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Rapid report

Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide

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Key words: alternative oxidase (AOX), *Nicotiana tabacum* (tobacco), nitric oxide (NO), plant mitochondrial electron transport chain, superoxide (O_2^-).

Summary

- The nonenergy-conserving alternative oxidase (AOX) has been hypothesized to modulate the amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plant mitochondria but there is sparse direct *in planta* evidence to support this.
- Laser scanning fluorescent confocal microscopy and biochemical methods were used to directly estimate *in planta* leaf concentrations of superoxide (O_2^-), nitric oxide (NO), peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) in wildtype (Wt) tobacco (*Nicotiana tabacum*) and transgenic tobacco with altered amounts of AOX.
- We found that plants lacking AOX have increased concentrations of leaf mitochondrial-localized O_2^- and leaf NO in comparison to the Wt, while leaf concentrations of H_2O_2 were similar or lower in the AOX-suppressed plants.
- Based on our results, we suggest that AOX respiration acts to reduce the generation of ROS and RNS in plant mitochondria by dampening the leak of single electrons from the electron transport chain to O_2 or nitrite. This may represent a universal role for AOX in plants. More work is now needed to establish the functional implications of this role, such as during abiotic and biotic stress.

Introduction

The plant mitochondrial electron transport chain (ETC) is unique because of the presence of two terminal oxidases. In addition to the usual cytochrome (cyt) oxidase (complex IV), an alternative oxidase (AOX) is present that directly couples the oxidation of ubiquinol with the reduction of oxygen to water. AOX is an interfacial membrane protein oriented toward the matrix side of the inner mitochondrial membrane. Notably, AOX dramatically reduces the energy yield of respiration since it is nonproton-pumping and bypasses proton-pumping complexes III and IV (Finnegan *et al.*, 2004). AOX is encoded by a small nuclear gene family (Considine *et al.*, 2002). In both *Arabidopsis* and tobacco (*Nicotiana tabacum*), the expression of a single gene family member (*Aox1a*) is strongly responsive to stress conditions such as cold or drought, while other gene family members display tissue and/or developmental specificity in their expression (Considine *et al.*, 2002; Vanlerberghe *et al.*, 2009). However, the actual partitioning of electrons to AOX is not simply a function of its protein content, but rather is subject to complex biochemical control (Guy & Vanlerberghe, 2005; Vidal *et al.*, 2007).

Since AOX reduces the otherwise tight coupling between carbon metabolism, electron transport and ATP turnover, it could have a general role in the optimization of respiratory metabolism, as well as the integration of this metabolism with other pathways that impact the supply of, or demand for, carbon skeletons, reducing power and ATP (Finnegan *et al.*, 2004). In addition, Purvis & Shewfelt (1993) suggested that AOX could act to dampen reactive oxygen species (ROS) generation by the ETC. The rate of such ROS generation is strongly dependent upon 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 of single electron leak to O_2, generating the ROS superoxide (O_2^-), which can then act as a substrate for the generation of other ROS species such as hydrogen peroxide (H_2O_2) and hydroxyl radical. The magnitude of membrane potential is dependent upon the activity of the energy-dissipating systems, particularly oxidative phosphorylation. Hence, when ADP is being actively phosphorylated, membrane potential and ROS generation are lower than when ADP is limiting. Increased energy dissipation, resulting in reduced ROS generation, can similarly be achieved by artificial uncouplers or by the action of uncoupling proteins (Møller, 2001). Since electron flow from ubiquinol to O_2 via AOX is not coupled to proton translocation (hence dissipating the energy as heat) and since this activity also reduces electron flow via the energy-conserving cyt pathway, AOX represents another potential means to control O_2^- generation by the ETC (Purvis & Shewfelt, 1993).

More recently, AOX has also been implicated in influencing the generation of nitric oxide (NO), a reactive nitrogen species (RNS). A number of studies have shown that NO is produced by animal and plant mitochondria from nitrite and that its synthesis involves the ETC (Modolo *et al.*, 2005; Planchet *et al.*, 2005; Poyton *et al.*, 2009; Gupta *et al.*, 2011). Further, at least three different mechanisms have been suggested as means by which NO concentration in plant mitochondria could be modulated by AOX.

Here, we used transgenic tobacco with modified amounts of AOX to directly test the hypotheses that AOX modulates the generation of ROS and RNS. For the first time, we establish that a lack of AOX increases the steady-state *in planta* concentrations of both leaf mitochondrial O_2^- and leaf NO. Our findings also establish the most likely of the suggested mechanisms by which AOX influences NO generation. Our work indicates an important and perhaps universal role of AOX to manage ROS and RNS generation by mitochondria.

