a ROS-based signaling pathway and that at least one cellular function being influenced by this signal pathway is the capacity of ROS-scavenging systems in the cell (Figs 1 and 2). The hypothesis is based on the following observations:

1. Both in organello (Popov et al. 1997) and in vivo (Maxwell et al. 1999) experiments strongly suggest that, at least under some metabolic conditions, AOX activity dampens ROS generation within the mitochondrion and presumably at the level of the mitochondrial ETC. Hence, AOX is uniquely positioned to modulate a mitochondrial ROS-based signal pathway.
2. Evidence continues to accumulate that ROS (particularly H_2O_2) can indeed act as signaling molecules within plant cells (Apel and Hirt 2004; Foyer and Noctor 2005, Gechev et al. 2006). Such results underline the importance of controlling rates of ROS generation from all potential sources, including the mitochondrial ETC. The ROS generated at the plasma membrane by NADPH oxidase is a well-established example of ROS that have numerous signaling roles in

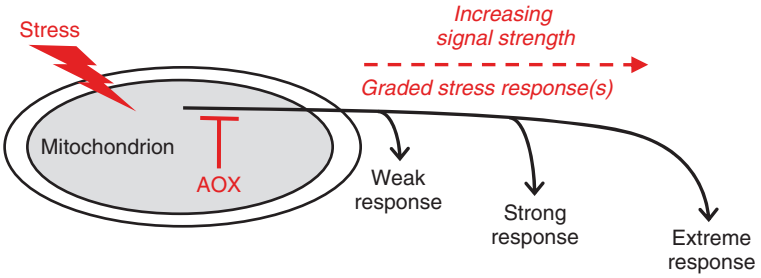

Fig. 1. A working model for the control of mitochondrial stress signaling by AOX. Mitochondria are able to produce an integrated signal (perhaps a ROS-based signal) in response to diverse abiotic and biotic stress conditions. The signal path is important to initiate or amplify appropriate cell responses to the stress. Both the intensity of responses (e.g. weak or strong induction of stress-response genes) and the type of response (e.g. survival response vs PCD response) will be dependent upon signal strength, and AOX activity is an important modulator of this signal strength. In this way, the level of AOX influences cellular responses to stress and may provide a degree of homeostasis to this signal path(s) during changing conditions.

plants, ranging from control of the hypersensitive response (Torres et al. 2005) to guard cell abscisic acid signal transduction (Kwak et al. 2003) and root cell expansion (Foreman et al. 2003). Given these important roles, it is not surprising that NADPH oxidases are subject to strict biochemical control (Sumimoto 2008). Similarly, AOX (at least the stress-induced gene family members) is subject to strict biochemical control (see Introduction), perhaps a requisite feature for an enzyme whose activity will define the strength of a signaling pathway.

3. Evidence is accumulating that the plant mitochondrion can act as a signaling organelle during various biotic and abiotic stress, although the biochemical and molecular details remain largely unknown (Amirsadeghi et al. 2007, Kim et al. 2006, Kuzmin et al. 2004, Millar et al. 2003, Noctor et al. 2007, Rhoads et al. 2006, Rikhvanov et al. 2007, Sweetlove et al. 2007, Takabatake et al. 2007, Vidal et al. 2007, Yao and Greenberg 2006, Yao et al. 2002, Zsigmond et al. 2008). Interestingly, a number of these studies document or imply increased ROS generation from the mitochondrion in response to stress. Presumably, this ROS originates from the mitochondrial ETC and hence its level may be modulated by AOX level.
4. Microarray and other experiments have shown that AOX is one of the most stress-responsive genes in the plant genome (being rapidly and strongly upregulated by a diverse range of biotic and abiotic stresses) and that it is by-far the most stress responsive of mitochondrial ETC proteins (Clifton et al. 2006, Rosso et al. 2006). These observations are often taken as evidence that increased AOX is important to provide *metabolic homeostasis* when metabolic conditions are being perturbed by stress. Another fundamentally different interpretation would be that the increased AOX is (also) acting to provide *signaling homeostasis*. Our hypothesis that AOX negatively regulates this signaling path(s) suggests that the typically seen upregulation of AOX during stress acts to moderate the stress signal, which may be otherwise being amplified by the stress imposition. In many cases, such a response might be necessary to prevent achieving a 'point of no return' signal threshold that induces an extreme response such as PCD. This could provide some explanation for the increased susceptibility to PCD of plants and suspension cells lacking AOX (Amirsadeghi et al. 2006, Robson and Vanlerberghe 2002, Vanlerberghe et al. 2002).
5. Reverse genetic experiments have provided some evidence in support of several of the most prevailing hypotheses regarding the role of AOX in plants (see above). Nonetheless, the results to date appear to fall short of allowing the development of a comprehensive mechanistic model that can explain all of the observations being made. For example, a reasonable hypothesis would be that the mitochondrion is that part of the cell which would be first and foremost impacted by altered AOX expression. Surprisingly perhaps, the studies to date do not give a clear indication that this is the case and several studies in fact conclude that other parts of the cell have been most affected (Giraud et al. 2008, Umbach et al. 2005). This may be a reflection of the ability of AOX to influence other parts of the cell through modulation of a mitochondrial-signaling pathway.
6. Reverse genetic experiments have provided conflicting results regarding the impact of AOX levels on cellular levels of ROS, the expression or activity of ROS-scavenging enzymes and levels of oxidative damage (see above). The variable findings

