



RESEARCH PAPER

# The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells

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## Abstract

When wild-type (wt) tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) cells are grown under macronutrient (P or N) limitation, they induce large amounts of alternative oxidase (AOX), which constitutes a non-energy-conserving branch of the respiratory electron transport chain. To investigate the significance of AOX induction, wt cells were compared with transgenic (AS8) cells lacking AOX. Under nutrient limitation, growth of wt cell cultures was dramatically reduced and carbon use efficiency (g cell dry weight gain g<sup>-1</sup> sugar consumed) decreased by 42–63%. However, the growth of AS8 was only moderately reduced by the nutrient deficiencies and carbon use efficiency values remained the same as under nutrient-sufficient conditions. As a result, the nutrient limitations more severely compromised the tissue nutrient status (P or N) of AS8 than wt cells. Northern analyses and a comparison of the mitochondrial protein profiles of wt and AS8 cells indicated that the lack of AOX in AS8 under P limitation was associated with increased levels of proteins commonly associated with oxidative stress and/or stress injury. Also, the level of electron transport chain components was consistently reduced in AS8 while tricarboxylic acid cycle enzymes did not show a universal trend in abundance in comparison to the wt. Alternatively, the lack of AOX in AS8 cells under N limitation resulted in enhanced carbohydrate accumulation. It is concluded that AOX respiration provides an important general mechanism

by which plant cells can modulate their growth in response to nutrient availability and that AOX also has nutrient-specific roles in maintaining cellular redox and carbon balance.

Key words: Alternative oxidase, growth, nutrient stress, oxidative stress, plant mitochondria, proteomics, respiration, tobacco cells.

## Introduction

Carbon oxidation in plant respiratory pathways [glycolysis, oxidative pentose phosphate pathway, tricarboxylic acid (TCA) cycle] is coupled to reduction of pyridine nucleotides. An important route by which these reducing equivalents are subsequently oxidized is by the mitochondrial electron transport chain (ETC). Electron transport to O<sub>2</sub> is coupled (through the generation of a proton motive force) to the synthesis of ATP from ADP and inorganic P (P<sub>i</sub>) by the process of oxidative phosphorylation. The ATP generated, along with reduced pyridine nucleotides and carbon intermediates produced within the respiratory pathways, then provides the energy and carbon to support biosynthesis and growth. Plant growth also depends upon the availability of mineral nutrients, and the macronutrients that most often limit growth in natural and agricultural settings are N and P. Plants have numerous physiological, morphological,

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biochemical, and molecular mechanisms that are induced under nutrient limitation and which act to improve the acquisition and/or use of nutrients. These include changes in root architecture, the expression of transporter proteins and numerous changes in metabolism (Theodorou and Plaxton, 1995; Raghothama, 1999; Wang *et al.*, 2001; Vance *et al.*, 2003). One relatively poorly understood adaptive response is that the growth rate of different plant parts is adjusted in relation to nutrient availability (Stitt and Scheible, 1998). More generally, numerous stress conditions are associated with reduced growth, a response that may have some adaptive advantage (Thomas and Sadras, 2001).

The plant mitochondrial ETC includes an inner membrane protein called alternative oxidase (AOX) (Simons and Lambers, 1999; Siedow and Umbach, 2000; Moore *et al.*, 2002). AOX catalyses the O<sub>2</sub>-dependent oxidation of ubiquinol, producing ubiquinone and water. Electron flow from ubiquinol to AOX is not coupled to the generation of a proton motive force and hence is a non-energy-conserving branch of the ETC, bypassing the last two sites of proton pumping associated with the cytochrome (cyt) pathway. Hence, the activity of AOX reduces the energy yield associated with respiration. This has prompted the question of what impact AOX may have on plant growth and productivity (Amthor, 2000; Moore *et al.*, 2002).

There is debate regarding the physiological role(s) of AOX respiration. The non-energy-conserving nature of AOX, along with the ability biochemically to regulate AOX activity in a sophisticated manner (Siedow and Umbach, 2000) presumably provides the mitochondrion with considerable metabolic flexibility. This flexibility could allow the mitochondrion to (i) modulate the rate of ATP production; (ii) maintain electron flux to oxygen when other downstream ETC components or ADP/P<sub>i</sub> are limiting; or (iii) modulate the reduction state of ETC components, and hence the rate of generation of reactive oxygen species (ROS). This flexibility may be particularly important during periods of abiotic and biotic stress (Simons and Lambers, 1999). It has been shown that transgenic cells lacking AOX have increased amounts of ROS emanating from the mitochondrion (Maxwell *et al.*, 1999) and that such cells are more susceptible to programmed cell death (Robson and Vanlerberghe, 2002).

Studies indicate that the capacity for electron transport to AOX and/or the level of AOX protein increases in response to a limited supply of mineral nutrients (Lambers *et al.*, 1981; Rychter and Mikulska, 1990; Parsons *et al.*, 1999; Gonzalez-Meler *et al.*, 2001). To investigate the physiological significance of this response, growth and other characteristics of wild-type (wt) tobacco cells have been compared with those of transgenic tobacco cells lacking AOX. Cells were grown under nutrient-sufficient conditions or under conditions of limiting P or N.

## Materials and methods

### *Plant material and growth conditions*

Suspension cells were derived from leaves of wt or transgenic tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) and were cultured for 8 years prior to this study. The transgenic cells (AS8) express an antisense construct of the nuclear gene *Aox1*, encoding a tobacco AOX. Expression of AOX in these cells is severely impaired (Vanlerberghe *et al.*, 1994). Cell cultures (200 ml culture in a 500 ml Erlenmeyer flask) were grown in the dark on a rotary shaker (140 rpm) at 28 °C and were subcultured every 7 d by dilution in fresh growth medium containing 88 mM sucrose as the carbon source (Vanlerberghe *et al.*, 1994). Low P medium contained 250 µM P (rather than the 2.5 mM found in complete medium) while low N medium contained 2.4 mM N (rather than the 60 mM found in complete medium).

