

Mitochondrial alternative oxidase acts to dampen the generation of active oxygen species during a period of rapid respiration induced to support a high rate of nutrient uptake

Justine Y. H. Yip and Greg C. Vanlerberghe*

Division of Life Science and Department of Botany, University of Toronto at Scarborough, 1265 Military Trail, Scarborough, Ontario M1C 1A4, Canada

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

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When wild type (wt) tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) suspension cells were grown under phosphate (P) limitation, they contained large amounts of mitochondrial alternative oxidase (AOX). When these cells were resupplied with P, there was a large, immediate and sustained stimulation of respiration to support a period of rapid P uptake. Two lines of evidence suggest that the abundant level of AOX present in wt cells contributed to this stimulated rate of respiration. First, when P-limited transgenic antisense tobacco cells (AS8) lacking AOX were resupplied with P, the stimulation of respiration was much less dramatic even though these cells displayed similar rates of P uptake. Second, while the stimulated rate of respiration in AS8 cells was insensitive (as expected) to the AOX inhibitor *n*-propyl gal-

late (nPG), much of the stimulated rate of respiration in wt cells could be inhibited by nPG. Given the non-phosphorylating nature of AOX respiration, wt cells required higher rates of electron transport to O₂ than AS8 cells to support similar rates of P uptake. The utilization of AOX by wt cells during P uptake was apparently not occurring because the cytochrome (Cyt) pathway alone could not fully support the rate of P uptake, as the respiration of cells lacking AOX (either untreated AS8 cells or wt cells treated with nPG) supported similar rates of P uptake as wt cells with abundant AOX. Rather, we provide *in vivo* evidence that the utilization of AOX during the period of high respiration supporting P uptake was to dampen the mitochondrial generation of active oxygen species (AOS).

Introduction

Plants have two paths of mitochondrial electron transport from ubiquinone (Q) to O₂ (Lambers 1997). Electron transfer through the cytochrome (Cyt) pathway is coupled to ATP synthesis and the terminal oxidase (Cyt oxidase) is inhibited by cyanide (CN). Alternatively, electron flow from Q to alternative oxidase (AOX) is not coupled to ATP production (for recent reviews on AOX, see Vanlerberghe and McIntosh 1997, Wagner and Moore 1997, Simons and Lambers 1999). We recently showed that mitochondrial AOX protein and the capacity for CN-resistant respiration are dramatically induced in wild type (wt) tobacco suspension cells when grown under phosphate (P) limitation and that antisense (AS8) cells unable to induce AOX under these conditions (because of the presence of a constitutively expressed antisense transgene) have altered growth, morphol-

ogy, cellular composition, patterns of respiratory carbon flow and rates of generation of AOS (Parsons et al. 1999). These results suggest that induction of non-phosphorylating AOX respiration (like induction of adenylate and inorganic phosphate [P_i]-independent pathways in glycolysis; Plaxton 1996) is an important plant metabolic adaptation to P limitation. By preventing severe respiratory restriction, AOX acts to prevent both redirections in carbon metabolism and the excessive mitochondrial generation of harmful AOS (Parsons et al. 1999).

P_i uptake across the plant plasma membrane is an energy-dependent process postulated to occur by a proton/P_i symport mechanism (see recent reviews by Schachtman et al. 1998, Raghothama 1999). Hence, uptake is viewed to depend upon the activity of a plasma membrane H⁺-ATPase

Abbreviations – AOS, active oxygen species; AOX, alternative oxidase; Cyt, cytochrome; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; nPG, *n*-propyl gallate; P, phosphate; P_i, inorganic phosphate; Q, ubiquinone; SHAM, salicylhydroxamic acid; wt, wild type.

that pumps protons out of the cell to maintain the proton motive force driving P_i uptake. Studies have shown that plant cells (roots or suspension cultured cells) grown under P limitation display an enhanced capacity for P uptake, which appears to be primarily because of an increase in the total number of transport molecules (Muchhal and Raghothama 1999 and references therein). Hence, the resupply of P to P-limited cells results in high rates of P uptake and studies have shown that this rapid uptake is accompanied by a dramatic stimulation of respiratory O_2 consumption, reflecting the additional energy demand required to support increased H^+ -ATPase activity (Gauthier and Turpin 1994, Weger 1996, Sakano et al. 1998).

Here, we investigate the potential role of AOX in the high rate of respiration seen to support rapid P uptake by P-limited tobacco cells. We utilize both wt suspension cells and transgenic cells which lack AOX because of the presence of an antisense AOX transgene.

