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Research

Knockdown of mitochondrial alternative oxidase induces the 'stress state' of signaling molecule pools in *Nicotiana tabacum*, with implications for stomatal function

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Summary

- The mitochondrial electron transport chain (ETC) includes an alternative oxidase (AOX) that may control the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS act as signaling intermediates in numerous plant processes, including stomatal movement.
- The role of AOX in controlling ROS and RNS concentrations under both steady-state and different stress conditions was evaluated using *Nicotiana tabacum* plants lacking AOX as a result of RNA interference. A potential functional implication of changes in ROS and RNS homeostasis was also evaluated by examining stomatal function.
- The leaves of nonstressed AOX knockdowns maintained concentrations of H_2O_2 and nitric oxide (NO) normally seen in wildtype plants only under stress conditions. Further, guard cell NO amounts were much higher in knockdowns. These guard cells were altered in size and were less responsive to NO as a signal for stomatal closure. This, in turn, compromised the stomatal response to changing irradiance.
- The results reveal a role for AOX in stomata. A working model is that guard cell AOX respiration maintains NO homeostasis by preventing over-reduction of the ETC, particularly during periods when high concentrations of NO acting as a signal for stomatal closure may also be inhibiting cyt oxidase respiration.

Introduction

Mitochondria are energy powerhouses of the plant cell (Millar *et al.*, 2011). They use an electron transport chain (ETC) to oxidize NAD(P)H and generate a proton motive force across the inner mitochondrial membrane that drives phosphorylation of ADP to ATP. A secondary consequence of ETC activity is the leakage of single electrons to O_2, producing superoxide (O_2^-). This reactive oxygen species (ROS) may then be converted to another ROS, H_2O_2, by superoxide dismutase. The rate of electron leakage increases as membrane potential increases and ETC components become more highly reduced. Such an over-reduction can occur when substrate supply to the ETC is high, when electron transport is slowed by a slow rate of ATP turnover, or when the capacity of an ETC component(s) is compromised, perhaps by stress or an inhibitory molecule (Møller, 2001).

It is now recognized that mitochondria are also a source of reactive nitrogen species (RNS). For example, single electron leakage may occur from over-reduced ETC components to nitrite, producing nitric oxide (NO) (Modolo *et al.*, 2005; Poyton *et al.*, 2009; Gupta *et al.*, 2010). NO may subsequently react with O_2^-, producing another RNS, peroxynitrite. However, while single electron leakage at complex I and complex III is generally regarded as the major means of O_2^- generation by mitochondria, the site(s) and mechanism(s) of NO generation remain poorly understood (Gupta *et al.*, 2010).

Reactive oxygen species and RNS are signaling molecules involved in controlling a wide range of plant processes, often acting in conjunction with one another (Neill *et al.*, 2002; Apel & Hirt, 2004; Foyer & Noctor, 2009; Baudouin, 2011; Suzuki *et al.*, 2012; Scheler *et al.*, 2013). Reflective of this, the synthesis and degradation of ROS and RNS occur by multiple pathways and within multiple cell compartments. H_2O_2 and NO have emerged as the signature ROS and RNS signaling molecules. While the metabolism of H_2O_2 is reasonably well understood (Foyer & Noctor, 2009), the primary pathways responsible for synthesis and degradation of NO remain elusive (Gupta *et al.*, 2010).

Mitochondria are suggested to act as 'signaling organelles' able to influence processes such as nuclear gene expression and resistance to biotic and abiotic stresses (Noctor *et al.*, 2004; Sweetlove *et al.*, 2007; Cvetkovska *et al.*, 2013; Schwarzländer & Finkemeier, 2013). Certainly, mitochondria are capable of retrograde regulation, generating signals that control the expression of nuclear genes encoding mitochondrial proteins. While the primary signals responsible for mitochondrial signaling remain unknown, ROS and RNS are considered strong candidates.

A feature of the plant ETC is the presence of two terminal oxidases, the usual energy-conserving cytochrome (cyt) oxidase (complex IV) and another termed alternative oxidase (AOX) (Finnegan *et al.*, 2004; Vanlerberghe, 2013). Electron transport is bifurcated, such that electrons in the ubiquinone pool partition between the cyt pathway (consisting of complex III, cyt c and complex IV) and AOX. AOX directly couples ubiquinol oxidation with O_2 reduction to H_2O. This activity reduces the energy yield of respiration because, unlike complexes III and IV, AOX is not proton pumping and hence does not contribute to membrane potential.

Alternative oxidase is encoded by a small gene family, some members of which are induced by abiotic and biotic stresses (Considine *et al.*, 2002; Clifton *et al.*, 2006). In tobacco and *Arabidopsis*, the partitioning of electrons to the stress-inducible AOX1a isoform is subject to biochemical control (Millar *et al.*, 1993; Umbach & Siedow, 1993; Vanlerberghe *et al.*, 1995; Rhoads *et al.*, 1998; Guy & Vanlerberghe, 2005). Through covalent modification and allosteric mechanisms, AOX1a is biochemically activated by a high reduction state of matrix NAD(P)H combined with high concentrations of pyruvate. These are conditions expected to occur when rates of upstream carbon metabolism oversupply downstream electron transport to O_2.

The nonenergy-conserving nature of AOX1a, along with the biochemical controls that govern its activity, makes it well suited as a means of preventing over-reduction of the ETC. Supporting this, we recently showed that transgenic tobacco leaves lacking AOX as a result of an *AOX1a* RNA interference construct have increased concentrations of mitochondrial-localized O_2^- and NO, the products that can arise when an over-reduced ETC results in electron leakage to O_2 or nitrite (Cvetkovska & Vanlerberghe, 2012). This interpretation is further supported by experiments with the complex III inhibitor antimycin A. In wildtype (WT) plants, antimycin A treatment increased both mitochondrial O_2^- and NO, as the restriction of electron flow over-reduces ETC components. However, in plants overexpressing AOX, O_2^- and NO do not increase in response to antimycin A as these plants can maintain high rates of electron flow to O_2, even with the sudden reduction of complex III activity (Cvetkovska & Vanlerberghe, 2013).

These findings provoke numerous questions, such as what might be the dynamics of ROS and RNS in tobacco AOX knockdowns under both steady-state and stress conditions? Furthermore, what might be the functional implication(s) of changes in ROS and RNS in knockdown plants, given their roles in controlling numerous developmental, physiological and stress acclimation responses? To address these questions, the current study examines NO and H_2O_2 concentrations in knockdown plants in response to disparate short-term and long-term abiotic stresses. The study also examines a potential functional consequence of the altered ROS and RNS dynamics. In particular, stomatal function is examined, as both H_2O_2 and NO are implicated as important molecules in the signal transduction pathways that control stomatal movements in response to environmental cues (Bright *et al.*, 2006; Wilkinson & Davies, 2010; Hancock *et al.*, 2011; García-Mata & Lamattina, 2013; Joudoi *et al.*, 2013).

Materials and Methods

Plants and growth conditions

All experiments used tobacco plants (*Nicotiana tabacum* L. cv Petit Havana SR1), including transgenic lines (RI9, RI29) expressing an *AOX1a* RNA interference construct. We have previously shown that these knockdown lines have suppressed amounts of AOX protein relative to the WT, even under strongly inducing conditions (Amirsadeghi *et al.*, 2006; Wang *et al.*, 2011; Wang & Vanlerberghe, 2013). Seeds were germinated in vermiculite, and 2-wk-old seedlings were planted in a growth medium (four parts soil (Pro-mix BX, Premier Horticulture, Rivière-du-Loup, QC, Canada) and one part vermiculite) and transferred to controlled-environment chambers (Model PGR-15, Conviron, Winnipeg, MB, Canada) with 16 h photoperiod, temperature of 28 : 22°C (light : dark), relative humidity of 60% and photosynthetic photon fluence rate (PPFR) of 150 μmol m^{-2} s^{-1} (150 PPFR). Plants were irrigated daily and fertilized four times each week with 1/10th Hoagland's solution. Plants were used at 3 wk after transfer to soil, using the fully developed fourth or fifth leaves. To confirm that the knockdown lines had less AOX than WT under the growth conditions, we measured leaf O_2 consumption in the presence of 30 μM myxothiazol, an inhibitor of complex III. Under these conditions, O_2 uptake occurred at rates (mean ± SE, $n = 4$) of 27.2 ± 1.6 (WT), 13.6 ± 0.7 (RI9), and 7.5 ± 0.6 (RI29) nmol O_2 mg^{-1} DW h^{-1}.