### *Cell growth and respiratory characteristics*

To evaluate growth, an aliquot of cell culture was washed twice with water, frozen, and lyophilized to determine cell dry weight. Relative growth rate (RGR) was calculated from dry weight data according to Hunt (1990). Respiration and the capacity of cyt and AOX pathways of whole cells were determined as described by Robson and Vanlerberghe (2002).

### *Phosphate, nitrogen, metabolite, and carbon use efficiency (CUE) analyses*

Total cell P, cell P<sub>i</sub>, and P content of the growth media were determined as described previously (Parsons *et al.*, 1999). N:C ratios were measured using an elemental combustion analyser (Costech Analytical Technologies, Valencia, CA, USA).

For sugar analyses, lyophilized cells were extracted in H<sub>2</sub>O at 95 °C for 1 h. The sample was centrifuged (2000 g, 5 min, 4 °C) and the supernatant stored at -80 °C prior to analysis of soluble sugars. Starch in the pellet was degraded to glucose as described by Jones (1981). Sugars were analysed by coupled enzymatic assays (Stitt *et al.*, 1989) using a diode array spectrophotometer (Hewlett-Packard). CUE was determined as described by Vanlerberghe *et al.* (1997).

### *Mitochondrial isolation and analysis*

Mitochondria were isolated from suspension cells at 5 d after subculture (using 600–1200 ml of culture depending on cell line and growth condition) as described previously (Robson and Vanlerberghe, 2002). O<sub>2</sub> uptake by mitochondria (0.15–0.4 mg protein ml<sup>-1</sup>) was measured in a Clark-type oxygen electrode cuvette (Hansatech, King's Lynn, UK) at 28 °C in a medium containing 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (pH 7.2), 0.25 M Suc, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% w/v bovine serum albumin, and 0.1 mM each of NAD<sup>+</sup>, NADP<sup>+</sup>, ATP, and thiamine pyrophosphate.

AOX capacity was measured as the *n*-propyl gallate-sensitive activity in the presence of 2 mM NADH, 10 mM malate, 10 mM glutamate, 2 mM ADP, 10 mM dithiothreitol, 2 mM pyruvate, and 1 mM cyanide (CN). Cyt pathway capacity was measured as the CN-sensitive activity in the presence of 2 mM NADH, 10 mM malate, 10 mM glutamate, 2 mM ADP, 20 µM *n*-propyl gallate, and in the presence or absence of 50 µM cytochrome *c* (cyt *c*). Control experiments showed that antimycin A-insensitive NADH-cyt *c* reductase activity (in the presence of added cyt *c*) contributed only 10–20% of the cyt pathway capacity under these assay conditions and for all types of mitochondria. In other control experiments, the possibility of substrate limitation was tested by including 10 mM succinate and 10 mM 2-oxoglutarate (in addition to NADH, malate, and glutamate). The presence of these additional substrates increased

cyt pathway capacity by 0–20% and did not change the pattern between the different mitochondria.  $\text{NADH}_{\text{in}}$  activity was measured in the presence of 10 mM malate, 10 mM glutamate, 2 mM ADP, and 20  $\mu\text{M}$  rotenone.  $\text{NADH}_{\text{ex}}$  and  $\text{NADPH}_{\text{ex}}$  were measured in the presence of 2 mM ADP, 1 mM  $\text{CaCl}_2$ , 20  $\mu\text{M}$  rotenone, and either 2 mM NADH or 2 mM NADPH. For respiratory control and ADP/O measurements (using 10 mM malate and 10 mM glutamate or 2 mM NADH), values were determined from oxygen electrode traces after the third state 3/state 4 transition (using 100 nmol ADP each time) and in the presence of 20  $\mu\text{M}$  *n*-propyl gallate.

Separation of mitochondrial proteins by reducing SDS-PAGE and western blot analysis using monoclonal antibodies recognizing AOX, cyt oxidase subunit 2 and cyt c were done as described by Robson and Vanlerberghe (2002).

#### Two-dimensional (2-D) gel electrophoresis and identification of proteins by mass spectrometry

For two-dimensional electrophoresis, total mitochondrial protein (1.0 mg) was solubilized in 7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v dodecylmaltoside, 20 mM dithiothreitol, and a trace of bromophenol blue by agitating for 2 h at RT. Samples were centrifuged (50 000 g, 30 min, 20 °C) and 1% v/v immobilized pH gradient (IPG) pH 3–10 buffer (Amersham) was added to the supernatant. IPG isoelectric focusing pH 3–10, 18 cm gel strips (Amersham) were rehydrated overnight in the solubilized protein supernatant and focused for a total of 110 kVh on an IPGphor (Amersham) as described by the manufacturer. Second dimension Tricine 10–15% w/v gradient SDS-PAGE was then carried out as described by Schägger and von Jagow (1987). Gels were stained with Coomassie R-350 (Amersham), destained and then scanned on a density-calibrated flatbed scanner using ImageMaster Labscan v3.00 software (Amersham). Spot matching and relative quantitative comparisons were done using ImageMaster 2-D Elite v3.1 software (Amersham). Protein spots cut from 2-D gels were reduced, alkylated with iodoacetamide, and trypsin (Promega) digested as described by Wilm *et al.* (1996). Peptide extracts were dried in a speed-vac and frozen until analysis. Automated LC-MS/MS was done on a nanoscale capillary high-pressure liquid chromatography system (Famos, Switchos, Ultimate, LC-Packings, NL) equipped with a reverse phase trap column (300  $\mu\text{m}$  ID $\times$ 5 mm, PepMap C18, 5  $\mu\text{m}$ , LC-Packings), and a custom-made nanoscale reverse phase column that was interfaced via the Z-spray electrospray source to a Q-TOF tandem mass spectrometer (Q-TOF Ultima Global, Micromass UK). The nanoscale column (75  $\mu\text{m}$  ID $\times$ 35 mm) was packed with YMC ODS-A C-18, 3  $\mu\text{m}$  beads (YMC, Kyoto, Japan). Injected peptides were eluted by a 20 min gradient of 5–70% v/v acetonitrile in 1% v/v acetic acid, 1% v/v formic acid. The mass spectrometer employed data-dependent acquisition and was calibrated using Glu-fibrinopeptide B (Sigma). Data analysis was performed using MassLynx and ProteinLynx software, and the resulting MS/MS data set was analysed using the Mascot search engine (Matrix Science Ltd, London, UK). Tolerances for Mascot searches were 1 Da for the MS and 0.4 Da for the MS/MS matching. The mass error on peptides was always within 0.1 Da of the expected mass calculated from database entries.