Materials and methods

Plant material and growth conditions

The suspension cells used were derived from leaves of wt or transgenic tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) and had been in culture for approximately 5 years prior to this study (Vanlerberghe et al. 1994). The transgenic cells (AS8) constitutively express an antisense construct of the nuclear gene *Aox1*, which encodes a tobacco AOX. As a result, these cells lack any detectable AOX protein, even when grown under P limitation (Parsons et al. 1999).

Cells were grown on a rotary shaker (140 rpm and 28°C) and were subcultured every 7 days by 14-fold dilution in fresh growth medium. For experiments, cells grown in standard growth medium (which contains 2.5 mM KH_2PO_4 as the sole source of P) for 7 days were transferred to fresh low P growth medium containing only 0.25 mM KH_2PO_4 (Parsons et al. 1999).

Cell respiratory characteristics

Suspension cells (adjusted to 1.5–3.0 mg dry weight ml^{-1} in their culture medium) were placed in an oxygen electrode cuvette at 28°C. Steady rates of respiratory O_2 uptake were determined after about 2–4 min. Phosphate (0.5 mM KH_2PO_4) and inhibitors of Cyt oxidase (1 mM KCN) and AOX (2 mM salicylhydroxamic acid [SHAM] or 20 μM nPG) were then added as described in the figure legends. Throughout this paper, the term ‘AOX capacity’ refers to the oxygen uptake in the presence of CN which is sensitive to SHAM.

Phosphate uptake

Cells grown in low P medium for 5 days were resupplied with P (0.5 mM KH_2PO_4) and the rate of P uptake by the cells was determined by monitoring the disappearance of P from the medium over time. Samples for P determination were taken every 10–20 min between 20 and 100 min after

P addition to the cells. Over this time period, we observed linear rates of disappearance of P from the medium. P in the medium was measured by the method of Ames (1966). Phosphate uptake was determined under standard growth conditions (140 rpm, 28°C) except that in some cases cells were treated with inhibitors (1 mM KCN or 20 μM nPG) 2 min prior to addition of P.

Analysis of AOS

To examine the in vivo generation of H_2O_2 over time with high sensitivity, we used the cell-permeable probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, USA). The method used was similar to that described previously for these suspension cells (Parsons et al. 1999). Briefly, cells grown in low P medium for 5 days were taken from culture, washed two times in modified growth medium (half strength, minus KH_2PO_4 , pH 5.0) and then resuspended in the modified growth medium to a density of ~ 4 mg dry weight ml^{-1} . Cells were then incubated under standard growth conditions (140 rpm, 28°C) for 30 min, followed by the addition of 20 μM DCFH-DA (from a 20 mM stock in 100% ethanol). In some cases, 0.5 mM KH_2PO_4 was added immediately prior to addition of the DCFH-DA. In either case, an aliquot of cells (0.5 ml) was taken just following addition of the DCFH-DA, immediately mixed with KCN (5 mM final concentration) to inhibit peroxidase activity and rapidly frozen in liquid N_2 . Further samples of cells were then taken every 7.5 min for a 30-min period.

For further processing, the cell samples were thawed, centrifuged (16000 g, 3 min) and the supernatant diluted 10-fold with dH_2O . Fluorescence of the diluted sample was then measured immediately with a spectrofluorometer (Hitachi F-4000, Tokyo, Japan) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Using this method, we found that cell samples taken immediately after the addition of DCFH-DA yielded negligible fluorescence, that fluorescence yield increased linearly with time over the 30-min period and that no fluorescence over time was generated in the absence of either the cells or the DCFH-DA.

Fluorescence yield was normalized for a cell density of 4 mg dry weight ml^{-1} . Each experimental treatment was repeated four times with different subcultures of cells, each time generating similar results. Then, data for each time point were averaged and linear regression analysis was used to determine the rate of fluorescence increase over the 30-min period.

Other methods

Aliquots of cells were washed twice with water, frozen and lyophilized to determine cell dry weight. Cell viability was determined by microscopic observation of cells treated with Evans blue, which accumulates in dead cells as a blue protoplasmic stain (Turner and Novacky 1974). Cell dimensions were determined as previously described (Parsons et al. 1999).

Results

Respiration following phosphate resupply

Previously (Parsons et al. 1999) we showed that when wt tobacco cells are grown for 5 days in a low P growth medium, there is a large increase in AOX protein, resulting in a large capacity for CN-resistant, SHAM-sensitive respiration (AOX capacity). Alternatively, AS8 transgenic cells lack any induction of AOX protein or capacity under these growth conditions (Parsons et al. 1999). Fig. 1 shows similar data, except that AOX capacity in this case was determined immediately following the resupply of P to the cells. Nonetheless, the data show that AOX capacity is very high in wt cells ($652 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$) and very low in AS8 ($52 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$). Similar experiments using nPG rather than SHAM gave similar AOX capacities (wt, $595 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$; AS8, $38 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$; average from five experiments).