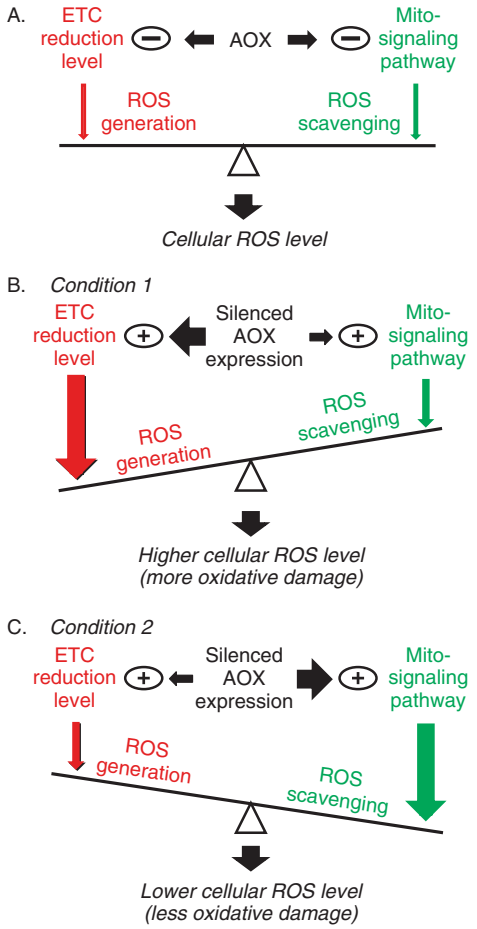

Fig. 2. A working hypothesis for the ability of AOX to influence cellular ROS levels and to provide a possible explanation for the disparate observations noted in reverse genetic experiments silencing AOX expression (see text for additional details). (A) In WT plants, AOX activity acts to lower the reduction level of ETC components, hence reducing the rate of ROS generation. However, AOX activity also lowers the signal intensity of a mitochondrial-initiated signaling pathway able to enhance the capacity of the cellular reactive oxygen network to scavenge ROS. By influencing the capacity for both ROS generation and ROS scavenging, AOX activity becomes an important determinant of cellular ROS level. The silencing of AOX expression in transgenic or mutant plants has the potential to enhance both ROS generation (by increasing ETC reduction levels) and ROS scavenging (by relieving inhibition of the mitochondrial-signaling pathway). However, the newly achieved balance between these two processes (and hence the new cellular ROS level) will depend upon the relative degree to which the lack of AOX promotes these two processes. For example, under Condition 1 (B), the lack of AOX has a much larger impact in enhancing ETC reduction level than in enhancing the mitochondrial-signaling pathway. Hence, this condition favors ROS generation over ROS scavenging, resulting in a higher cellular ROS level than those found in the WT. Alternatively, under Condition 2 (C), the lack of AOX has a much larger impact in enhancing the mitochondrial-signaling pathway than the ETC reduction level. Hence, this condition favors ROS scavenging over ROS generation, resulting in a lower cellular ROS level than those found in the WT. Note that 'ETC reduction level' in these models need not necessarily be limited to the mitochondrial ETC but could rather involve the photosynthetic ETC as well (see text for additional details).

may result from AOX activity having two functional roles. First, AOX is able to dampen ROS generation at the level of the mitochondrial ETC and perhaps at the level of the photosynthetic ETC as well (see discussion above). In this way, AOX activity impacts the *rate of cellular ROS generation*. However, we propose that AOX also plays a second functional role. In this role, the activity of AOX dampens the intensity of a signal transduction pathway from the mitochondrion that is able to enhance the ROS-scavenging network. In this way, AOX activity also impacts the *rate of cellular ROS scavenging*. Fig. 2 illustrates that, given this dual role of AOX, the impact of altering AOX expression on cellular levels of ROS levels will be variable, depending upon which functional role of AOX is most prevalent under a given set of metabolic conditions. Hence, such a model may explain some of the contrasting results seen in reverse genetic experiments evaluating the impact of AOX on the reactive oxygen network.