#### RNA isolation and analysis

Total RNA was extracted using TRIZOL (Invitrogen), according to the manufacturer's instructions. RNA was separated on formaldehyde-agarose gels and transferred to Hybond-N membrane (Amersham Pharmacia). Partial cDNAs were amplified from tobacco leaf RNA using an RT-PCR kit (Access RT-PCR, Promega) for use as hybridization probes. The cDNAs amplified were salicylic acid-binding catalase (SA-CAT; U03473), glutathione peroxidase (GPx; X60219), and pathogenesis-related protein 1a (PR-1a; X06361). The primer sequences used for RT-PCR were as follows: SA-CAT 5'-

CTTACCTGTGCTGATTTTCTCC-3' and 5'-TGTCCTCCGAAT-AGTAAAGACC-3'; GPx 5'-CCAGTCAATCCAGCAAGC-3' and 5'-TTCTTGATATCCTTCTCCATGC-3'; PR-1a 5'-ATTGCCTT-CATTTCTTCTTGCTCT-3' and 5'-GACTTTCGCCTCTATAAT-TACCTG-3'. Amplified fragments were of the expected sizes, were purified from agarose gels using a gel extraction kit (Qiagen), and were radiolabelled using the Rediprime II random priming labelling system (Amersham). Pre-hybridization and hybridization were each done overnight at 65–70 °C in 0.25 M  $\text{Na}_2\text{HPO}_4$  (pH 7.2) with 7% w/v SDS. Pre-hybridization also included denatured salmon sperm DNA (100  $\mu\text{g}$   $\text{ml}^{-1}$ ). Washes were performed according to Church and Gilbert (1984). Blots were analysed by autoradiography using CL-Xposure film (Pierce) and a Biomax Transcreen-HE intensifying screen (Kodak).

#### Other methods

Protein concentration was determined by a modified Lowry method (Larson *et al.*, 1986). Data were analysed by paired or unpaired two-tail *t*-tests.

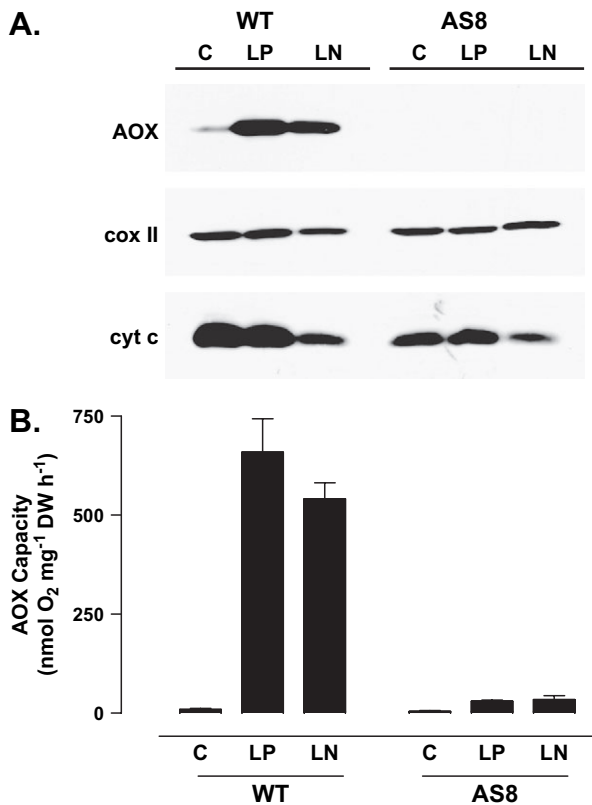
## Results

### Macronutrient limitation results in a large induction of AOX in wt tobacco cells

Wild-type tobacco cells were grown in three nutrient media: a complete medium, a low P medium, and a low N medium. On day 5 after subculture, complete medium-grown cells had a low level of AOX protein in their mitochondria (Fig. 1A) and a low AOX capacity as measured in whole cells (Fig. 1B). Growth of cells under P or N limitation resulted in a large induction of both AOX protein and capacity (Fig. 1). Capacity increased from a basal level of 9  $\text{nmol O}_2 \text{mg}^{-1} \text{DW h}^{-1}$  in complete medium to averages of 659 and 541  $\text{nmol O}_2 \text{mg}^{-1} \text{DW h}^{-1}$  in low P and low N media, respectively.

Transgenic (AS8) tobacco cells (expressing an antisense AOX transgene) had no detectable AOX protein in any of the nutrient media and so these cells maintained negligible AOX capacity in low P or low N conditions in comparison to the wt (Fig. 1B). By contrast, other ETC proteins [cyt oxidase subunit 2 (cox II), cyt c] were readily detected in the mitochondria of AS8 cells and were generally of similar abundance to that seen in wt cells (Fig. 1A), although more quantitative analyses did show differences in several ETC activities and ETC protein levels between the two cell lines (see later).