The data in Fig. 1 also show that the immediate stimulation of respiration by P resupply is much more dramatic in wt cells (an increase of $198 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$) than in AS8 (an increase of $45 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$).

In a different experiment, P addition to cells grown for 5 days in a low P growth medium was followed by the addition of the AOX inhibitor nPG (Fig. 2). For wt cells, P resupply brought about an immediate 1.5-fold increase in respiratory O_2 uptake, representing an increase of $184 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$ (Fig. 2). Subsequent addition of the AOX inhibitor nPG decreased this stimulated rate by 23%, representing a decrease of $127 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$. Alternatively, O_2 uptake of AS8 cells was stimulated only 1.2-fold by P addition (an increase of $80 \text{ nmol O}_2 \text{ mg}^{-1} \text{ dry weight h}^{-1}$) and this stimulated rate was slightly increased by nPG (Fig. 2).

The short-term immediate differences between wt and AS8 cells in respiratory stimulation by P (Figs 1, 2) were

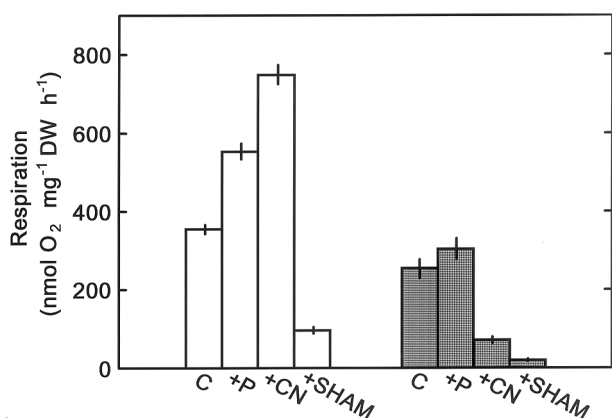


Fig. 1. Rates of respiratory O_2 consumption by wt (open bars) and AS8 transgenic (dark bars) tobacco suspension cells grown for 5 days in a low P growth medium. O_2 consumption was monitored under control conditions (C) and following the successive additions of $0.5 \text{ mM KH}_2\text{PO}_4$ (+P), 1 mM KCN (+CN) and 2 mM SHAM (+SHAM). Data is the average \pm SE from 9 separate cultures of cells ($n=9$).

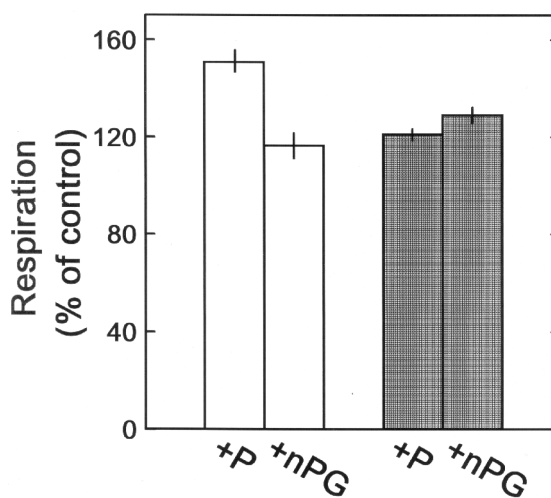


Fig. 2. Rates of respiratory O_2 consumption by wt (open bars) and AS8 transgenic (dark bars) tobacco suspension cells grown for 5 days in a low P growth medium. O_2 consumption was monitored prior to P addition and following the successive additions of $0.5 \text{ mM KH}_2\text{PO}_4$ (+P) and $20 \mu\text{M nPG}$ (+nPG). O_2 consumption rates are presented as a percentage of the rate prior to P addition. Data is the average \pm SE from 6 separate cultures of cells ($n=6$).

maintained in the longer term. Hence, when respiration was periodically monitored over a 120-min period of active P uptake, wt cells maintained respiration rates that were consistently 1.4–1.7-fold higher than in AS8 (Fig. 3).