Materials and Methods

Plants and growth conditions

Tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) was used for all experiments. Transgenic lines with suppressed amounts of AOX protein (lines RI9, RI29) as a result of the presence of an *Aox1a* RNA interference construct, or elevated amounts of AOX protein (line B7) as a result of the presence of an *Aox1a* transgene driven by a constitutive promoter, have been previously characterized (Amirsadeghi *et al.*, 2006; Wang *et al.*, 2011). Seed was germinated in vermiculite for 2 wk before transfer to pots containing a general purpose soil (Pro-mix BX; Premier Horticulture, Rivière-du-Loup, Quebec, Canada). Plants were raised in controlled-environment chambers (Model PGR-15; Conviron, Winnipeg, MB, Canada) with 16 h photoperiod, temperature of 28 : 22°C (light : dark), photosynthetic photon flux density of 130 μmol m^{-2} s^{-1} and relative humidity of 60%. Irrigation alternated between water and 10 × diluted Hoagland's solution. Plants were used 3 wk after transfer to soil.

Imaging of leaf mitochondrial O_2^- and leaf NO and $ONOO^-$

Imaging of O_2^- was performed using the MitoSOX Red mitochondrial O_2^- indicator (M36008; Invitrogen, Carlsbad, CA, USA) that is selectively accumulated by mitochondria, where it is oxidized by O_2^- and becomes fluorescent upon binding nucleic acid (Robinson *et al.*, 2008). Leaf NO was detected using a DAF-FM diacetate NO indicator (D23844; Invitrogen), which after crossing the plasma membrane is cleaved by esterases to generate intracellular DAF-FM, which is then oxidized specifically by NO to produce a fluorescent product (Kojima *et al.*, 1999). Leaf peroxynitrite ($ONOO^-$) was detected using APF (A36003; Invitrogen), which becomes fluorescent after reacting with $ONOO^-$ (Setsukinai *et al.*, 2003). In some cases, the leaf was also labeled with Mitotracker Red (M7512; Invitrogen), which selectively accumulates in mitochondria.

Tobacco leaves were removed and the lower epidermis peeled off. The remaining leaf segments were then floated (30 min, room temperature (RT), dark) on either 3 μM MitoSOX Red (in water), 4 μM DAF-FM diacetate (in water), or 10 μM APF (in 10 mM KH_2PO_4, pH 7.4). In some cases, leaf segments were double-labeled by also including 0.35 μM MitoTracker Red in the floating solution. Samples were then mounted on microscope slides and the mesophyll cell layer immediately examined with a LSM510 META laser-scanning confocal microscope (Carl Zeiss) with appropriate excitation/detection settings (MitoSOX Red, 488/585–615 nm; MitoTracker Red, 543/585–615 nm; DAF-FM diacetate, 488/500–530 nm; APF, 488/500–530 nm).

All images were acquired under similar acquisition settings. Z-series, typically 8–16 μm in depth with 2 μm step size, were combined in a maximum intensity projection image using LSM510 imaging software. Colocalization (after double-labeling experiments) was analyzed after thresholding for image background noise using JACoP, a plugin for the software Image J (Bolte & Cordelieres, 2006). In this analysis, a Pearson's coefficient of 1 corresponds to complete colocalization of two fluorophores, a value of 0 indicates random localization, and a value of −1 indicates a complete mutual exclusion of the two signals. In our analysis, the Manders' coefficient represents the fraction of pixels from the red fluorescence channel that overlap with the green fluorescence channel. All images were analyzed as Z-series to ensure that the areas of colocalization existed in all three dimensions.

Biochemical analysis of NO and H_2O_2

Nitric oxide was assayed using the method described by Murphy & Noack (1994), with some modifications. Leaf tissue (0.15 g FW) was flash-frozen and ground under liquid N_2 in 100 mM potassium phosphate buffer (pH 7.0) containing 0.6% (w/v) insoluble polyvinylpyrrolidone (PVP). Following the addition of powdered activated carbon, the sample was centrifuged (11 000 ***g***, 10 min, 4°C) and the clarified supernatant was pretreated for 5 min at RT with catalase (100 U) and superoxide dismutase (50

U) to remove ROS. Oxyhemoglobin was prepared fresh on the day of use by converting 20 mg metHb to Hb using an excess of sodium dithionite (2 mM) in 50 mM Tris pH 7.0. The solution was then oxidized with a stream of O_2 and desalted by passing it through a Sephadex G-25 column (GE Healthcare, Mississauga, ON, Canada). Following elution with 50 mM Tris, pH 7.0, the concentration of HbO_2 was determined by absorbance at 415 nm (extinction coefficient 131 mM^{-1} cm^{-1}). HbO_2 (10 μM) was then added directly to the pretreated leaf samples and incubated (5 min, RT). The conversion of HbO_2 to metHb was measured by absorbance at 401 and 421 nm. The concentration of NO was calculated as ($A_{401}HbO_2 - A_{421}$ metHb) using an extinction coefficient of 77 mM^{-1} cm^{-1}.