Stomatal measurements

To examine the effect of different signaling molecules on stomatal closure, epidermal peels taken from the abaxial side of well-watered plants during the light period were floated in an 'opening buffer' (10 mM MES-KOH, pH 6.15, 10 mM KCl, 50 μM $CaCl_2$) for 1 h at 28°C and 150 PPFR. The peels were then incubated for 1 h under the same conditions and buffer, but including different concentrations of sodium nitroprusside (SNP), H_2O_2, ABA or salicylic acid (SA). Stomata were then immediately observed and imaged by bright-field microscopy using an Axiophot epifluorescent microscope (Zeiss) fitted with a PCO 12-bit charge-coupled device (CCD) camera and 40× objective (40X Plan Neofluar NA 0.75). Pore length, width, and area were measured using Northern Eclipse v. 5.0 software (EMPIX Imaging, Mississauga, ON, Canada). Stomatal aperture is defined as the ratio of width to length. Peels treated with only opening buffer were also used to estimate guard cell size (area) using image analysis software (Volocity 6.1.1, Perkin Elmer, Woodbridge, ON, Canada). Within each independent experiment, at least 30 stomata were analyzed for each plant line and treatment, and all data represent the mean of three independent experiments. The influence of drought on stomata was studied similarly, except that peels were imaged immediately, without any preincubation.

For stomatal density measurements, a thick layer of clear nail polish was applied to the abaxial side of fresh detached leaves and

left to dry 30 min. The nail polish was then carefully peeled from the leaf and mounted on a slide. Slides were viewed and imaged by bright field microscopy as described earlier but with a 5× objective (5X Plant Neofluar NA 0.15). Densities were determined using Image J (National Institutes of Health, Bethesda, MD, USA) with the Cell Counter plugin. In each independent experiment, at least two individuals were observed for each plant line and treatment. At least five different fields of view (total area of 4.7 mm^2) were counted per individual. Stomatal index was calculated as: number of stomata × 100/(number of stomata + number of epidermal cells). All data are the means of three independent experiments.

Stomatal NO was estimated using DAF-FM diacetate NO indicator (D23844, Invitrogen) (Kojima *et al.*, 1999). Epidermal peels were loaded with 10 μM DAF-FM (in 10 mM KH_2PO_4, pH 7.4) in the dark at room temperature for 30 min. The peels were then washed twice for 10 min with 10 mM KH_2PO_4 (pH 7.4) under the same conditions. The peels were then immediately observed with a LSM510 META laser-scanning confocal microscope (Carl Zeiss) and appropriate excitation/detection settings (488/500–530 nm). All images were acquired using the same microscope settings. The intensity of DAF-FM fluorescence of individual guard cells was then estimated by image analysis software (Volocity 6.1.1), using the threshold values set during image acquisition and a minimum object size of 1 μm^2. In a control experiment, when epidermal peels were pretreated with SNP (2 mM, 30 min, 28°C), it dramatically increased subsequent guard cell DAF-FM fluorescence, while cotreatment with both SNP 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 (Supporting Information, Fig. S1).

Gas-exchange analyses

A portable system (Model GFS-3000; Heinz Walz GmbH, Effeltrich, Germany) with leaf cuvette was used to measure CO_2 exchange at 28°C and 60% relative humidity in attached leaves at 4–5 h into the light period. Light was provided through red and blue LEDs (Model 3055-FL, Heinz Walz GmbH). Gas flow rate was set to 750 μmol s^{-1} and impeller speed to step 7. For light response curves, net CO_2 assimilation rate (A_n) was measured at intervals over the range of 0–2000 PPFR, with CO_2 supplied at a concentration of 400 μmol C mol^{-1}. A_n was determined after 6 min at a particular PPFR. Stomatal conductance (g_s) was estimated as: 1.6 $(A_n)/(C_a - C_i)$, where C_a and C_i represent CO_2 concentrations in air and leaf intercellular space, respectively (Farquhar & Sharkey, 1982). For CO_2 response curves, A_n was measured at saturating irradiance (1600 PPFR), and with CO_2 supplied at a range of concentrations (with 2 min at each concentration) in the following sequence: 400, 50, 100, 200, 300, 400, 400, 600, 800, 1000 and 1200 μmol C mol^{-1}. The initial measure at 400 was not used and the following two repeated measures at 400 were averaged.

Quantitative real-time PCR

For each independent experiment, equal FWs of leaf from two individuals were combined and used for RNA extraction, following the method described by Vanessa *et al.* (2008). DNA was eliminated from RNA samples using RNase-free DNaseI. Extracted RNA had A_{260}/A_{280} ratios > 1.9. First-strand cDNA was synthesized using SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). Quantitative real-time PCR utilized SYBR Green (Invitrogen). The efficiency and specificity of each gene-specific primer pair (listed in Table S1) was confirmed beforehand. *EF1* was the internal reference gene used to normalize between samples. Three technical replicates were used per sample. All data are the means of five independent experiments.

Other methods

An oxyhemoglobin-based biochemical assay to measure leaf NO and a xylenol orange-based biochemical assay to measure leaf H_2O_2 were performed exactly as previously described (Cvetkovska & Vanlerberghe, 2012). To measure leaf water loss, leaves were detached from plants, weighed immediately and then incubated at 150, 500 or 900 PPFR. Leaves were then weighed periodically over 8 h. Statistical analyses were done using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Using biochemical assays, we measured leaf concentrations of NO and H_2O_2 in WT tobacco and two knockdown lines (RI9, RI29) with suppressed amounts of AOX. At their growth irradiance (150 PPFR), well-watered plants lacking AOX maintained significantly higher concentrations of NO and significantly lower concentrations of H_2O_2 than WT (Fig. 1). NO was 2.5-fold higher in RI9 and 2.4-fold higher in RI29 than WT (Fig. 1a). Conversely, H_2O_2 was only 43 and 51% of WT in RI9 and RI29, respectively (Fig. 1b).

As NO and H_2O_2 act as signal molecules in the control of stomatal opening and closing, we examined whether their altered amount in knockdown plants was impacting stomatal function. At their growth irradiance (150 PPFR), WT and knockdown plants showed no differences in g_s (Fig. 1c) or A_n (Fig. 1d). Further, there was no obvious difference between lines in the rate of FW loss from detached leaves incubated at 150 PPFR (Fig. S2a).