Phosphate uptake

Despite the dramatic differences in respiratory O_2 consumption between the wt and AS8 during P uptake (Fig. 3), we found no differences between the wt and AS8 in the actual rate of P uptake over this time period. When wt cells, grown for 5 days in low P growth medium, were resupplied with $0.5 \text{ mM KH}_2\text{PO}_4$, the measured rate of P uptake was

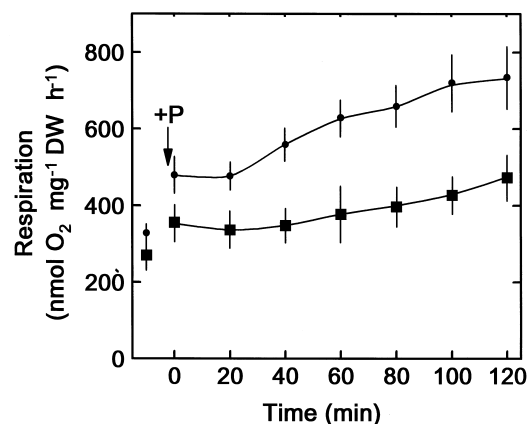


Fig. 3. Rates of respiratory O_2 consumption by wt (circles) and AS8 transgenic (squares) tobacco suspension cells grown for 5 days in a low P growth medium. The data points to the left of time 0 represent the rates of oxygen consumption prior to P addition. Respiratory O_2 consumption was then periodically monitored following the addition of $0.5 \text{ mM KH}_2\text{PO}_4$ (+P) at time 0. Data is the average \pm SE from 3 separate cultures of cells ($n=3$).

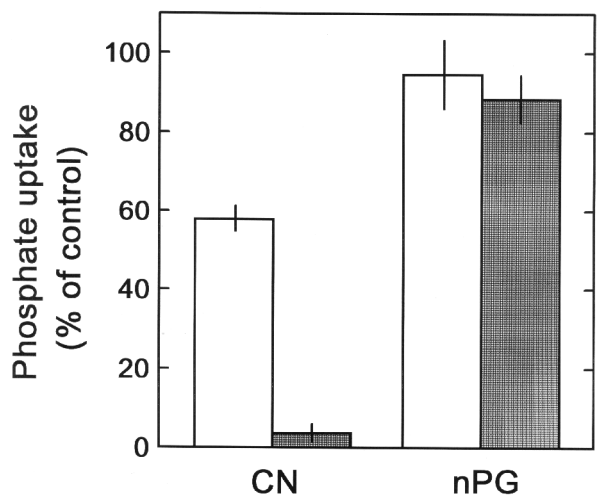


Fig. 4. Rates of P uptake by wt (open bars) and AS8 transgenic (dark bars) tobacco suspension cells grown for 5 days in a low P growth medium and then resupplied with 0.5 mM KH_2PO_4 . Rates of P uptake were determined in the presence of 1 mM KCN (CN) or 20 μM nPG (nPG), both of which were added to cells 2 min prior to P addition. Rates are expressed as a percentage of the rate of P uptake by untreated control cells. The CN data is the average \pm SE from 4 separate cultures of cells ($n = 4$) while the nPG data is the average \pm SE from 3 separate cultures of cells ($n = 3$).

29.5 ± 2.3 nmol P mg^{-1} dry weight h^{-1} . For AS8, the rate of uptake was 29.1 ± 1.2 nmol P mg^{-1} dry weight h^{-1} . In each case, data are the average \pm SE from 7 separate cultures of cells ($n = 7$). These rates of P uptake are similar to those measured in other P-limited plant cell culture systems (Brodelius and Vogel 1985, Mitsukawa et al. 1997).

Treatment with nPG had little effect on P uptake, reducing the rate of uptake by approximately 5% in wt cells and by approximately 12% in AS8 (Fig. 4). Alternatively, CN reduced the rate of P uptake by 42% in wt cells, and by 96% in AS8 (Fig. 4).

Generation of active oxygen species during phosphate uptake

The cell-permeable probe DCFH-DA can be used to examine the *in vivo* generation of H_2O_2 over time with high sensitivity in these tobacco suspension cells (Maxwell et al. 1999, Parsons et al. 1999). This method was used to determine the rate of generation of H_2O_2 by wt and AS8 cells after 5 days growth in low P medium and during the period of stimulated respiration immediately following P resupply. After 5 days growth in low P medium, wt cells displayed a rate of H_2O_2 generation of 1.7 relative fluorescence units min^{-1} (as measured by DCFH-DA fluorescence) while AS8 cells displayed a rate of 3.4 relative fluorescence units min^{-1} (Table 1). This result is similar to that reported previously, although the previous study used cells grown in low P medium for 3 days rather than 5 days (Parsons et al. 1999). Following P resupply, both wt and AS8 cells displayed increased rates of generation of H_2O_2 . The wt rate increased by 3.3 relative fluorescence units min^{-1} , while the AS8 rate increased by 5.1 relative fluorescence units min^{-1} (Table 1).

Viability, growth and morphology following phosphate resupply

In both the wt and AS8 cultures, cell viability remained high both prior to and following P resupply (Table 2).