H_2O_2 was extracted and measured as previously described (Jiang *et al.*, 1990) with some modifications. Leaf tissue (0.2 g FW) was flash-frozen and ground under liquid N_2 in 1 ml of 0.2 M $HClO_4$. The homogenate was incubated (5 min, 4°C), centrifuged (10 000 ***g***, 10 min, 4°C) and the supernatant then removed and neutralized to pH 7–8 with 0.2 M NH_4OH, pH 9.5. After centrifugation (3000 ***g***, 2 min, 4°C) the supernatant was passed through an AG 1-X8 resin column (Bio-Rad). Following elution from the column with ice-cold distilled water, 0.2 ml of the eluate was combined with 0.5 ml of an assay reagent (0.5 mM $(NH_4)_2Fe(SO_4)_2$, 50 mM H_2SO_4, 0.2 mM xylenol orange, 200 mM sorbitol). Following incubation (45 min, RT), the absorbance at 560 nm was measured using a spectrophotometer. To evaluate the % recovery of H_2O_2, 5 μmol of H_2O_2 was added directly to the tissue before extraction. Recovery ranged from 90 to 95%.

Results

MitoSOX Red fluorescence was used to directly visualize mitochondrial O_2^- superoxide in tobacco leaf. To ensure no confounding effects as a result of autofluorescence, images of unlabeled tissue were obtained using the acquisition settings and the longest exposure time being used for labeled samples. Chlorophyll autofluorescence was detected through a 650 nm long-pass filter but no signal was detected in the 585–615 and 500–530 nm range (Supporting Information, Fig. S1). As another control, leaf segments with the lower epidermis removed were treated with the complex III inhibitor antimycin A (10 μM, 1 h, RT) before loading with MitoSOX Red and, as expected, this dramatically elevated MitoSOX Red fluorescence. Further, a cotreatment with antimycin A and the cell-permeable O_2^- scavenger superoxide dismutase-polyethylene glycol (SOD-PEG, 100 U, 1 h, RT) dramatically reduced the subsequent MitoSOX Red signal (Fig. S2). There are reports that MitoSOX Red can cause mitochondrial rupture and signal relocation to the nucleus at higher concentrations (Robinson *et al.*, 2008) and we did observe labeling of nuclei. However, we observed no visible difference in the number of mitochondria in antimycin A-treated leaf labeled with either MitoSOX Red or Mitotracker Red (Fig. S3).

MitoSOX Red was used to compare O_2^- concentrations between Wt and transgenic plants. Transgenic line B7 has elevated amounts of AOX (compared with Wt), RI9 has only very low amounts compared with Wt, and RI29 has no detectable AOX protein, even under strongly inducing conditions (Amirsadeghi *et al.*, 2006; Wang *et al.*, 2011). We found that the two knockdowns each had consistently much higher concentrations of O_2^- than Wt (Fig. 1). Meanwhile, the AOX overexpressor (B7) had similar (or lower) O_2^- to the Wt. Distinguishing between these two lines was difficult because of the relatively low signal in both.

DAF-FM fluorescence was used to directly visualize NO concentration in tobacco leaves. Several controls were first observed. When leaf samples were treated with the NO donor sodium nitroprusside (2 mM, 1 h, RT, 130 μmol m^{-2} s^{-1}), it dramatically increased subsequent DAF-FM fluorescence, while cotreatment with both sodium nitroprusside and the cell-permeating NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 200 μM) dramatically reduced the subsequent DAF-FM signal (Fig. S4). Finally, single-labeled control experiments with DAF-FM and Mitotracker Red established that there was no bleed-through and crosstalk between channels (data not shown).

The AOX overexpressor (B7) had similar NO (or lower) to the Wt, although, as with the O_2^- results, distinguishing between these two lines was difficult because of the relatively low signal in both. Furthermore, we found that the two knockdowns each had