Given the large differences in AOX capacity between wt and AS8 cells, other ETC activities were also compared using mitochondria isolated from cells at 5 d after subculture. Respiratory control (RC) and ADP/O ratios (with either NADH or malate as substrate) confirmed that the isolated mitochondria were of reasonable quality. Average values obtained were: RC (NADH), 2.7; RC (malate), 2.6; ADP/O (NADH) 1.5; ADP/O (malate) 2.1. It was also seen that these values did not differ appreciably between cell lines or across growth conditions (data not shown). Measurements of AOX capacity in these mitochondria



**Fig. 1.** The level of AOX in wild-type (wt) and transgenic (AS8) tobacco cells grown in complete (C), low P (LP), or low N (LN) nutrient media for 5 d. (A) The level of AOX protein and two other ETC proteins in mitochondria isolated from wt or AS8 cells. Mitochondrial proteins (80  $\mu$ g) were separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to AOX, cox II, or cyt c. The experiment was repeated several times and representative results are shown. (B) The level of AOX capacity measured in intact cells. Data are the average  $\pm$  standard error from three to four independent experiments.

confirmed what had been seen with whole cell measurements (see above). That is, there was a large AOX capacity in mitochondria isolated from low P and low N-grown wt cells (compared with complete-medium-grown cells) and no substantial AOX capacity in any AS8 mitochondria (Fig. 2C).

In wt mitochondria, the capacity of the cyt pathway was increased slightly by growth under low P (in comparison to complete medium), while it was significantly reduced ( $P=0.01$ ) by low N (Fig. 2A). AS8 mitochondria displayed significantly less cyt pathway capacity than the wt in low P medium and, to a lesser extent, in complete medium also. However, both cell lines displayed similar cyt pathway capacity in low N medium. These activities (Fig. 2A) are in the presence of added cyt c (see Materials and methods), and despite similar respiratory control and ADP/O ratios, cyt c addition consistently increased the capacity of AS8 more so than the wt, particularly when mitochondria from low P-grown cells are compared (Fig. 2B).

Similar trends in cyt pathway capacity were also seen when measured in whole cells (data not shown). In complete

medium, wt cells had a capacity that was slightly higher (but not statistically different;  $P=0.09$ ) than that of AS8 cells. Growth in low P medium increased the capacity of wt cells but reduced the capacity of AS8 cells (data not shown). As a result, low P-grown wt cells had a significantly higher (1.8-fold) cyt path capacity than did low P-grown AS8 cells ( $P < 0.01$ ), similar to the results observed with isolated mitochondria (Fig. 2A).

In wt mitochondria, internal rotenone-resistant NADH dehydrogenase activity ( $\text{NADH}_{\text{in}}$ ) was increased significantly under low P (in comparison to complete medium,  $P < 0.001$ ), while it was reduced significantly by low N ( $P=0.001$ ) (Fig. 2D). Interestingly,  $\text{NADH}_{\text{in}}$  activity in AS8 was similar under all growth conditions, lacking the large changes observed in the wt. The external rotenone-resistant NAD(P)H dehydrogenase activities ( $\text{NADH}_{\text{ex}}$  and  $\text{NADPH}_{\text{ex}}$ ) were each much higher in the wt than AS8 in both complete and low P (Fig. 2E, F). In low N, these activities were reduced in the wt and were similar between cell lines.

#### *Under macronutrient limitation, transgenic cells lacking AOX display enhanced growth in comparison to wt cells*

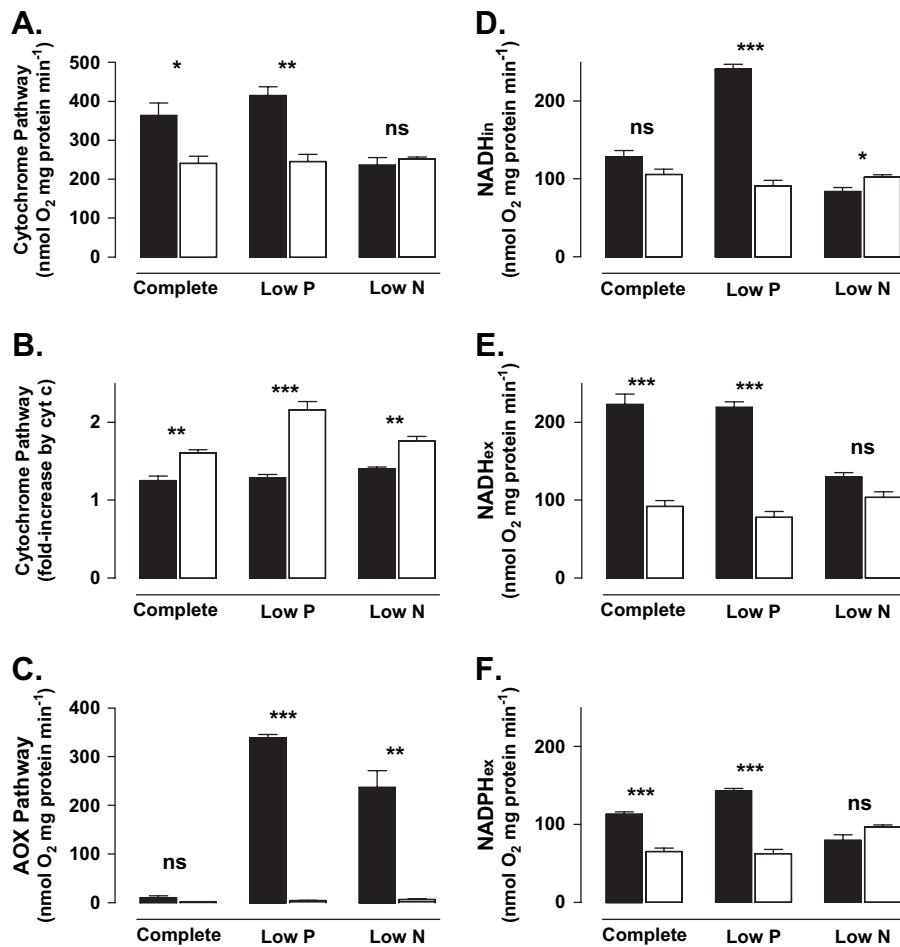
The growth (change in culture density) of wt and AS8 cells was compared in each of the nutrient media. In complete medium, wt cells displayed slightly more growth (though not statistically different) than did AS8 cells over the culture period (Fig. 3A). However, in both low P and low N media, AS8 cells displayed significantly more growth than did wt cells in the same medium (Fig. 3B, C). AS8 culture density was 2.3-fold higher than wt after 7 d growth in low P (Fig. 3B) and 2.1-fold higher than wt after 6 d growth in low N (Fig. 3C).