After 5 days growth in a low P medium, the dry weight of the AS8 culture was significantly higher than in the wt (Table 2), as previously observed (Parsons et al. 1999). Both cultures responded to P resupply with a dramatic stimulation of growth over the following 2-day period (Table 2).

After 5 days growth in low P medium, the average length of AS8 cells was approximately 2-fold that of wt cells (Fig. 5). This difference became more pronounced after 6 and 7 days in low P medium because of the further increases in the average length of AS8 cells. Alternatively, when AS8 cells were resupplied with P after 5 days growth in low P medium, average cell length significantly decreased over the following 2-day period (Fig. 5).

Discussion

Previously, we showed that mitochondrial AOX protein and the capacity for AOX respiration are dramatically induced in wt tobacco suspension cells when grown under P limitation (Parsons et al. 1999). Figs 1–3 show that when P-limited wt cells are resupplied with P, there is a large immediate stimulation of respiratory O_2 uptake. Given the high capacity for AOX respiration in these cells (Fig. 1), we hypothesized that AOX might contribute to this high rate of respiration. Two different approaches were taken to investigate this possibility.

A first approach to investigate whether AOX contributes to the high rate of respiration seen in wt cells following P resupply was to compare the respiratory response of such cells with that of AS8 transgenic tobacco suspension cells. These transgenic cells lack any significant AOX capacity, even when grown under P limitation (Fig. 1; Parsons et al. 1999). Hence, if AOX does contribute to the high rate of respiration seen when P-limited wt cells are resupplied with P, then one might expect that P-limited AS8 cells would show a less dramatic stimulation of respiration following P resupply. Indeed, this is what we observed. In both short-term (Figs 1, 2) and long-term (Fig. 3) experiments, the stimulation of respiration was much less dramatic in AS8 than in wt. This was the case regardless of whether the respiratory increase was expressed on a dry weight basis

Table 1. Rates of AOS generation by wt and AS8 transgenic tobacco suspension cells after 5 days growth in low P medium (–P) and immediately following P resupply (+P). AOS generation was monitored using the cell-permeable probe DCFH-DA. Results for each treatment are based on 4 separate experiments with different subcultures of cells (see Materials and methods for further explanation).

Treatment	Rate of AOS generation (relative fluorescence min^{-1})	
	wt	AS8
–P	1.7	3.4
+P	5.0	8.5

Table 2. Viability and growth (culture density) of wt and AS8 transgenic tobacco suspension cells after 5 days in a low P medium and following P resupply (2.5 mM KH₂PO₄) after 5 days in the low P medium. Viability results are the average \pm SE from 7 separate cultures of cells (n = 7) while growth results are the average \pm SE from 6 separate cultures of cells (n = 6).

Time	Viability (% live cells)		Culture density (g dry weight l ⁻¹)	
	wt	AS8	wt	AS8
After 5 days in low P	97.8 \pm 0.3	95.7 \pm 0.5	4.13 \pm 0.14	6.74 \pm 0.56
18 h after P resupply	97.5 \pm 0.2	94.8 \pm 0.5	4.40 \pm 0.17	7.80 \pm 0.29
48 h after P resupply	97.9 \pm 0.3	94.9 \pm 0.4	7.34 \pm 0.43	10.77 \pm 0.48

(Fig. 1) or as a percentage of the respiration rate just prior to P addition (Fig. 2). Also, this difference in stimulation was not due to the cells exhibiting different rates of P uptake (see below).

A second approach to investigate whether AOX contributes to the high rate of respiration seen in wt cells following P resupply was to examine whether the stimulated rate of respiration in the wt cells was sensitive to the AOX inhibitor nPG. Sensitivity to nPG (in the absence of a Cyt pathway inhibitor) would be an indication that AOX was actively engaged in respiration. Fig. 2 shows that, indeed, the stimulated rate of respiration of wt cells was sensitive to nPG. Although such inhibitor-based results must be interpreted with caution, the data suggest that AOX is engaged in the respiration of wt cells during rapid P uptake. In fact, the major concern with using such an inhibitor-based approach to examine whether AOX is contributing to respiration is that it may actually underestimate the contribution of AOX or even indicate a lack of AOX involvement when AOX is in fact being utilized. This is because it is now recognized that electrons can likely be diverted from the AOX pathway to the Cyt pathway during such an assay, leading to a systematic underestimate of AOX activity (see Day et al. 1996 for a critical discussion of these points). In any event, the sensitivity of respiration to nPG is consistent with some contribution of AOX to respiration. One must also be cautious of any non-specific effects (unrelated to the inhibition of AOX), which such inhibitors might have on O₂ uptake in such assays. However, the result with AS8 cells is consistent with there being little non-specific effect of nPG on O₂ uptake by these tobacco cells. In the absence of non-specific effects of nPG on O₂ uptake, we expect that the rate of O₂ uptake of AS8 cells (which lack AOX) should not be significantly affected by the inhibitor in such an assay and this is indeed what we observed (Fig. 2).