These results indicated that knockdown plants were able to maintain normal stomatal function at their growth irradiance (150 PPFR) despite the altered concentrations of NO and H_2O_2, suggesting potential compensating mechanisms. We therefore compared the stomata of well-watered WT and knockdown plants. First, we found no differences in stomatal density, epidermal cell density, or stomatal index between plant lines (Fig. S3). Next, epidermal peels were incubated in an 'opening buffer' under conditions that induce stomatal opening. After incubation, we found that stomatal pore area was *c.* 23% lower in

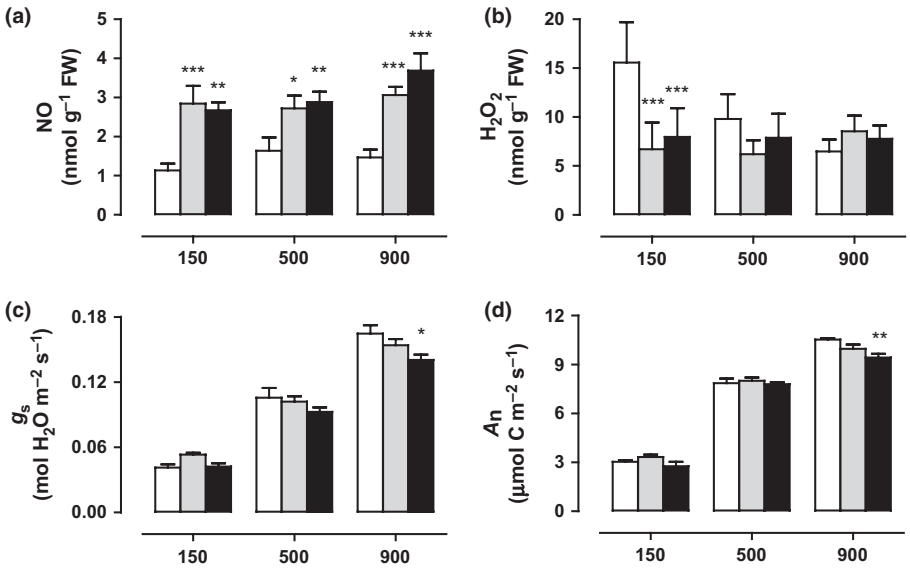

Fig. 1 Leaf concentration of nitric oxide (NO) (a), leaf concentration of H_2O_2 (b), leaf stomatal conductance (g_s) (c), and leaf net CO_2 assimilation rate (A_n) (d) of wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. These were well-watered plants grown at a photosynthetic photon fluence rate (PPFR) of 150 µmol m^{-2} s^{-1} (150 PPFR). Measurements were done in the light period from plants kept at 150 PPFR, plants transferred from 150 to 500 PPFR for 30 min, or plants subsequently transferred from 500 to 900 PPFR for an additional 30 min. WT, white bars; RI9, gray bars; RI29, black bars. Data are the means ± SE of three (g_s, A_n) or four (NO, H_2O_2) independent experiments ($n = 3$ or 4). Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare, at each irradiance, the WT with each transgenic line. Level of significant difference: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bars without an asterisk are not significantly different from the WT.

knockdowns than in WT plants (Fig. 2a). Guard cell area, pore length, and pore width were each also significantly reduced in the knockdowns (Fig. 2b–d). Nonetheless, stomatal aperture (pore width : length ratio), the usual measure of degree of stomatal opening, was similar between WT and knockdowns after incubation in the opening buffer (Fig. 2e).

Next, stomatal closing assays were used to establish the responsiveness of guard cells to signaling molecules known to promote closure. These signaling molecules included NO and H_2O_2 as well as SA and ABA. Epidermal peels preincubated with opening buffer were subsequently treated for 1 h with different concentrations of signal molecule and analyzed.

As expected, incubation of WT peels with increasing concentrations of NO (0, 0.1, 1, 5, 10 mM SNP) induced closure, defined by the decline in stomatal aperture (Fig. 3a). However, the knockdown lines were largely unresponsive to NO, with even the highest concentration resulting in little closure (Fig. 3a). After incubation in opening buffer, the pore area of both knockdown lines was significantly less than that of the WT (Fig. 3b) as a result of the differences in guard cell size (see earlier). However, owing to the differential response of stomatal aperture of WT and knockdown plants to NO, their pore areas were similar after treatment with 10 mM SNP (Fig. 3b).

Incubation of WT peels with increasing concentrations of H_2O_2 (0, 0.1, 0.5, 1.0, 5.0 mM) also induced closure, as seen by the drop in stomatal aperture (Fig. 3c). However, unlike the case with NO, the stomata of knockdown plants showed a similar decline in stomatal aperture in response to H_2O_2 as the WT. Hence, the pore area of the knockdowns remained significantly lower than WT, even at the highest H_2O_2 concentration (Fig. 3d).

The stomata of WT and knockdown lines also responded similarly to increasing concentrations of ABA (0.0, 0.05, 0.1, 1.0, 5 µM), which induced some closing even at the lowest concentration tested (Fig. 4a). Given the similar stomatal response across plant lines, the knockdowns maintained a significantly smaller pore area than WT across all but the highest ABA concentration tested (Fig. 4b). At 5 µM ABA, the pore area was still highest in the WT, although the difference between it and the knockdowns was no longer statistically significant.

Stomatal aperture also responded to SA, tested at concentrations of 0.0, 0.1, 1.0, 5.0, and 10 mM. All lines appeared to respond similarly to low concentrations of SA, while the stomatal aperture of WT declined more than in knockdowns at high SA (5.0 and 10.0 mM) (Fig. 4c). Hence, at low concentrations of SA (up to 1 mM), pore area was significantly higher in the WT than in both knockdowns, while at high concentrations of SA (5 and 10 mM), pore area no longer differed between lines (Fig. 4d).

Overall, the results suggested that AOX knockdowns with increased steady-state leaf concentrations of NO (Fig. 1a) were compensating for this, at least in part, through reduced sensitivity of their stomata to closure by NO (Fig. 3a). We next evaluated whether this change in stomatal signaling properties would impact stomatal responses to environmental cues. First, we examined responses to short-term irradiance shifts, where plants grown at 150 PPFR were transferred to 500 PPFR for 30 min, and then from 500 to 900 PPFR for another 30 min.

The 30 min irradiance treatments resulted in changes in NO and H_2O_2 across the plant lines (Fig. 1). H_2O_2 declined in the WT with each irradiance shift, while remaining relatively stable in the knockdowns. Hence, the large difference in H_2O_2 concentration between WT and knockdowns at their growth irradiance