Figure 3D summarizes the growth responses by a comparison of culture density on day 5. The growth of wt cells was severely reduced by the nutrient limitations. Culture density was only 29% (low P) and 31% (low N) of that seen in complete medium. The growth of AS8 cells was only moderately reduced by these growth conditions, with culture density maintained at 75% (low P) and 82% (low N) of that seen in complete medium (Fig. 3D).

It has been consistently observed that low P-grown AS8 cell cultures were noticeably darker in colour than all other cultures by 5 d after subculture. Nonetheless, both the wt and AS8 cell cultures maintained  $>90\%$  viability (determined using Evan's Blue; Robson and Vanlerberghe, 2002) through 7 d growth in all three nutrient media (data not shown).

#### *Under macronutrient limitation, a lack of AOX increases the CUE of respiration*

The average respiration rate and RGR of wt and AS8 cells was compared in each of the three nutrient media. In low P and low N media, the RGR of wt cells is severely reduced



**Fig. 2.** Electron transport chain activities in mitochondria isolated from wt (filled columns) and AS8 (open columns) tobacco cells grown in a complete, low P or low N nutrient media for 5 d. See Materials and methods for an explanation of assay conditions. Activities measured included cyt path capacity (A, B), AOX capacity (C), NADH<sub>in</sub> (D), NADH<sub>ex</sub> (E), and NADPH<sub>ex</sub> (F). Data are the average  $\pm$  standard error from three to four independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant.

(in comparison to complete medium) yet the cells maintain a respiration rate similar to that in complete medium (Fig. 4A). In complete medium, the relationship between respiration and RGR is similar in wt and AS8. AS8 cells maintain both a slightly lower growth rate and respiration rate than do wt cells. However, the relationship between respiration and growth differs dramatically between the two cell lines when grown under nutrient limitation. In either low P or low N, AS8 maintained a lower respiration rate yet higher RGR than the wt. In summary, the ratio of respiration rate to RGR in AS8 was relatively constant in all three nutrient media, while in the wt this ratio increased dramatically with P or N limitation (Fig. 4B).

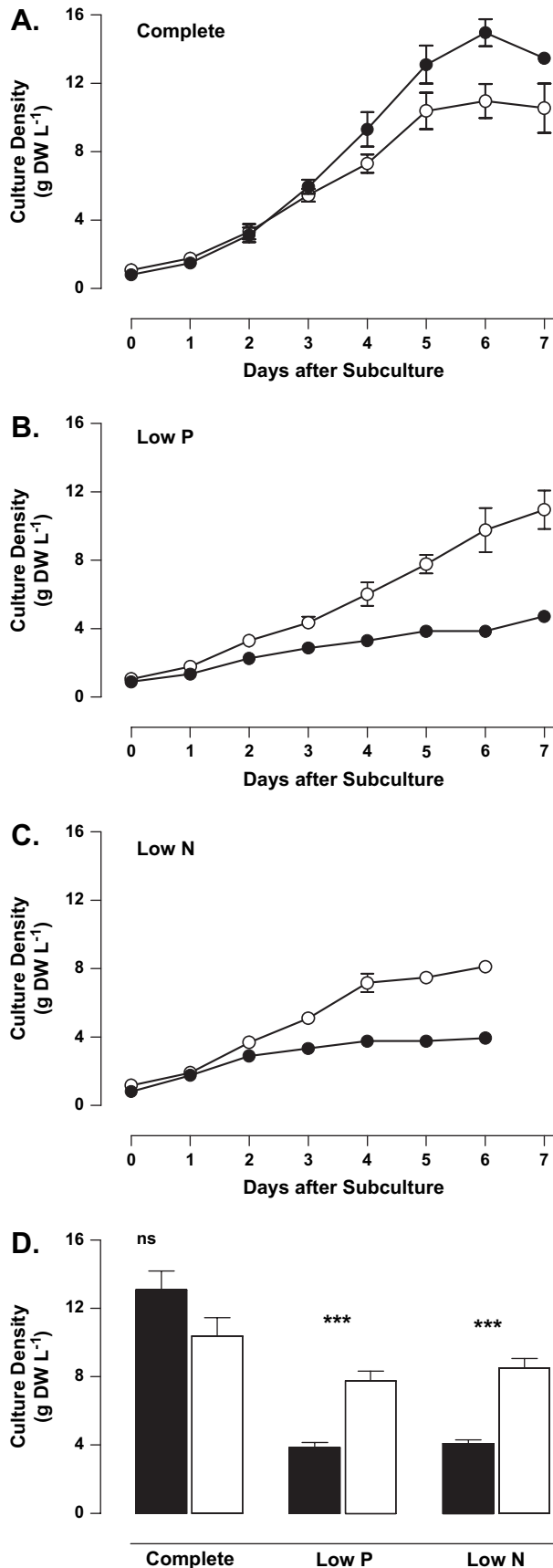
The CUE (g cell DW gain g<sup>-1</sup> sugar consumed) of wt and AS8 cells was compared under each of the growth conditions (Fig. 4C). In complete medium, both cell types displayed a CUE value of 0.51. When wt cells were grown under nutrient limitation, their CUE values dropped dramatically (by 63% in low P and 42% in low N). However,

AS8 cells in low P or low N maintained a CUE value of 0.49, similar to that in complete medium (Fig. 4C).

#### *The 'overgrowth' of cells lacking AOX during macronutrient limitation severely compromises tissue nutrient status*

The depletion of P from complete and low P medium was compared over a subculture period. Wt and AS8 cells each depleted medium P at identical rates. Complete medium P was depleted by day 4 (Fig. 5A) while low P medium P was depleted by day 1 (Fig. 5B).