Two lines of evidence, one based on a comparison of wt and transgenic cells and one based on the use of inhibitors, suggest that AOX is being utilized by P-limited wt tobacco cells during the high rates of respiration seen following P resupply (see above). Nonetheless, two observations also suggest that, while AOX is being utilized, the Cyt pathway alone could compensate for a lack of AOX to support the rapid P uptake. First, despite the lack of AOX, AS8 cells maintained similar rates of P uptake to those seen in wt (see 'Results'). Second, when AOX in wt cells was eliminated by the addition of nPG, it had only a minor inhibitory effect on the rate of P uptake (Fig. 4). Also, this minor inhibitory effect was seen in AS8, suggestive of a minor non-specific effect of the inhibitor on P uptake, unrelated to the inhibi-

tion of AOX. These observations suggest that the utilization of AOX by wt cells was not occurring because the Cyt pathway alone could not fully support the rate of P uptake, but rather that AOX activity plays some other role (see below).

Preliminary studies indicated that the AOX inhibitor SHAM had serious non-specific effects on P uptake since it inhibited uptake in AS8 cells (which lack AOX) by approximately 60% (data not shown). This observation illustrates some of the potential limitations in using inhibitor-based data alone to establish the role of AOX in complex physiological processes such as ion uptake.

We found that, while AS8 cells were unable to utilize AOX during the period of rapid P uptake following P resupply, this had no catastrophic effects on the cell culture. This is based upon 3 different criteria. First, cell viability following P resupply remained high in both the wt and AS8 (Table 2). Second, both the wt and AS8 cultures responded to P resupply with a dramatic stimulation of growth (Table 2). The third criterion is based on observations of average cell length. The average length of AS8 cells increases with increased time in low P medium (Fig. 5, see also Parsons et al. 1999). However, this trend was reversed by P resupply (Fig. 5). Presently, we do not understand why a lack of AOX during P limitation results in this altered morphology, but it is clear that this morphology is largely reversed by P resupply. While the resupply of P to AS8 cells grown in low P medium for 5 days did not appear to have any catastrophic effects on the culture (as judged at least by these 3 criteria), we have found that AS8 cells maintained for longer periods of time in low P medium do show a marked loss of viability following P resupply, while wt cells

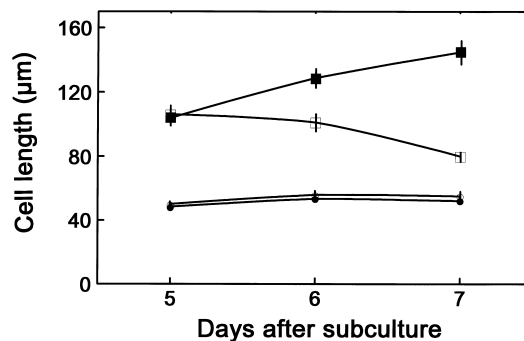


Fig. 5. Cell length of wt (circles) and AS8 transgenic (squares) tobacco suspension cells after growth in a low P medium for 5–7 days. In some cases (open symbols), cells were resupplied with P (2.5 mM KH₂PO₄) at day 5. Data is the average \pm SE from 3 separate cultures of cells (n = 90).

do not (EWY Eng and GC Vanlerberghe, unpublished data). At present, however, we do not believe that this is necessarily a direct consequence of P uptake in the absence of AOX.

Rapid P uptake is an energy-demanding process, dependent upon high rates of respiratory energy production to support increased plasma membrane H^+ -ATPase activity (see Introduction). Hence, when the mitochondrial respiration of AS8 cells was completely compromised by the addition of CN, P uptake was almost completely abolished (Fig. 4). Alternatively, when CN was added to wt cells, the high capacity AOX pathway (able to maintain very high respiration rates in the presence of CN; Fig. 1) could partially compensate for the lack of Cyt pathway respiration and hence the rate of P uptake was only reduced by 42% (Fig. 4). In this case, the high rate of AOX respiration could not completely compensate for the lack of Cyt pathway activity because electron transport from Q to O_2 via AOX is not coupled to proton translocation and hence decreases the ATP yield of oxidative phosphorylation by approximately two-thirds compared with the Cyt pathway (Lambers 1997). Hence, support of an energy-demanding process by AOX alone would require approximately 3 times more O_2 uptake than if only the Cyt pathway were utilized.