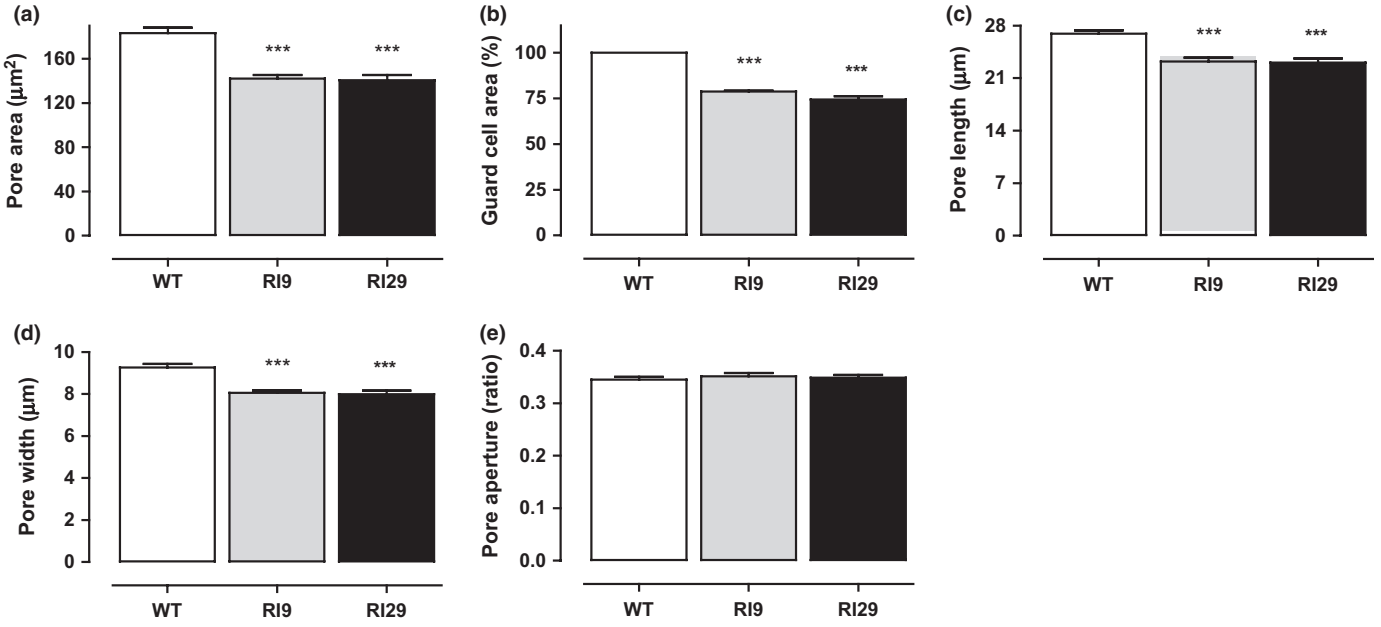

Fig. 2 Stomatal characteristics of wildtype (WT) tobacco (*Nicotiana tabacum*) and two transgenic lines (RI9, RI29) with reduced alternative oxidase. These were well-watered plants grown at a photosynthetic photon fluence rate (PPFR) of 150 μmol m^{-2} s^{-1} (150 PPFR). Stomatal pore area (a), guard cell area (b), pore length (c), pore width (d), and stomatal aperture (e) were measured as described in the Materials and Methods section following incubation in an opening buffer. WT, white bars; RI9, gray bars; RI29, black bars. At least 30 stomata were analyzed in each experiment and for each plant line and treatment. Data are means ± SE from 12 independent experiments ($n = 12$), except for panel (b) which is from three independent experiments ($n = 3$). Data were analyzed by one-way ANOVA followed by a Dunnett post-test to compare the WT with each transgenic line. Level of significant difference: ***, $P < 0.001$. Bars without an asterisk are not significantly different from WT.

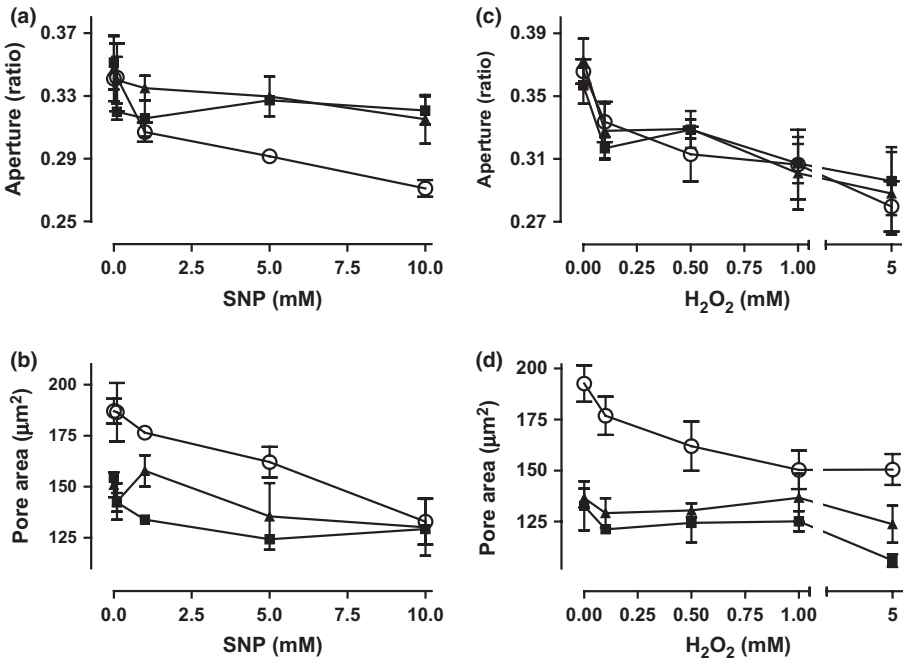

Fig. 3 Effects of nitric oxide (NO) (a, b) and H_2O_2 (c, d) on stomatal aperture (a, c) and stomatal pore area (b, d) of wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. WT, open circles; RI9, closed triangles; RI29, closed squares. Epidermal peels were treated to induce stomatal opening and then incubated with different concentrations of NO (supplied as sodium nitroprusside (SNP)) or H_2O_2 for 1 h, after which time stomatal aperture and pore area were measured, as described in the Materials and Methods section. At least 30 stomata were analyzed in each experiment and for each plant line and treatment. Data are the means ± SE from three independent experiments ($n = 3$). Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare, at each concentration, the WT with each transgenic line.

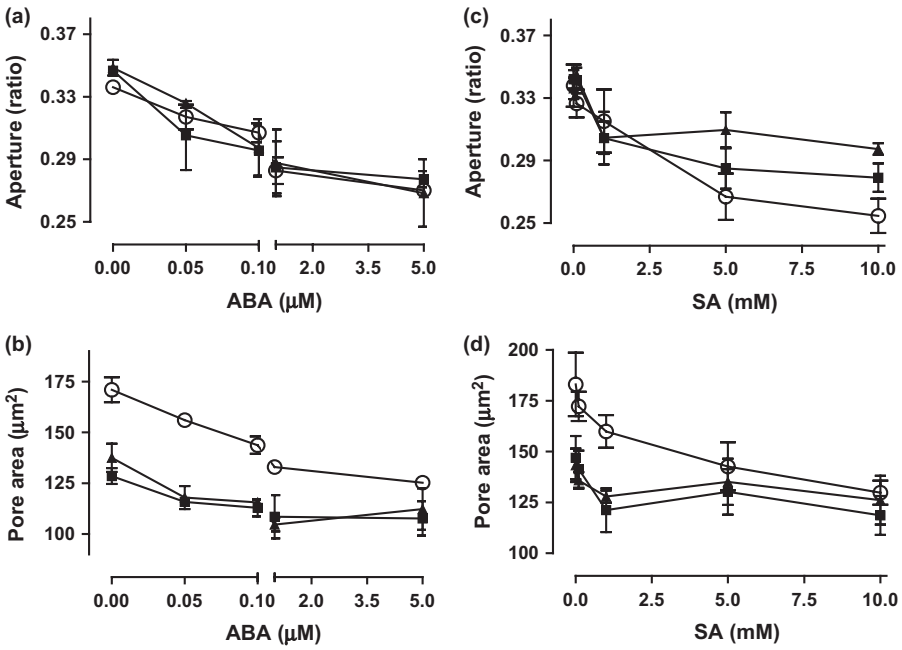

Fig. 4 Effects of ABA (a, b) and salicylic acid (SA) (c, d) on stomatal aperture (a, c) and stomatal pore area (b, d) of wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. WT, white circles; RI9, closed triangles; RI29, closed squares. Epidermal peels were treated to induce stomatal opening and then incubated with different concentrations of ABA or SA for 1 h, after which time stomatal aperture and pore area were measured, as described in the Materials and Methods section. At least 30 stomata were analyzed in each experiment and for each plant line and treatment. Data are the mean ± SE from three independent experiments ($n = 3$). Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare, at each concentration, the WT to each transgenic line.

was abolished at high irradiance (Fig. 1b). After the shift to 500 PPFR, NO increased 1.4-fold in WT and remained high after transfer to 900 PPFR (1.3-fold higher than at growth irradiance) (Fig. 1a). On the other hand, NO in knockdowns was stable after transfer to 500 PPFD, while increasing at 900 PPFR. This was particularly the case in RI29, where NO was 1.4-fold higher at 900 than at 150 PPFR. Nevertheless, the large significant difference in NO between the WT and knockdowns was maintained at all irradiance intensities (Fig. 1a).

In order to easily compare the relative changes in NO and H_2O_2 in response to irradiance, Fig. S4 normalizes the NO and H_2O_2 concentrations of each plant line at 150 PPFR to values of 1. This figure also includes summing the relative amount of these signal molecules (i.e. relative $NO + H_2O_2$). This analysis shows that while the shift to 900 PPFR resulted in a 14% decline in $NO + H_2O_2$ in WT (compared with 150 PPFR), conversely it saw an 18% increase in $NO + H_2O_2$ in knockdowns.