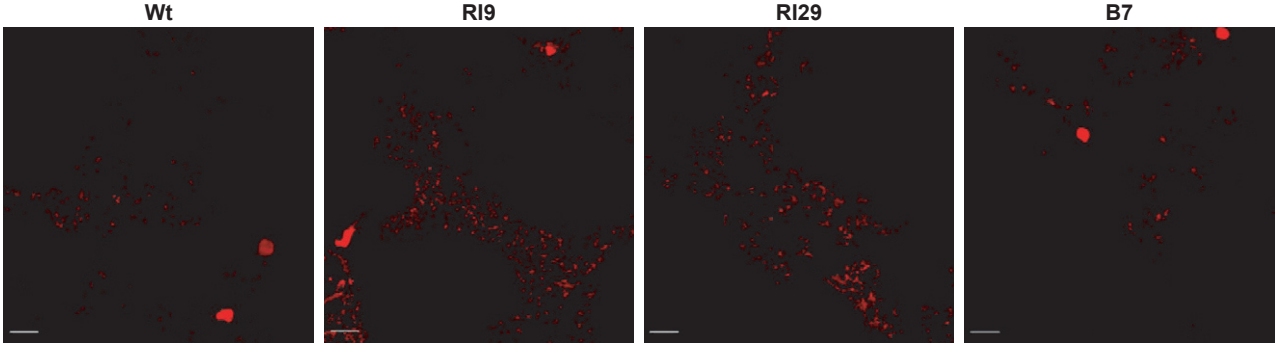

Fig. 1 Mitochondrial O_2^- in mesophyll cells of wildtype (Wt) and transgenic tobacco (*Nicotiana tabacum*) plants with suppressed amounts of alternative oxidase (AOX) (RI9, RI29) or elevated AOX (B7) under steady-state conditions. All images are maximum-intensity projections of Z-series (8–16 μm in depth) and are representative of four independent experiments, each of which showed similar results. Bars, 20 μm.