The level of total cell P and cell P<sub>i</sub> (both expressed on a cell DW basis) was examined after growth of cells in complete or low P medium. In complete medium, neither total cell P (Fig. 5C) nor the level of cell P<sub>i</sub> (Fig. 5D) differed significantly between wt and AS8. Growth in low P medium reduced the level of total P in both cell lines, but the reduction was more severe in AS8 (75% reduction) than



in the wt (50% reduction). As such, the level of total P in low P-grown AS8 cells was only 55% of that seen in low P-grown wt cells (Fig. 5C).

In the wt, cell P<sub>i</sub> was unchanged after 7 d growth in low P medium versus complete medium (Fig. 5D). In AS8, cell P<sub>i</sub> was 59% lower after growth in low P medium versus growth in complete medium. As a result, the level of cell P<sub>i</sub> in low P-grown AS8 cells was only about 53% of that in low P-grown wt cells.

The N:C ratios (% N in cell/% C in cell) of wt and AS8 cells were compared in each of the growth conditions (Fig. 6). In complete medium, the N:C ratios of wt and AS8 were not significantly different. In low N, the N:C ratio of both cell lines declined compared with complete medium, but the decline was more severe in AS8 (64% reduction) than in the wt (48% reduction). As such, AS8 now had a significantly lower N:C ratio than the wt. In low P, the N:C ratio of the wt increased 1.4 fold (in comparison to complete medium) but this was not seen in AS8 (Fig. 6).

#### *Cells lacking AOX during P limitation exhibit increased expression of antioxidant enzymes*

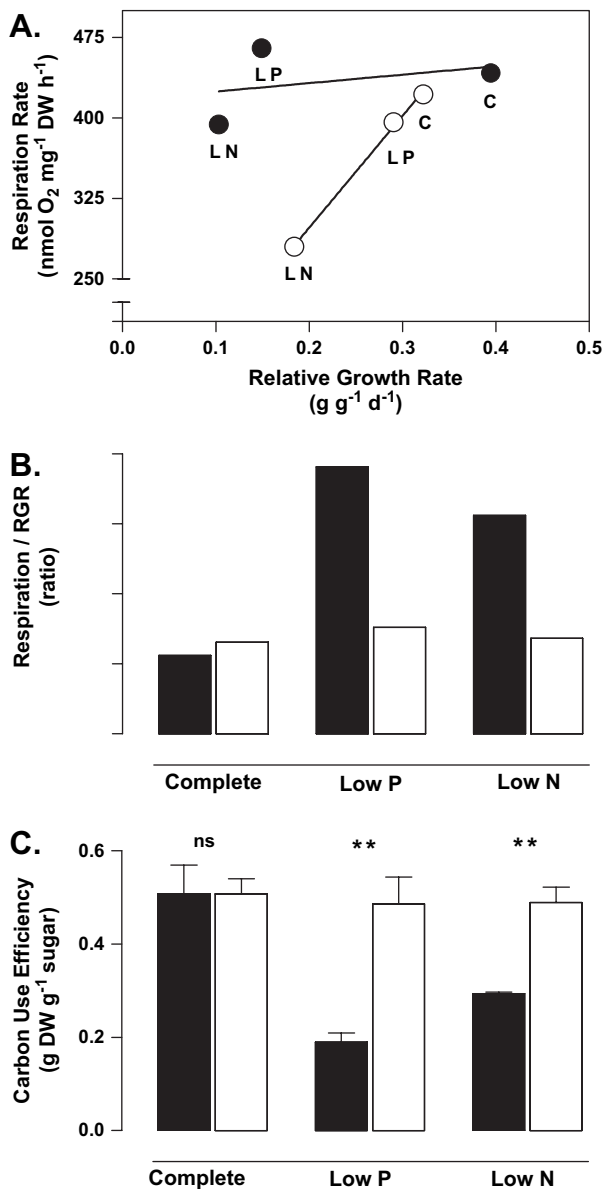
Catalase (Cat) and glutathione peroxidase (GPx) are key enzymes for scavenging of H<sub>2</sub>O<sub>2</sub> in plant cells (Mittler, 2002) while expression of pathogenesis-related (PR) proteins is mediated by ROS induced by numerous biotic and abiotic factors (Green and Fluhr, 1995).

A Cat gene transcript was nearly undetectable in wt cells, regardless of whether the cells were grown in complete, low P, or low N medium (Fig. 7). In AS8, the expression of this gene was strongly dependent upon growth conditions. Similar to wt, the transcript was nearly undetectable in low N-grown AS8. However, expression in AS8 was enhanced by growth in complete medium (in comparison to wt) and strongly enhanced by growth in low P medium.

The expression of a GPx gene transcript was also enhanced in complete medium-grown AS8 cells (in comparison to the wt) and strongly enhanced by growth in low P. In low N, transcript level was similar between the two cell lines (Fig. 7).

A PR protein (PR-1a) gene transcript was also strongly induced in low P-grown AS8 cells, while its level was relatively low in low N-grown AS8 and nearly undetectable in complete medium-grown AS8. In the wt, expression was also highest in low P but its level was much less than that of low P-grown AS8 cells. Moderate levels of

**Fig. 3.** Growth of wt (filled circles, filled columns) and AS8 (open circles, open columns) tobacco cells in different nutrient media. On day 0, wt and AS8 cells were transferred to fresh media, and growth (culture density) was assessed over a 6–7 d period. Cells were grown in complete (A), low P (B) or low N (C) media. (D) A summary of the growth responses, showing culture density on day 5. Data are the average  $\pm$  standard error from six independent experiments. In some cases, error bars are smaller than the data symbols. \*\*\*,  $P < 0.001$ , ns, not significant.