The shift to 500 PPFR resulted in a similar increase in A_n in each plant line (Fig. 1d). In each line, A_n increased further at 900 PPFR but was now slightly lower in RI9 than in WT and significantly lower in RI29 than in WT. Changes in g_s mirrored the changes in A_n (Fig. 1c). g_s was similar across lines at 500 PPFR (albeit slightly lower in knockdowns) while at 900 PPFR, g_s was significantly lower in RI29 than in WT. These results were corroborated by a comparison of light response curves (0–2000 PPFR). At 900 PPFR and higher irradiances, A_n was lower in RI29 than in WT, while RI9 showed an intermediate response (Fig. S5a). Similarly, g_s was lower in RI29 than in WT at high irradiances, with RI9 again showing an intermediate response (Fig. S5b).

The lower A_n in knockdowns at 900 PPFR and above suggested either a stomatal or biochemical limitation of photosynthesis in the plants lacking AOX. To distinguish between these possibilities, we measured A_n as a function of C_i under saturating light (1600 PPFR). The A_n/C_i curves were indistinguishable between plant lines, evidence that the differences in A_n between WT and knockdowns at high PPFR results from a stomatal limitation in knockdowns (Fig. S6).

These analyses were supplemented by examining FW loss of detached leaves. While no differences were evident between lines at 150 PPFR (see earlier), WT leaves tended to dry out slightly faster than the knockdowns at 500 PPFR (Fig. S2b) and this effect became more pronounced at 900 PPFR (Fig. S2c).

Besides examining short-term responses of stomata to irradiance, we also evaluated a longer-term response of stomata to environmental cues by subjecting plants growing at 150 PPFR to a progressive decline in water availability. This was done by ceasing irrigation of plants for up to 4 d. We have previously shown that this treatment reduces the leaf relative water content (RWC) of each plant line from *c.* 88% in well-watered plants (day 1, 1 d after last watering) to 79% in drought-stressed plants (day 4, 4 d after last watering) (Wang & Vanlerberghe, 2013).

In well-watered plants grown at 150 PPFR, there were significant differences in the amount of both NO and H_2O_2 in the two knockdown lines compared with the WT (Fig. 1). However, after 4 d of drought, the NO amount at 150 PPFR was similar between lines as a result of a large increase in NO in WT but only a modest increase in knockdown lines, compared with well-watered plants (Fig. 5a). The amount of H_2O_2 in drought-stressed plants at 150 PPFR was also now similar across lines, in this case as a result of a large decline in WT, combined with increase in the knockdowns, compared to well-watered plants (Fig. 5b). Despite these changes, g_s and A_n at 150 PPFR did not differ across plant lines (Fig. 5c,d), similar to the case in well-watered plants.

Epidermal peels were used to determine stomatal aperture and pore area of both well-watered and drought-stressed plants (Fig. 6). In this case, the peels were analyzed directly, without any preincubation. Nonetheless, the aperture of well-watered plants (day 1) was similar to that of peels first incubated in opening

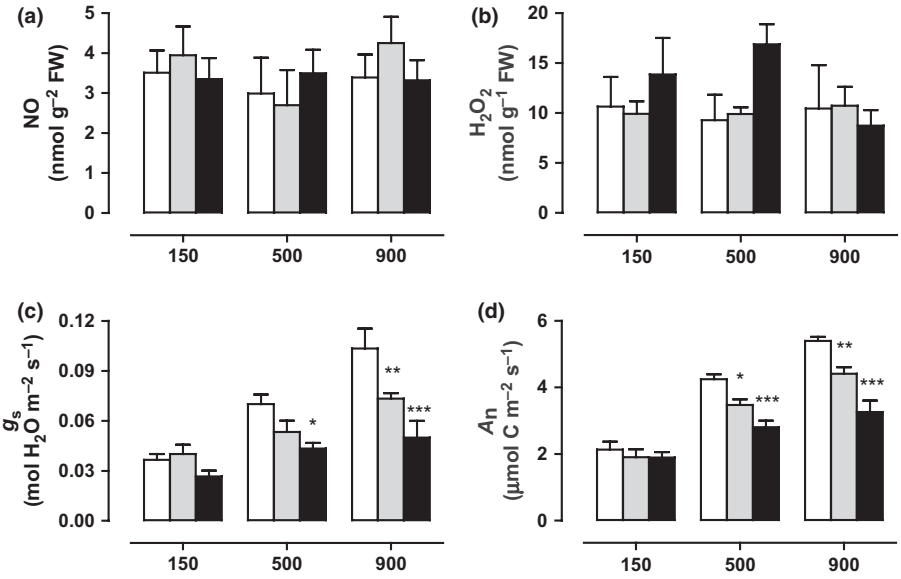

Fig. 5 Leaf concentration of nitric oxide (NO) (a), leaf concentration of H_2O_2 (b), leaf stomatal conductance (g_s) (c), and leaf net CO_2 assimilation rate (A_n) (d) of wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. These were drought-stressed plants grown at a photosynthetic photon fluence rate (PPFR) of 150 µmol m^{-2} s^{-1} (150 PPFR). Measurements were done in the light period from plants kept at 150 PPFR, plants transferred from 150 to 500 PPFR for 30 min, or plants subsequently transferred from 500 to 900 PPFR for an additional 30 min. WT, white bars; RI9, gray bars; RI29, black bars. Data are the means ± SE of three (g_s, A_n) or four (NO, H_2O_2) independent experiments ($n = 3$ or 4). Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare, at each irradiance, the WT with each transgenic line. Level of significant difference: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bars without an asterisk are not significantly different from WT.

buffer (compare Fig. 2e with Fig. 6a). As before, there was no significant difference in the aperture between knockdown lines and the WT (Fig. 6a) and hence the pore area of knockdowns was significantly lower than that of the WT as a result of the smaller guard cell size of knockdowns (Fig. 6b). As expected, stomatal aperture and pore area of WT plants decreased progressively between day 1 and day 4 as drought severity increased. Stomatal aperture in the knockdown lines acted similarly to the WT throughout the 4 d time course and, as such, these plants tended to maintain their slightly lower pore area, relative to WT (Fig. 6). This result was significant on all days comparing WT with RI29 and on 2 d (day 1 and day 3) comparing WT with RI9.

To study the possible interactive effects of drought and irradiance on stomatal responses, we shifted day 4 drought-stressed plants at 150 PPFR to higher irradiances, similar to the experiment on well-watered plants. In drought-stressed plants, the shifts to higher irradiance (30 min at 500 PPFR followed by another 30 min at 900 PPFR) had little impact on leaf NO and H_2O_2 concentrations and no significant differences were found between lines (Fig. 5a,b). However, large differences in A_n and g_s were now evident between lines at both 500 and 900 PPFR (Fig. 5c,d). Lower A_n in the knockdowns at high irradiance again suggested either a stomatal or biochemical limitation of photosynthesis, so we again examined A_n as a function of C_i. Unlike with well-watered plants, the A_n of drought-stressed knockdown plants remained much lower than that of WT plants, even at equivalent C_i (data not shown). These results suggest a biochemical rather than a stomatal limitation of photosynthesis in drought-stressed plants lacking AOX. As a biochemical limitation of photosynthesis is likely to generate additional feedback signaling effects on stomata (e.g. as a result of decreased CO_2 demand), this experiment was not pursued further.