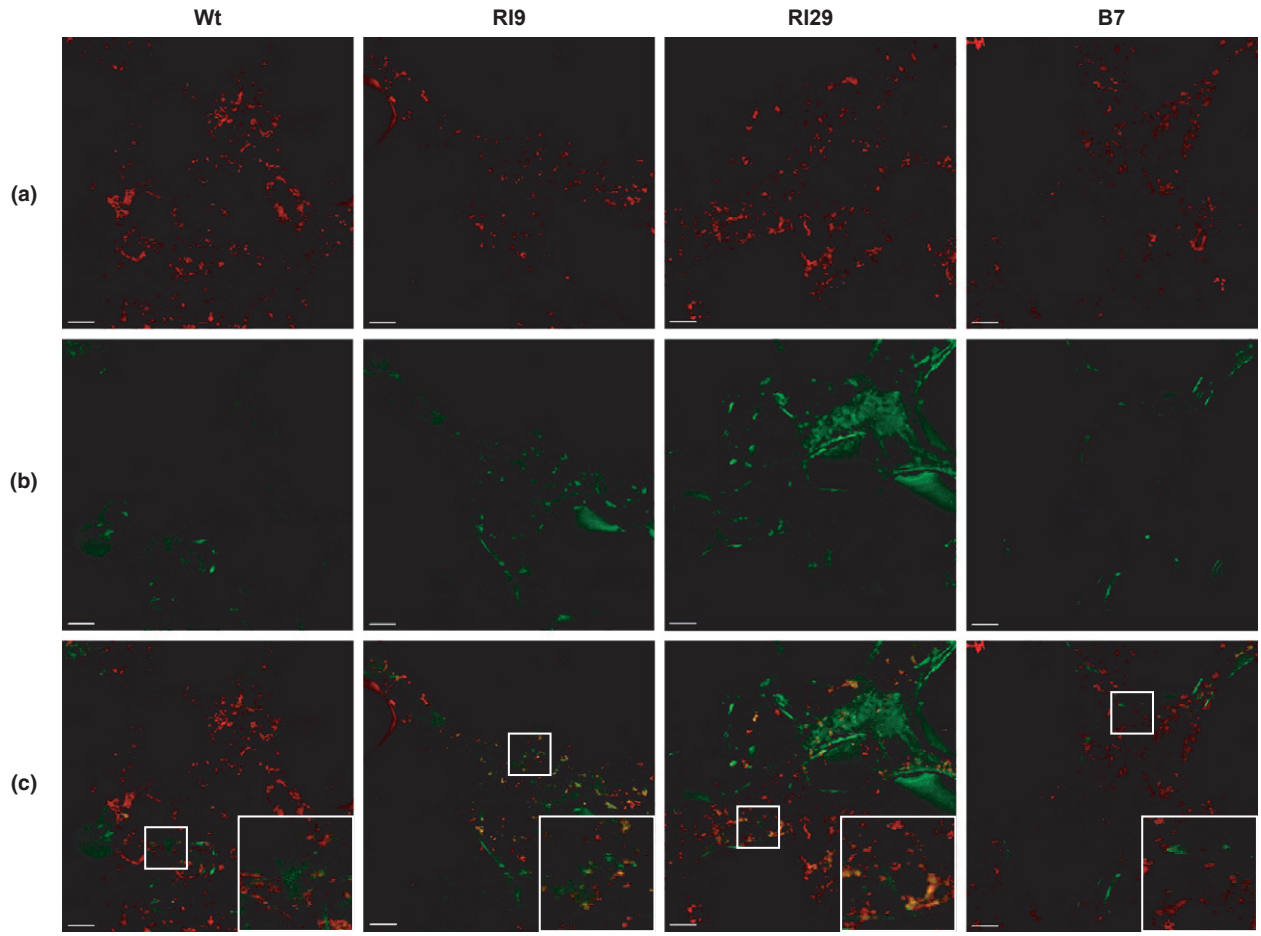

Fig. 2 Concentrations of cellular NO in mesophyll cells of wildtype (Wt) and transgenic tobacco (*Nicotiana tabacum*) plants with suppressed amounts of alternative oxidase (AOX) (RI9, RI29) or elevated AOX (B7) under steady-state conditions. (a) Mitotracker Red fluorescence (red), localizing mitochondria; (b) DAF-FM fluorescence (green), indicating NO concentration; (c) merged images, showing that NO localizes to both mitochondria (yellow) and other parts of the cell (green). The images in the bottom right corners of the panels in (c) are close-ups of the boxed areas. All images are maximum-intensity projections of Z-series (8–16 μm in depth) and are representative of three independent experiments, each of which showed similar results. Bars, 20 μm.

consistently higher concentrations of NO than the Wt (Fig. 2). In this case, the concentration of NO was also considerably higher in the stronger knockdown (RI29) than the slightly leaky AOX knockdown (RI9).

Unlike the MitoSOX probe, DAF-FM diacetate does not localize specifically to mitochondria and hence will react with NO throughout the cell. Further, unlike O_2^-, NO can readily traverse membranes and hence move from its compartment of origin. Also, it is probable that there are multiple locations and mechanisms of NO synthesis in plant cells. Given these factors, it was not surprising that DAF-FM fluorescence was seen throughout the cell (Fig. 2). Nonetheless, colocalization analyses of DAF-FM and Mitotracker (from double-labeling experiments) clearly showed that some of the NO was localized to the mitochondria, that NO concentrations in the mitochondria were higher in the knockdowns than in the Wt, and that a greater fraction of the mitochondria in knockdowns colocalized with the NO signal (Fig. 3).

To confirm these results, we also measured leaf NO concentration by an independent, biochemical method. Similar to the microscopy results, NO concentration in RI29 was much higher than in the Wt (and RI9), while the mean NO concentration in RI9 was only slightly higher (and not significantly different) than the Wt (Fig. 4a).

$ONOO^-$ (a product of the reaction of O_2^- and NO) can be visualized in cells using APF fluorescence. As expected, treating leaf samples (0.5 mM, 1 h, RT) with 3-morpholino sydnonimine (SIN1), which spontaneously releases NO and O_2^-, increased APF fluorescence, while a cotreatment with the $ONOO^-$ scavenger Ebselen (40 μM, 1 h, RT) reduced the signal (Fig. S5). Using APF, we could only detect very low concentrations of $ONOO^-$ in our experiments, with no observable differences between Wt and transgenic lines (Fig. S6).

We also used a biochemical assay to compare H_2O_2 concentration between Wt and transgenic plants. This analysis showed that the knockdowns tended to have less H_2O_2 (though not statistically different) than the Wt (Fig. 4b).

Discussion

Alternative oxidase has been hypothesized to have an impact on the generation of ROS and RNS by plant mitochondria. To