**Fig. 4.** Relationships between respiration, growth, and carbon use for wt (filled symbols, filled columns) and AS8 (open symbols, open columns) tobacco cells grown in complete (C), low P (LP) or low N (LN) nutrient media. (A) A plot of respiration rate versus relative growth rate (RGR). Respiration and RGR are each the average rate obtained between days 3 and 5 after subculture and are based on data from four to six independent experiments. For each cell line, a line of best fit is drawn through the data points corresponding to each of the different nutrient media. (B) The same data as in (A) but plotted simply as the ratio of respiration:RGR. (C) Carbon use efficiency of cells determined between days 1 and 6 after subculture. Data are the average  $\pm$  standard error from three to four separate experiments. \*\*,  $P < 0.01$ ; ns, not significant.

transcript were present in complete medium-grown wt cells and nearly undetectable levels were present in low N-grown wt cells (Fig. 7).

In summary, all three ROS-related gene transcripts were strongly enhanced in low P-grown AS8 cells in comparison to low P-grown wt. Such a strong differential expression of

these genes between the two lines was not particularly evident in complete or low N media.

#### *Cells lacking AOX during P limitation exhibit changes in their mitochondrial protein profile*

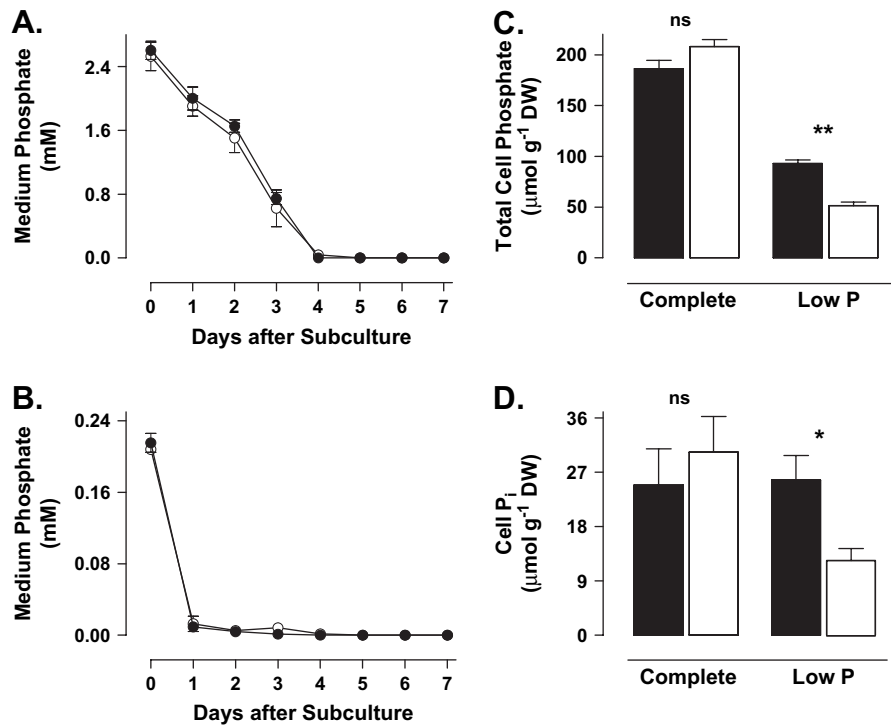
Given the large differences in numerous ETC activities between low P-grown wt and AS8 cells (Fig. 2A), the protein complement of these two contrasting mitochondria was chosen for closer examination. Mitochondria were isolated from low P-grown cells on three separate occasions and the mitochondrial proteins separated by 2-D gel electrophoresis. For each experiment, a pair-wise comparison of the 2-D gels showed that the overall protein pattern was very similar between wt and AS8 mitochondria (Fig. 8A, B). For the three experiments, a total of 280, 391, and 305 protein spots were found that co-localized between the two gels. These individual spots were quantified, and those showing at least a 2-fold difference in abundance between the two cell lines were numbered (Figs 8C, 9) and then identified by mass spectrometry. Table 1 lists proteins of decreased abundance in AS8 compared with the wt (a total of 40 proteins) while Table 2 lists proteins of increased abundance in AS8 compared with the wt (a total of 32 proteins). In each table, proteins have also been broadly classified into functional groups.

Of the 72 proteins listed in Tables 1 and 2, 55 were found in all three experiments to have at least a 2-fold difference in abundance between the cell lines. The remaining 17 protein spots differed at least 2-fold in two of the three experiments (while not differing or differing up to 1.9-fold in the third experiment), thus giving confidence to the quantitative comparisons (Tables 1, 2).

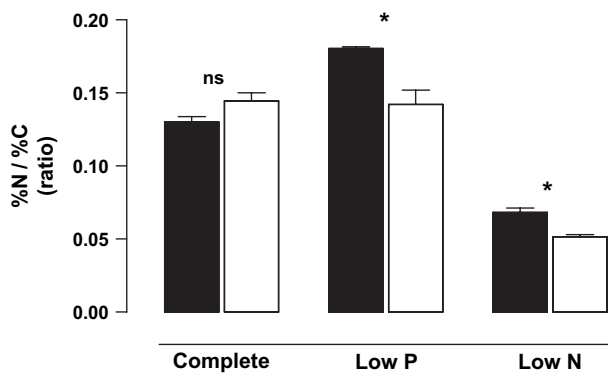
A majority of the identified proteins are known or were predicted by TargetP (Emanuelsson *et al.*, 2000) to be localized to the mitochondrion. However, some of the proteins are generally considered to be extramitochondrial, while others were predicted by TargetP to be extramitochondrial or to have an unclear localization (Tables 1, 2). It is likely that some of these proteins represent contaminants of the mitochondrial preparation.

Of all the proteins identified, nine clearly represented ETC components, including components of Complex I, III, and IV. Strikingly, all of these proteins were less abundant in AS8 than in the wt (Table 1). In most cases, abundance declined by 2–6-fold.