We examined the expression of select genes whose expression has been previously linked in *N. tabacum* to changes in mitochondrial function or changes in the concentration of NO or H_2O_2. The transcript of a gene encoding catalase and previously identified as being specifically induced in tobacco by NO (Zago *et al.*, 2006) had similar relative abundance across plant lines and in both well-watered and drought-stressed plants (data not shown). The relative transcript abundance of a gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase, previously identified in tobacco suspension cells as associated with mitochondrial dysfunction (Maxwell *et al.*, 2002) was reduced by drought, but again was similar across lines, in both well-watered and drought-stressed plants (data not shown). In both tobacco suspension cells (Maxwell *et al.*, 1999) and *Arabidopsis* leaves (Giraud *et al.*, 2008), it was shown that reduced AOX results in increased expression of genes encoding pathogenesis-related (PR) proteins. Similarly, we found that *PR1a* transcript abundance in well-watered plants (day 1) was *c.* eightfold higher in the knockdowns than in the WT (Fig. 7a). This difference in transcript abundance between WT and knockdown plants was magnified further in response to drought stress (day 4) (Fig. 7a). Despite the clear trends, the relative expression of PR-1 was also highly variable between experiments compared with each of the other genes examined. Reduced mitochondrial function as a result of hypoxia typically increases the amount of fermentation enzymes (Bailey-Serres *et al.*, 2012). We found that transcripts encoding pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were perhaps slightly elevated by drought, but there was little

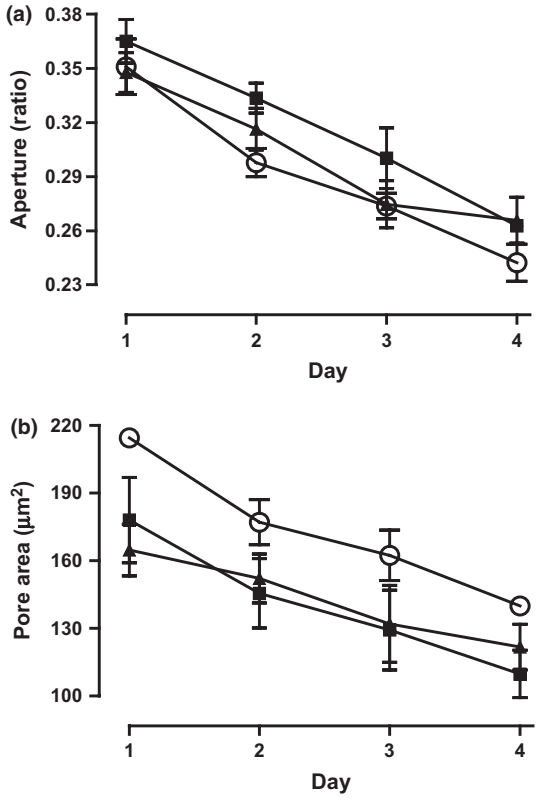

Fig. 6 Stomatal aperture (a) and stomatal pore area (b) of wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. Well-watered plants were not watered for 1–4 d, with measurements taken each day. WT, open circles; RI9, closed triangles; RI29, closed squares. At least 30 stomata were analyzed in each experiment and for each plant line and treatment. Data are the means ± SE from three independent experiments ($n = 3$). Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare, at each time point, the WT with each transgenic line.

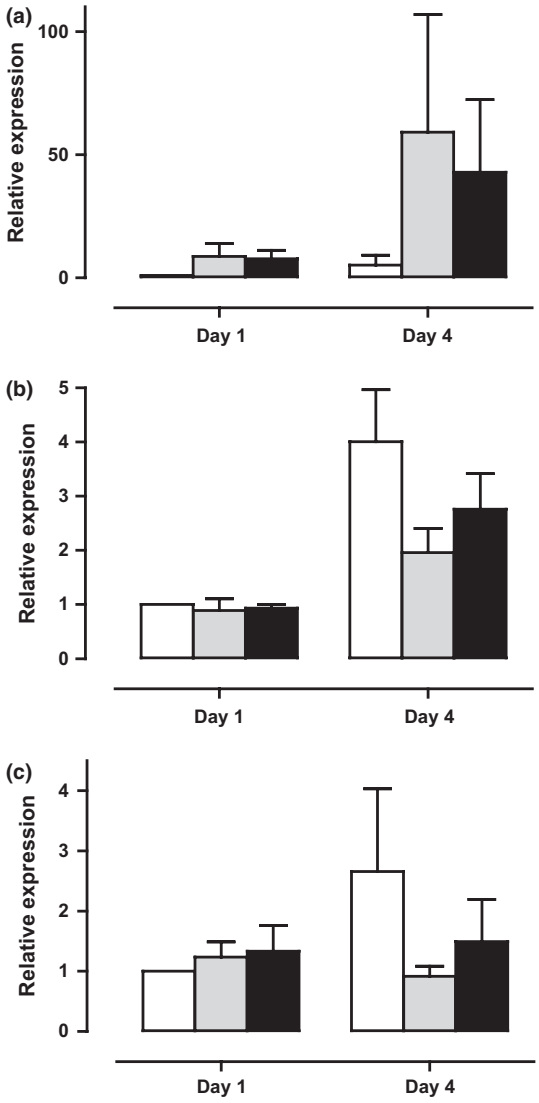

Fig. 7 Leaf relative transcript abundance for genes encoding pathogenesis-related protein 1a (a), pyruvate decarboxylase (b), and alcohol dehydrogenase (c) in wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. WT, white bars; RI9, gray bars; RI29, black bars. Relative transcript abundance was measured by quantitative PCR from both well-watered (day 1) and drought-stressed (day 4) plants. Data are normalized to the well-watered WT sample, which was set to 1. Data are the means ± SE from five independent experiments ($n = 5$). Data were analyzed by two-way ANOVA followed by a Bonferroni post-test to compare, at each time point, the WT with each transgenic line.

difference between WT and knockdowns, in either well-watered or drought-stressed plants (Fig. 7b,c).

Confocal microscopy and the NO-specific fluorescent probe DAF-FM were used to directly evaluate the NO amount of guard cells in well-watered plants. This analysis showed that DAF-FM fluorescence (a measure of NO amount) was much higher in knockdown that in WT guard cells (Fig. 8).

Discussion

Knockdown of alternative oxidase induces the 'stress state' of signaling molecule pools in tobacco leaf

Our analyses showed that, under nonstress conditions, AOX knockdown plants maintained higher NO and lower H_2O_2 concentrations than WT plants. Significantly, the concentrations in AOX knockdown plants fell within the range normally seen in WT plants during disparate stresses (dotted lines in Fig. 9). In other words, knockdown of AOX induced the 'stress level' of these signaling molecules, even under nonstress conditions. After application of stress (increased irradiance, drought, or the combination of both), H_2O_2 and NO concentrations were now comparable between WT and AOX knockdown plants. The exception to this was NO concentration in response to the short-term irradiance stress. This stress maintained, and perhaps even exaggerated further, the difference in NO concentration between WT and knockdown plants.

In broad terms, the results suggest that AOX is important to maintaining H_2O_2 homeostasis under nonstress conditions, but not necessarily during stress. The reduced H_2O_2 concentration in nonstressed knockdown plants compared with WT plants seems paradoxical, as these plants exhibit increased mitochondrial O_2^- (Cvetkovska & Vanlerberghe, 2012, 2013), providing substrate