Despite the energy demands of rapid P uptake, our results suggest that wt cells maintain a level of AOX respiration during this process. As such AOX activity decreases the ATP yield associated with the coupled processes of carbon oxidation and O_2 reduction, one would expect that wt cells (utilizing a combination of the Cyt pathway and AOX) would require greater overall rates of carbon oxidation and electron transport to O_2 than AS8 cells (utilizing only the Cyt pathway) to support similar rates of P uptake. This is indeed what we observed (Figs 1–3) and is further evidence consistent with AOX being utilized by wt cells during rapid P uptake.

Given the non-phosphorylating nature of AOX respiration, it has been suggested that it may have a negative impact on plant productivity (by decreasing the carbon-use efficiency of respiration) and that its elimination might increase crop yield (Gifford et al. 1984). Nonetheless, given the ubiquitous nature of AOX in the plant kingdom, it is also possible that the negative effects of such respiration on carbon-use efficiency may be offset by other positive effects. Two such potential positive effects of AOX during the rapid respiration supporting P uptake are discussed below.

Sakano (1998) presented a hypothesis that AOX may function as part of a biochemical pH-stat during periods of rapid cytosolic acidification, such as might occur during rapid P uptake by a proton/ P_i symport. It was suggested that AOX respiration, with its concomitant protonation of oxygen to water would act as a 'sink' for protons, with the advantage that, unlike the Cyt pathway, AOX would not be subject to adenylate control. Our study does suggest the involvement of AOX respiration during rapid P uptake in wt cells. However, this component of respiration was also not critical to P uptake as cells lacking AOX (either wt cells treated with nPG or AS8 cells) maintained similar rates of P uptake to untreated wt cells with abundant AOX. If AOX respiration was critical to maintaining intracellular

pH during P uptake, a lack of AOX might be expected to have compromised P uptake because of dissipation of the proton motive force across the plasma membrane. It appears likely that within our system, increased H^+ -ATPase activity (and possibly other intracellular pH-stat mechanisms) was able to sufficiently maintain proton motive force during rapid P uptake without the necessary involvement of AOX. Nonetheless, if H^+ -ATPase activity were impaired, such as might occur during environment stress, it is still conceivable that AOX could play an active role in pH regulation during a period of rapid cytosolic acidification. In this regard, it is noteworthy that AOX expression is upregulated in response to various stress conditions (Simons and Lambers 1999).

Recent *in vivo* studies suggest that AOX acts to prevent over-reduction of electron transport chain components, hence preventing the excessive generation of harmful AOS in the mitochondrion (Maxwell et al. 1999, Parsons et al. 1999). Organello studies also support such a role (Popov et al. 1997, Purvis 1997). We hypothesized that this function of AOX may be important during P uptake, when a high rate of respiration is induced. Indeed, we found that following P resupply, both wt and AS8 cells displayed increased rates of H_2O_2 generation, coincident with the increased rates of respiration (Table 1). Nonetheless, while the stimulation of respiration during P uptake is much less pronounced in AS8 than in the wt (Figs 1–3), the increase in rate of generation of H_2O_2 was more in AS8 (a rate increase of 5.1 relative fluorescence units min^{-1}) than it was in the wt (a rate increase of 3.3 relative fluorescence units min^{-1}). This is an indication that the presence of AOX in wt cells can dampen to some extent the generation of AOS, which accompanies high rates of respiration. Presumably, this is because of the ability of AOX to prevent over-reduction of electron transport chain components by accepting electrons from the Q pool, and without this process being subject to the same level of adenylate control as is the Cyt pathway. For example, Millar et al. (1998) did simultaneous measures of O_2 consumption and the level of Q pool reduction in isolated soybean root mitochondria. They showed that under the ADP-limiting conditions likely to prevail *in vivo* (particularly in cells grown under P limitation; see Parsons et al. 1999), the activity of AOX allowed high rates of respiration without large increases in Q-pool reduction level (Millar et al. 1998).

AOX may thus represent an important mechanism of defense against oxidative stress. This role of AOX may be particularly relevant during periods when there are rapid changes in respiration rate, such as occurs here to support rapid nutrient uptake. Such rapid changes in respiration rate do not allow for coarse adjustment (at the gene expression level) of respiratory components. Nonetheless, rapid mechanisms of fine regulation of AOX activity (Vanlerberghe et al. 1995) could provide the metabolic flexibility necessary to prevent excessive AOS generation under the new respiratory conditions. This may help to explain why this apparently wasteful pathway (at least in terms of energy conservation) remains ubiquitous in the plant kingdom.