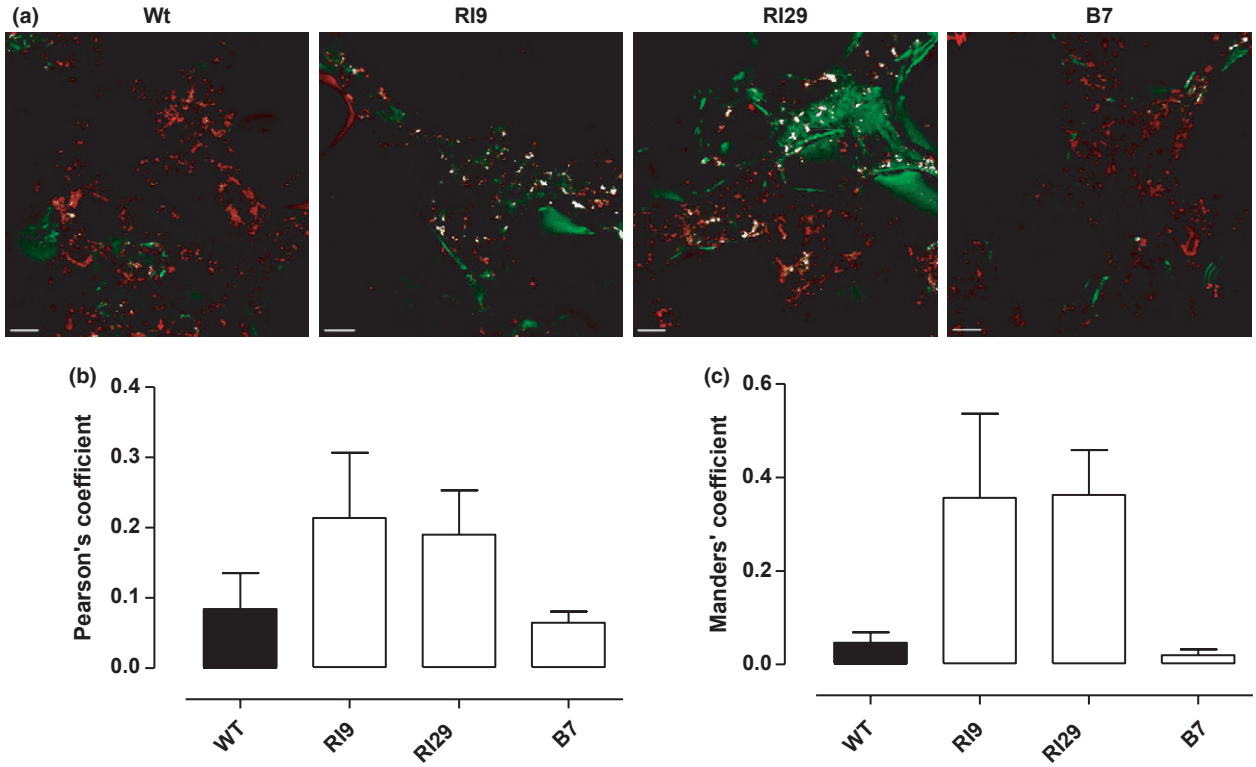

Fig. 3 Colocalization analyses of DAF-FM and Mitotracker Red in wildtype (Wt) and transgenic tobacco (*Nicotiana tabacum*) plants with altered amounts of alternative oxidase (AOX) amounts. (a) A colocalization map where the pixels overlapping in the red and green channels are shown in white, for better clarity. Bars, 20 μm. The Pearson's coefficient (b) and the Manders' coefficient (c) indicate that plants with decreased amounts of AOX (RI9 and RI29) have a greater fraction of mitochondrial signal overlapping with the NO signal. Data are the means ± SE of three independent experiments.

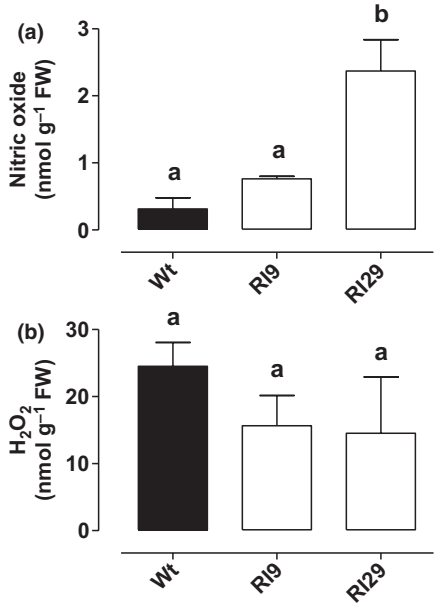

Fig. 4 Leaf concentration of nitric oxide (NO) (a) and hydrogen peroxide (H_2O_2) (b) in wildtype (Wt) and transgenic tobacco (*Nicotiana tabacum*) plants with suppressed amounts of alternative oxidase (AOX) protein (RI9, RI29) under steady-state conditions. Data are the means ± SE of three independent experiments. Each set of data was analyzed by one-way ANOVA followed by a Bonferroni multiple comparison test. Bars not sharing a common letter (a or b) are significantly different from one another ($P < 0.05$).

evaluate this critically, we directly imaged mitochondrial O_2^- and NO in leaf mesophyll cells of Wt tobacco and transgenic tobacco with either increased (B7) or decreased (RI9, RI29) amounts of AOX. Leaf NO concentrations were also estimated by a biochemical approach, as was H_2O_2.

Vanlerberghe *et al.* (1994) reported the generation of transgenic tobacco suspension cells with modified amounts of AOX and these were subsequently used to show that cells lacking AOX had higher concentrations of ROS (imaged using the general ROS fluorescent probe DCF-DA (D399; Invitrogen)) emanating from the mitochondrion (Maxwell *et al.*, 1999). That work was followed by studies aimed to establish whether the suspension cell results can be extended to whole plants. In some cases, lack of AOX was shown to increase the amount of ROS-scavenging systems or the degree of oxidative modification of macromolecules, indirect evidence suggesting an increased rate of ROS generation in such plants (Amirsadeghi *et al.*, 2006; Giraud *et al.*, 2008; Watanabe *et al.*, 2008; Wang *et al.*, 2011). Lack of AOX in *Arabidopsis* during severe stress was shown to increase the whole leaf concentration of O_2^-, visualized using the nitroblue tetrazolium staining method (Giraud *et al.*, 2008). In this case, however, the authors suggest that the increased O_2^- is likely of chloroplast origin because of the strong impact that lack of AOX was having on photosynthesis under the stress conditions. The current study is thus the first to directly establish a mitochondrial-localized increase in O_2^- in plants lacking AOX.