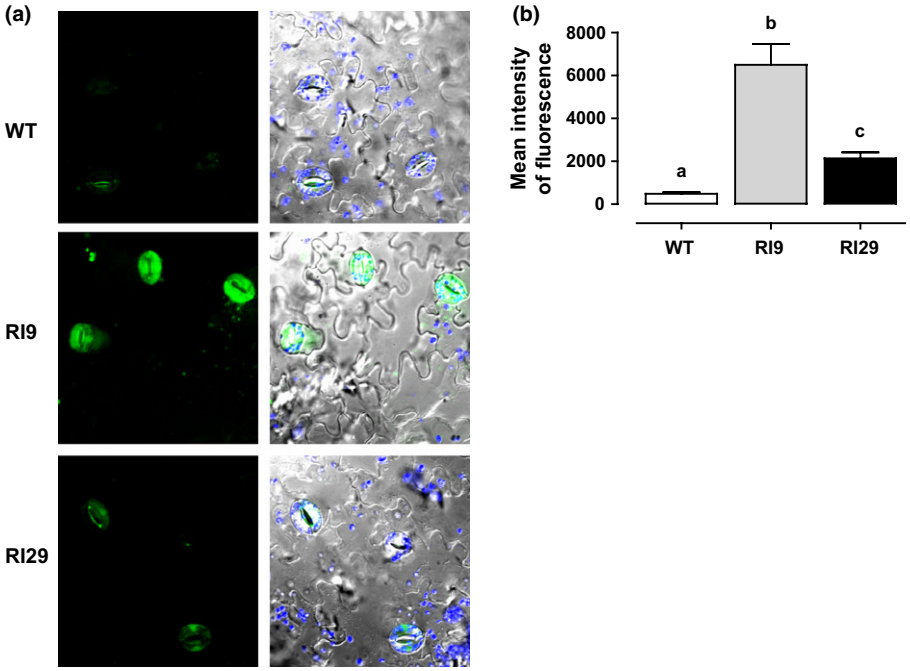

Fig. 8 Nitric oxide amount in guard cells of wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. These were well-watered plants grown at a photosynthetic photon fluence rate (PPFR) of 150 μmol m^{-2} s^{-1} (150 PPFR). (a) Representative guard cell images. The left-hand panels show DAF-FM fluorescence (green color) while the right-hand panels show combined DAF-FM fluorescence (green) and Chl autofluorescence (false coloration blue). (b) The total DAF-FM fluorescence associated with individual guard cells. WT, white bars; RI9, gray bars; RI29, black bars. The data in (b) are the means ± SE of three independent experiments and the analysis of *c*. 50 different stomata of each plant line. Data were analyzed by one-way ANOVA followed by a multiple comparison test. Bars not sharing a common letter (a, b, c) are significantly different from one another ($P < 0.05$).

for conversion to H_2O_2. Nonetheless, the H_2O_2 concentration is governed by both its rate of synthesis and its degradation, and knockdown of AOX in both tobacco and *Arabidopsis* is reported to induce multiple H_2O_2-scavenging systems under normal growth conditions (Amirsadeghi *et al.*, 2006; Giraud *et al.*, 2008). In tobacco this may overcompensate for the lack of AOX, in terms of H_2O_2 scavenging (Amirsadeghi *et al.*, 2006; this study). Conversely, overexpression of AOX in tobacco can suppress H_2O_2-scavenging systems, resulting in elevated H_2O_2 (Pasqualini *et al.*, 2007).

Our results also indicate that AOX is important to maintaining NO homeostasis under both nonstress and short-term irradiance stress conditions, but not necessarily during drought stress or when drought is combined with increased irradiance. Hence, it is interesting that stomatal function was not compromised in knockdowns during drought but was compromised by the irradiance stress (see the following section).

Knockdown of AOX alters stomata and impairs stomatal responses to increased irradiance

H_2O_2 and NO are implicated as key signaling molecules in the signal transduction paths responsible for stomatal movements (see the Introduction section). As AOX knockdown plants had altered concentrations of these molecules, including much higher NO in guard cells, we examined stomatal function. When measured under normal growth conditions, knockdowns had similar g_s, A_n, stomatal density, stomatal index, stomatal aperture, and rate of water loss from detached leaves as the WT. However, their guard cells were significantly smaller than those of WT plants (meaning that knockdowns had a smaller measureable pore area for gas exchange, despite the similar stomatal aperture) and were less responsive to NO as a signal for closure.

The reduced responsiveness of the knockdown stomata to NO is presumably to compensate for the constitutive high concentration of NO present in the guard cells of these leaves. We hypothesized that knockdowns would similarly compensate for their constitutive low H_2O_2 by increasing their responsiveness to H_2O_2. However, we found no evidence for this, suggesting that NO concentration prevails over H_2O_2 in terms of being a key signal for closure. This interpretation aligns with prevailing models of stomatal signaling for closure, where NO acts downstream of H_2O_2 (Bright *et al.*, 2006; Srivastava *et al.*, 2009; He *et al.*, 2013).

Salicylic acid is also described as a signaling molecule capable of inducing stomatal closure, such as during plant–pathogen interactions (Mateo *et al.*, 2004; Melotto *et al.*, 2006). The AOX knockdowns were less responsive to SA as a signal for stomatal closure and also showed elevated abundances of *PR1a* transcript. These findings hint that SA (like NO) may be constitutively higher in knockdowns. Interestingly, the regulation of *PR1a* expression by SA involves the establishment of a more reduced cellular redox state (high NADPH) and enhanced *S*-nitrosylation of a key transcription factor (Spoel & Loake, 2011; Fu & Dong,

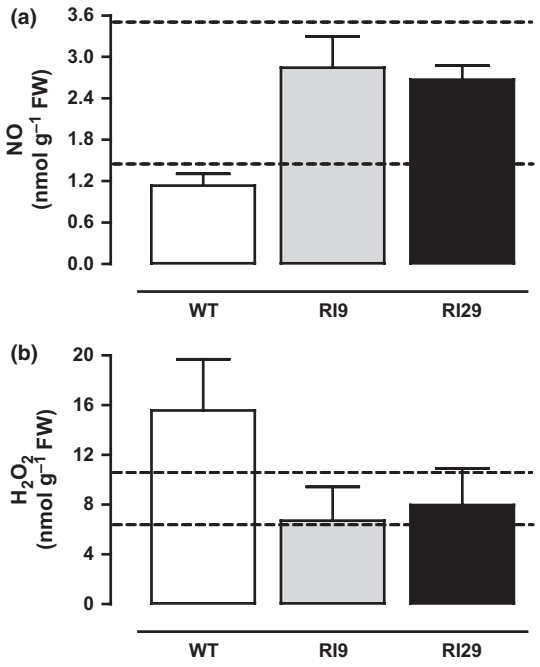

Fig. 9 Leaf concentration of nitric oxide (NO) (a) and H_2O_2 (b) in wildtype (WT) tobacco (*Nicotiana tabacum*) and two knockdown lines (RI9, RI29) with reduced alternative oxidase. WT, white bars; RI9, gray bars; RI29, black bars. The bars show the NO and H_2O_2 concentrations measured at a photosynthetic photon fluence rate (PPFR) of 150 μmol m^{-2} s^{-1} (150 PPFR) in well-watered plants that had been grown at 150 PPFR (i.e. nonstress conditions). The lower dotted lines in (a) and (b) represent the concentrations of NO and H_2O_2, respectively, measured in WT plants following an irradiance stress treatment (i.e. 30 min at 500 PPFR followed by another 30 min at 900 PPFR). The upper dotted lines in (a) and (b) represent the concentrations of NO and H_2O_2, respectively, measured in WT plants after 4 d of drought stress. (Note that we have not shown a third dotted line, to represent WT plants after the combined drought and irradiance stress, as NO and H_2O_2 concentrations in both these cases were almost identical (just slightly lower) to those found in the drought-alone treatment. This model figure is meant to illustrate that the concentrations of NO and H_2O_2 found in nonstressed RI9 and RI29 plants fall within the range normally seen in WT plants during disparate stresses. These data are from Figs 1 and 5.

2013). Cellular redox state is conceivably more reduced in the AOX knockdowns, and the high NO in these plants might promote *S*-nitrosylation reactions. To examine whether altered cellular redox state is impacting gene expression in the AOX knockdowns, we determined the transcript abundance of genes encoding the fermentation enzymes PDC and ADH. The expression of these genes is enhanced by hypoxia/anoxia, and while the mechanism for these inductions is not fully understood, it may result from changes in cellular redox state (Bailey-Serres *et al.*, 2012). However, while PDC and ADH expression did increase slightly in response to drought, transcript abundances in each of the plant lines were similar under both well-watered and drought conditions. Despite the limited analysis, our data indicate that some stress-related genes, in this case the SA-responsive *PR1a* gene, are altered in plants lacking AOX. It would be interesting to further examine the underlying mechanism for this change in *PR1* expression, which was also seen in both tobacco suspension cells (Maxwell *et al.*, 1999) and *Arabidopsis* leaves (Giraud *et al.*, 2008) lacking AOX.