Some thoughts on testing this hypothesis

Presumably, the activity of a signaling pathway from the mitochondrion that is able to modulate cellular responses to stress will be most active in the period immediately following the imposition of the stress. For this reason, study of the initial 'response period' following a stress may be the most informative in terms of establishing a role for AOX. For example, does AOX level in transgenic lines influence the timing or scale of initial responses to stress? For such experiments, it will be beneficial if the initial growth conditions (prior to the stress) are conditions in which the WT and transgenic or mutant lines are showing minimal differences in their baseline cellular physiology. For example, if one wishes to examine the response of ROS-scavenging enzymes across plant lines, it will be beneficial that their levels are similar prior to stress imposition. Determination of the initial growth conditions necessary to establish these baseline conditions would therefore be very useful. If AOX is acting to dampen this stress signal path, then suppression of AOX may allow for an accelerated and enhanced stress response that may provide at least short-term benefit. For example, if the signal path is responsible for induction of the ROS-scavenging network, then a more rapid and/or stronger induction of the network might result in less short-term oxidative damage (despite more ROS generation at the ETC due to lack of AOX). This represents a readily testable hypothesis.

If AOX can be shown to impact short-term responses to stress, a greater challenge will be to elucidate

the mitochondrial 'signal' being modulated by AOX. Although we have hypothesized here that the signal may be a ROS, AOX activity has the potential to modulate other mitochondrial parameters (e.g. membrane potential and ubiquinone reduction state) or mitochondrial products (e.g. reducing equivalents and ATP) that might also act as the initial signal. The challenge then will be to correlate the strength of one of these potential signals with the strength of stress responses. Once positive correlations are established, it will then be possible to focus efforts toward detailed elucidation of the signal path. It may also be informative to look for parallels between such a mitochondrial signal path and signal paths thought to arise from the chloroplast. A common theme for signal transduction from the chloroplast involves redox signals related to photosynthetic electron transport (Pogson et al. 2008). Such signal paths are just beginning to be elucidated and are suggested to be important in the activation of numerous biotic and abiotic stress responses (Lee et al. 2007, Mühlenbock et al. 2008).

Another major challenge will be to test the hypothesis that changes in AOX expression and activity in response to stress represents a programmed response by the cell to modulate a stress-signaling pathway(s) rather than simply a passive consequence of stress. Stress responses are often regarded as graded responses whereby the severity of the stress determines the *level of response* (e.g. the level of induction of ROS-scavenging enzymes). Another aspect of this graded response, however, is that the *type of response* can shift depending upon the severity of the stress (Fig. 1). An excellent example of this is the shift from responses that promote cell survival toward a cell PCD response. This may be a useful model to evaluate whether changes in AOX are acting to determine cell fate. A useful system for this may be bacterial pathogenesis, in which some bacterial strains cause plant defense responses that do not include PCD while other strains elicit defense responses that do include PCD. The more extreme PCD response may be dependent upon stronger induction of the stress-signaling pathway and hence a downregulation (or lack of induction) of AOX in comparison to the defense response that does not include PCD. In some cases, such downregulation could be due to inactivation of AOX by high levels of ROS. ROS may be able to convert AOX to its oxidized (low activity) form (see Introduction and Vanlerberghe et al. 1999) or inhibit AOX by means of the lipid peroxidation product 4-hydroxy-2-nonenal (Winger et al. 2005). Also, reverse genetic experiments could be used to see whether manipulation of AOX level alters the 'appropriate' defense response to the particular strain of bacteria.

We have emphasized the role that AOX might play to control a mitochondrial 'stress'-signaling pathway. However, changes in AOX activity could also be acting continuously to control the strength of signals that provide the cell with information about mitochondrial status, which is then used to appropriately maintain the organelle. In this way, AOX might be thought not just to provide metabolic homeostasis to the cell but also signaling homeostasis from the mitochondrion.

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Edited by V. Hurry