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References

- Ames BN (1966) Assays of phosphate and phosphatases. *Methods Enzymol* 8: 115–117
- Brodelius P, Vogel HJ (1985) A phosphorus-31 nuclear magnetic resonance study of phosphate uptake and storage in cultured *Catharanthus roseus* and *Daucus carota* cells. *J Biol Chem* 260: 3556–3560
- Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM, Wiskich JT (1996) The cyanide-resistant oxidase: To inhibit or not to inhibit, that is the question. *Plant Physiol* 110: 1–2
- Gauthier DA, Turpin DH (1994) Inorganic phosphate (P_i) enhancement of dark respiration in the P_i -limited green alga *Selenastrum minutum*: Interactions between H^+ / P_i cotransport, the plasma-membrane H^+ -ATPase, and dark respiratory carbon flow. *Plant Physiol* 104: 629–637
- Gifford RM, Thorne JH, Hitz WD, Giaquinta RT (1984) Crop productivity and photoassimilate partitioning. *Science* 225: 801–808
- Lambers H (1997) Oxidation of mitochondrial NADH and the synthesis of ATP. In: Dennis DT, Turpin DH, Lefebvre DD, Layzell DB (eds) *Plant Metabolism*, 2nd edn. Addison-Wesley Longman, Essex, pp 200–219
- Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen species production in plant cells. *Proc Natl Acad Sci USA* 96: 8271–8276
- Millar AH, Atkin OK, Menz I, Henry B, Farquhar G, Day DA (1998) Analysis of respiratory chain regulation in roots of soybean seedlings. *Plant Physiol* 117: 1083–1093
- Mitsukawa N, Okumura S, Shirano Y, Sato S, Kato T, Harashima S, Shibata D (1997) Overexpression of an *Arabidopsis thaliana* high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. *Proc Natl Acad Sci USA* 94: 7098–7102
- Muchhal US, Raghothama KG (1999) Transcriptional regulation of plant phosphate transporters. *Proc Natl Acad Sci USA* 96: 5868–5872
- Parsons HL, Yip JYH, Vanlerberghe GC (1999) Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. *Plant Physiol* 121: 1309–1320
- Plaxton WC (1996) The organization and regulation of plant glycolysis. *Annu Rev Plant Physiol Plant Mol Biol* 47: 185–214
- Popov VN, Simonian RA, Skulachev VP, Starkov AA (1997) Inhibition of the alternative oxidase stimulates H_2O_2 production in plant mitochondria. *FEBS Lett* 415: 87–90
- Purvis AC (1997) Role of the alternative oxidase in limiting superoxide production by plant mitochondria. *Physiol Plant* 100: 165–170
- Raghothama KG (1999) Phosphate acquisition. *Annu Rev Plant Physiol Plant Mol Biol* 50: 665–693
- Sakano K (1998) Revision of biochemical pH-stat: Involvement of alternative pathway metabolisms. *Plant Cell Physiol* 39: 467–473
- Sakano K, Kiyota S, Yazaki Y (1998) Degradation of endogenous organic acids induced by P_i uptake in *Catharanthus roseus* cells: Involvement of the biochemical pH-stat. *Plant Cell Physiol* 39: 615–619
- Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: From soil to cell. *Plant Physiol* 116: 447–453
- Simons BH, Lambers H (1999) The alternative oxidase: Is it a respiratory pathway allowing a plant to cope with stress? In: Lerner HR (ed) *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization*. Marcel Dekker, Inc, New York, NY, pp 265–286
- Turner JG, Novacky A (1974) A quantitative relationship between plant and bacterial cells involved in the hypersensitive reaction. *Phytopathology* 64: 885–890
- Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: From gene to function. *Annu Rev Plant Physiol Plant Mol Biol* 48: 703–734
- Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1994) Molecular genetic alteration of plant respiration: Silencing and overexpression of alternative oxidase in transgenic tobacco. *Plant Physiol* 106: 1503–1510
- Vanlerberghe GC, Day DA, Wiskich JT, Vanlerberghe AE, McIntosh L (1995) Alternative oxidase activity in tobacco leaf mitochondria: Dependence on tricarboxylic acid-cycle mediated redox regulation and pyruvate activation. *Plant Physiol* 109: 353–361
- Wagner AM, Moore AL (1997) Structure and function of the plant alternative oxidase: Its putative role in the oxygen defense mechanism. *Biosci Rep* 17: 319–333
- Weger HG (1996) Interactions between respiration and inorganic phosphate uptake in phosphate-limited cells of *Chlamydomonas reinhardtii*. *Physiol Plant* 97: 635–642

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