It should also be noted that, at least in animal mitochondria, it has been shown that complex III-generated O_2^- is released to both sides of the inner mitochondrial membrane (Muller *et al.*, 2004). Also, plants have external NAD(P)H dehydrogenases that are another potential source of O_2^- in the intermembrane space (de Oliveira *et al.*, 2008). This raises the possibility that in plants lacking AOX, superoxide concentrations are not only higher in the matrix (as demonstrated here using MitoSOX) but perhaps within the intermembrane space as well.

Research over the last decade has established the mitochondrion as a source of NO in both animal and plant cells, although the details of its synthesis remain poorly understood (Poyton *et al.*, 2009; Gupta *et al.*, 2010). Significantly, at least three distinct hypotheses have been put forth regarding mechanisms by which AOX might control the plant mitochondrial concentration of NO. First, some studies have suggested that AOX might directly catalyze the reduction of nitrite to NO. This hypothesis is based on the observation that nitrite-dependent NO generation by mitochondria could be inhibited by AOX inhibitors such as salicylhydroxamic acid or *n*-propyl gallate (Tischner *et al.*, 2004; Gupta *et al.*, 2005; Planchet *et al.*, 2005). Secondly, it has been suggested that AOX might control NO concentrations indirectly by controlling the generation of the NO scavenger O_2^- (Wulff *et al.*, 2009). It is well established that NO can be effectively scavenged by O_2^-, generating peroxynitrite ($ONOO^-$), and our results in this work show that AOX activity does indeed influence the mitochondrial generation of O_2^-. Thirdly, it has been suggested that NO generation results from electron leak to nitrite, analogous to the means by which O_2^- is generated (Kozlov *et al.*, 1999; Igamberdiev *et al.*, 2010). In this case, AOX could control NO production in a manner similar to controlling O_2^- generation. That is, the nonenergy-conserving nature of AOX will act to moderate membrane potential, which in turn should lessen electron leak by preventing overreduction of ETC components. Also, electron flow to AOX would bypass complex III, cyt c and complex IV, all sites which have been implicated, at least in animals, to generate NO (Poyton *et al.*, 2009).

Our experiment distinguishes between these three mechanisms by which AOX might control NO concentration and our results support the third mechanism. If AOX acts to directly catalyze the generation of NO, we would have expected lower concentrations of NO in RI29 and RI9 than in the Wt. Similarly, if AOX influences NO concentration by controlling the generation of the NO scavenger O_2^-, we would again expect the knockdowns to have lower concentrations of NO, as more O_2^- is available in these plants to scavenge NO. However, if AOX controls NO generation by directly influencing the rate of electron leak to nitrite, then RI29 and RI9 would be predicted to have elevated concentrations of NO. Using two independent methods, we found that the concentrations of NO are indeed higher in the absence of AOX. This lends support to the literature suggesting that an overreduced ETC can be a source of NO, and establishes a role for AOX in controlling NO generation, in addition to O_2^- (Fig. 5). It should be noted that the studies suggesting that AOX might directly catalyze the reduction of nitrite to NO were done primarily under anoxia or hypoxia, while our studies were performed under normoxic conditions. This raises the intriguing possibility of a dual role of AOX to either generate or dampen NO production, depending upon oxygen tension.

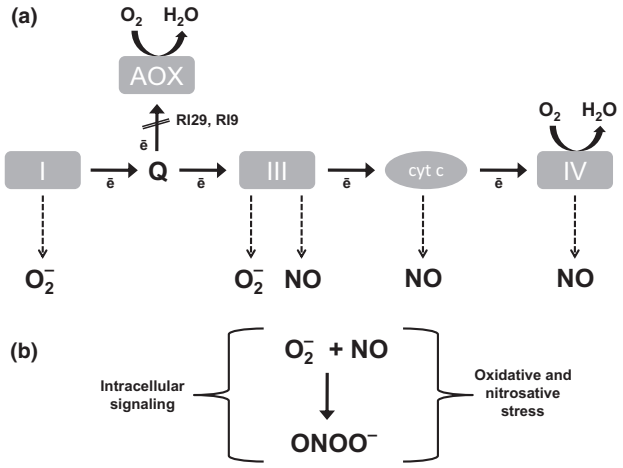

Fig. 5 (a) The plant mitochondrial electron transport chain (ETC), showing its two terminal oxidases (alternative oxidase (AOX), IV) and the most likely sites of superoxide (O_2^-) and nitric oxide (NO) generation, based on other studies. The results of this study suggest that electron flow to AOX dampens the electron leak from the ETC that generates O_2^- and NO at these sites. (b) O_2^-, NO and the product of their reaction ($ONOO^-$) are molecules implicated in oxidative and nitrosative stress and various intracellular signaling pathways. See the text for further details. I, complex I; III, complex III; IV, complex IV (cytochrome oxidase); cyt c, cytochrome c; ē, electron; Q, ubiquinone pool.