Abscisic acid is a dominant signal for stomatal closure in response to drought, and ABA-induced closure is reported to occur by both NO-dependent and NO-independent pathways (Lozano-Juste & León, 2009; Hancock *et al.*, 2011; Daszkowska-Golec & Szarejko, 2013). The robustness of the ABA signal may explain why both the responsiveness of knockdown stomata to ABA in closure assays and the response of knockdown stomata to progressive drought stress were both similar to WT, despite the altered dynamics of NO and H_2O_2 in these plants.

The stomatal deficiency of AOX knockdown plants was only revealed in response to the short-term irradiance stress (shifts to higher PPFR). Under normal steady-state growth conditions or during a longer-term stress (4 d of increasing drought severity), the plants were able to compensate for this deficiency, probably by multiple mechanisms including both developmental changes (i.e. guard cell size) and physiological/biochemical changes (i.e. altered responsiveness to some signal molecules). The inability of stomata of knockdowns to respond normally to the irradiance stress may be down to the rapidity of this treatment, which resulted in significant short-term changes in H_2O_2 and NO. It is probably too simplistic to directly compare the changes in these signal molecules with stomatal responses, and such an analysis is further complicated by the altered responsiveness of the stomata of knockdowns to NO. Nonetheless, the reduced g_s of knockdown plants compared with the WT after transfer to 900 PPFR may be explained, at least in part, by the altered response of the H_2O_2 and NO pools. While the sum of H_2O_2 + NO declined in the WT after transfer to 900 PPFR (primarily because of the drop in H_2O_2), the sum of these pools increased in knockdowns (because of some increase in both H_2O_2 and NO). Hence, these 'closing signals' were declining in the WT in response to higher PPFR while conversely increasing in the knockdowns.

Photosynthesis has been examined in *Arabidopsis aox1a* mutants and knockdowns (Strodtkötter *et al.*, 2009; Zhang *et al.*, 2010; Florez-Sarasa *et al.*, 2011; Yoshida *et al.*, 2011; Gandin *et al.*, 2012). To our knowledge, stomata were not examined in detail. One study did report that g_s was similar between the WT and *aox1a* mutant, while A_n/C_i curves showed a slightly reduced A_n in *aox1a*, suggestive of a biochemical rather than a stomatal limitation of photosynthesis in the mutant (Gandin *et al.*, 2012). Our finding of a stomatal defect in tobacco lacking AOX1a may be the result of species differences or may indicate that a stomatal defect in the *Arabidopsis aox1a* mutant has not been revealed, because of the particular growth conditions or experiments performed.

Does composition of the mitochondrial electron transport chain impact stomatal function through changes in ROS and RNS dynamics?

Several disparate mutants with impaired complex I are reported to display altered stomatal function. These include an *Arabidopsis* DEXH box RNA helicase mutant (He *et al.*, 2012), an

Arabidopsis RNA editing mutant (Yuan & Liu, 2012), and the CMSII mutant of tobacco (Djebbar *et al.*, 2012). Each of the mutants displays altered ROS dynamics, suggesting a link between ROS and stomatal function, reminiscent of the current study. However, plants with defective complex I all showed enhanced stomatal closure compared with WT during drought, which improved their 'drought tolerance' by reducing water loss. In the AOX knockdowns, however, changes in stomatal aperture in response to drought were similar to WT plants, water loss from detached leaves of drought-stressed knockdowns was similar to the WT (data not shown), and all plant lines maintained similar progressive changes in leaf RWC over a 10 d drought treatment (Wang & Vanlerberghe, 2013). These observations suggest normal stomatal function and rates of water loss from AOX knockdown plants under drought conditions. The cause of this difference between complex I and AOX mutants is unknown, but may relate to NO. Our work suggests that NO supersedes ROS in terms of controlling stomatal function, and finds that NO concentrations are similar in drought-stressed WT and AOX knockdowns. It would be interesting to examine the NO concentration in the different complex I mutants under nonstress and stress conditions.

Alternative oxidase may play a unique role in guard cell NO homeostasis

Guard cells maintain a high overall respiratory activity (Vani & Raghavendra, 1994), but relatively little else is known regarding respiration in these specialized cells. It was recently reported that several disparate mutants with increased SA display reduced stomatal aperture, which was attributed to increased ROS from a source other than NADPH oxidase (Miura *et al.*, 2013). Interestingly, the study also found high abundances of *AOX1a* transcript in guard cells and, through cluster analysis of several microarray datasets, identified *AOX1a* as a gene of interest in the regulation of stomatal movement.

Our results show that reduced AOX dramatically increases guard cell NO, impacting stomatal function. We propose the following hypothetical working model, whereby AOX promotes guard cell NO homeostasis in two related ways. First, AOX may have a general role to prevent over-reduction of ETC components, which might otherwise promote nitrite reduction to NO. There is already evidence that this is a role of AOX in tobacco mesophyll cells (Cvetkovska & Vanlerberghe, 2012, 2013). Secondly, as guard cells generate transient high concentrations of NO to act as a key signal molecule controlling stomatal aperture, these cells may be particularly prone during these transients to inhibition of cyt oxidase. NO is a reversible inhibitor of cyt oxidase at nanomolar concentration (Brown, 2001; Cooper, 2002). Partial inhibition of cyt oxidase and electron flow by the high NO being generated for signaling could then cause over-reduction of the ETC, favoring the generation of additional NO by the respiratory chain itself, and more cyt oxidase inhibition (i.e. a vicious circle promoting increased cyt oxidase inhibition). The additional NO, and probably O_2^-, from the respiratory chain could then disrupt normal guard cell signaling. However, unlike cyt oxidase, AOX is not inhibited by NO (Millar & Day, 1996), meaning that it could act to maintain electron flow during any transient period of cyt oxidase inhibition. AOX would essentially act as an auxiliary respiratory pathway in cells normally subject to periodic inhibition of cyt oxidase.

The location and biosynthetic pathway of NO synthesis for guard cell signaling are unknown. Nonetheless, given the ease with which NO crosses membranes, even an extramitochondrial location of synthesis could elevate mitochondrial NO and inhibit cyt oxidase, necessitating the need for AOX. There is even some speculation that the mitochondrial ETC is the source of NO for stomatal signaling (Wang *et al.*, 2010). If this is the case, the necessity of having AOX as an auxiliary respiratory pathway seems even more apparent. In *Arabidopsis*, AOX expression is increased by exogenous ABA (Ghassemian *et al.*, 2008; Giraud *et al.*, 2009; Liu *et al.*, 2010; He *et al.*, 2012; Miura *et al.*, 2013) and a molecular link between ABA and *AOX1a* expression is defined (Giraud *et al.*, 2009). In guard cells, this molecular link may ensure that sufficient AOX is present to maintain electron flow when ABA elevates NO concentration to promote stomatal closure. Further experimentation, in particular using isolated guard cells with different amounts of AOX, could be used to test this model.

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Supporting Information

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

Fig. S1 The effect of pretreatment of epidermal peels with an NO donor and/or NO scavenger on the subsequent DAF-FM fluorescence associated with tobacco guard cells.

Fig. S2 Fresh weight loss from detached leaves incubated at different irradiances, in WT tobacco and knockdown lines with reduced AOX.

Fig. S3 Stomatal density, epidermal cell density, and stomatal index of well-watered and drought-stressed plants of WT tobacco and knockdown lines with reduced AOX.

Fig. S4 Relative changes in NO, H_2O_2, and $NO + H_2O_2$ in response to short-term changes in irradiance, in WT tobacco and knockdown lines with reduced AOX.

Fig. S5 A_n rate and g_s at measurement irradiances of 0–2000 PPFR for WT tobacco and knockdown lines with reduced AOX.

Fig. S6 A_n rate as a function of C_i for WT tobacco and knockdown lines with reduced AOX.

Table S1 Primer sequences used for quantitative PCR

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