There are multiple potential locations and mechanisms of NO synthesis in plants, although these are still poorly understood (Gupta *et al.*, 2010). This, combined with the lack of compartment-specific NO probes and the ability of NO to readily traverse membranes (and hence move away from its site of synthesis), limits the degree to which an *in planta* analysis of the location of NO synthesis can be undertaken. Nonetheless, we were able to establish significant colocalization of NO with mitochondria, as well as demonstrating that, in AOX knockdown plants, both the NO signal from mitochondria and the fraction of mitochondria colocalizing with NO were increased compared with the Wt. While these results infer an increased *mitochondrial* generation of NO in the absence of AOX, we should not exclude the possibility that the lack of AOX has indirectly increased the rate of NO synthesis by other mechanisms than a mitochondrial ETC reduction of nitrite.

Studies have shown that H_2O_2 can induce the synthesis of NO (Bright *et al.*, 2006; Wang *et al.*, 2010a,b). If the AOX knockdown plants had higher concentrations of H_2O_2 (a reasonable possibility given their higher concentrations of O_2^-), it could provide another explanation for their high NO. However, we found that the knockdowns actually tended to have less H_2O_2 than the Wt, as measured using a biochemical assay. This is consistent with previous results in both tobacco and *Arabidopsis,* and likely relates to a subtle overcompensating induction of the H_2O_2-scavenging systems of the cell in the absence of AOX (Amirsadeghi *et al.*, 2006; Watanabe *et al.*, 2008). We attempted

use of the fluorescent probe DCF-DA (often used as an indicator of H_2O_2 concentration) to confirm the results of the biochemical assay of H_2O_2. Two problems with this approach became clear. First, DCF-DA has poor specificity for H_2O_2, and other ROS (including NO, O_2^- and $ONOO^-$) are actually able to oxidize the probe much faster than H_2O_2 (Hempel *et al.*, 1999). Secondly, even at concentrations as low as 2 μM, we surprisingly found that the probe's fluorescence emission was strong across a wide range of the visible spectrum (~500–650 nm), overlapping strongly with the emission signal of Mitotracker Red. Therefore, caution must be exercised in interpreting the results of colocalization experiments involving these two probes, such as in the case of Maxwell *et al.* (1999), where there is a striking apparent colocalization of these fluorescence signals.

Despite the higher concentrations of O_2^- and NO in the AOX knockdown plants, we found that the concentrations of $ONOO^-$ remained low and similar to that seen in the Wt. This suggests that much higher concentrations of O_2^- and NO, such as might be seen during biotic stress, are required to produce $ONOO^-$ in quantities that can be readily detected using confocal microscopy.

During stress, both ROS and RNS can act as signal molecules, when produced in a discreet and controlled fashion; or as toxic byproducts of metabolism, when produced in an uncontrolled manner (Apel & Hirt, 2004; Foyer & Noctor, 2005; Moreau *et al.*, 2010; Beaudouin, 2011; Mittler *et al.*, 2011). That AOX can have an impact on the concentration of these molecules has important and broad implications for plant stress biology and signal transduction research (Fig. 5). For example, a mitochondrial burst of ROS may be important during programmed cell death (Yao & Greenberg, 2006; Amirsadeghi *et al.*, 2007), the activity of glycine decarboxylase (a key mitochondrial enzyme for photorespiration) is regulated by NO (Palmieri *et al.*, 2010), and the mitochondrion has been implicated as a major source of the increased NO seen during responses to biotic stress (Modolo *et al.*, 2005). Recently, we reported that a suppression of AOX might be important in supporting a mitochondrial O_2^- burst seen before a hypersensitive response to bacterial pathogen (Cvetkovska & Vanlerberghe, 2012). Given the results found here, such a suppression of AOX might also support a mitochondrial generation of NO.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Confocal microscope images of Wt tobacco mesophyll cells, showing the lack of chlorophyll fluorescence signal in the 500–530 and 585–615 nm ranges.

Fig. S2 Confocal microscope images of mitochondrial O_2^- in Wt tobacco mesophyll cells either left untreated or treated with antimycin A and/or SOD-PEG.

Fig. S3 Confocal microscope images of Wt tobacco mesophyll cells, showing the comparable number of mitochondrial signals generated by MitoSOX Red and MitoTracker Red.

Fig. S4 Confocal microscope images of cellular NO in Wt tobacco mesophyll cells either left untreated or treated with sodium nitroprusside and/or cPTIO.

Fig. S5 Confocal microscope images of cellular $ONOO^-$ in Wt tobacco mesophyll cells either left untreated or treated with SIN1 and/or Ebselen.

Fig. S6 Confocal microscope images of cellular $ONOO^-$ in Wt and transgenic plants with altered AOX concentrations.

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