Ten identified proteins represented TCA cycle enzymes. However, unlike the case with ETC components, here there was no clear trend in abundance between the two cell lines. Six proteins were less abundant in AS8 (Table 1), while four were more abundant (Table 2). Among these, examples of post-translational modifications, changes in the abundance of different isoforms, and changes in the amounts of specific enzymes were seen (Tables 1, 2; Fig. 9). For example, post-translational modification(s) changing the



**Fig. 5.** Phosphate levels in nutrient media and cells. The depletion of P from complete (A) and low P (B) medium during the growth of wt (filled circles) and AS8 (open circles) cells. Data are the average  $\pm$  standard error from three to four independent experiments. In some cases, error bars are smaller than the data symbols. (C) The level of total phosphate (inorganic and organic) in wt (filled columns) and AS8 (open columns) cells after growth in complete or low P nutrient medium. Data are the average  $\pm$  standard error from three independent experiments. For each experiment, total P level was determined by taking the average of the level after 5, 6, and 7 d of growth. (D) The level of inorganic P ( $P_i$ ) in wt (filled columns) and AS8 (open columns) cells after growth in complete or low P nutrient medium for 7 d. Data are the average  $\pm$  standard error from three to four independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; ns, not significant.



**Fig. 6.** The N:C ratio of wt (filled columns) and AS8 (open columns) cells after growth in complete, low P or low N nutrient medium for 3 d. Data are the average  $\pm$  standard error from five independent experiments. \*,  $P < 0.05$ ; ns, not significant.

on-gel localization (isoelectric point, pI) of citrate synthase was seen. In one experiment, spot D20 was 2.5-fold less abundant in AS8 than in the wt, while spot I18 was 2.3-fold more abundant in AS8 (Fig. 9E, F).

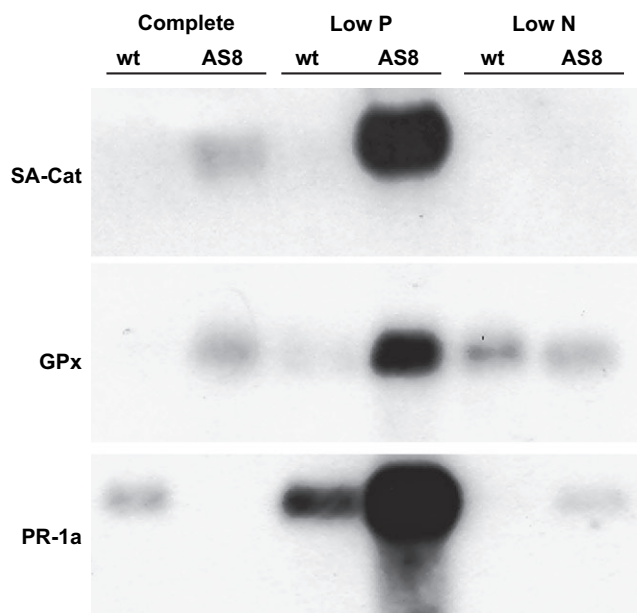
Many proteins identified have either been firmly established or suggested to play a role during cellular stresses. Most (but not all) of these proteins were increased in abundance in AS8. These included Cat, ascorbate peroxidase,

glutathione *S*-transferase (GST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), many molecular chaperones, and aldehyde dehydrogenase (ALDH) (Tables 1, 2).

Many identified proteins are enzymes in metabolism. Beside TCA cycle enzymes these included ALDH, glutamate dehydrogenase,  $\beta$ -cyanoalanine synthase (CAS), a NifS-related aminotransferase (cysteine desulphurase), a putative enoyl-CoA hydratase, a putative fumarylacetoacetate hydrolase (Table 1, hypothetical protein), GAPDH, transketolase, and nucleoside diphosphate kinase (Tables 1, 2; Fig. 9).

#### *Cells lacking AOX during nitrogen limitation accumulate excess carbohydrate*

Metabolite pools of wt and AS8 cells were examined under the different growth conditions. Figure 10A shows the total carbohydrate pools, defined here as the total of hexose equivalents found in sucrose, starch, Glu 6-P, Fru 6-P, Glu, and Fru. In complete medium, wt and AS8 maintained similar pool sizes. In low P, the carbohydrate pool of wt cells declined significantly in comparison to complete medium ( $P < 0.05$ ) while the AS8 pool remained unchanged. As such, the pool size of low P-grown AS8 cells was significantly higher than that of low P-grown wt cells. In



**Fig. 7.** Transcript levels in wt and AS8 cells after growth in complete, low P or low N nutrient medium for 5 d. Total cell RNA was separated by gel electrophoresis, transferred to a membrane, and hybridized with partial cDNAs recognizing salicylic acid-binding catalase (Cat, U03473), glutathione peroxidase (GPx, X60219), or pathogenesis-related protein 1a (PR-1a; X06361). Transcript sizes were estimated by comparison with an RNA ladder and were of the expected size (SA-Cat, 1860 bp; GPx, 816 bp; PR-1a, 783 bp). Ethidium bromide staining of rRNA bands confirmed equal loading of RNA between cell lines (not shown). Experiments were repeated and representative results are shown.

low N, both cell lines exhibited a significant increase in their carbohydrate pool (in comparison to complete medium), but the increase was more dramatic in AS8 (1.8-fold) than in the wt (1.4-fold). As such, the carbohydrate pool of low N-grown AS8 cells was significantly larger than that of low N-grown wt cells (Fig. 10A). Closer examination of sucrose levels indicated that AS8 maintained significantly higher levels than the wt, regardless of growth conditions (Fig. 10B). This analysis also indicated that sucrose alone could not account for the increase in total carbohydrate seen in low N-grown cells.

Given the large changes in the carbohydrate pools induced in both cell lines by low N (along with the large difference between the two cell lines in low N), a more detailed analysis of the low N carbohydrate pools was performed (Fig. 10D). Besides sucrose, the Fru+Fru 6-P pool was significantly higher in low N-grown AS8 cells than wt cells while the Glc+Glc 6-P and starch pools did not differ between lines.

## Discussion

### *Alternative oxidase respiration can have a negative effect on growth in cultured cells*

During nutrient-limited growth, wt tobacco cells induced large amounts of AOX and the significance of this induction

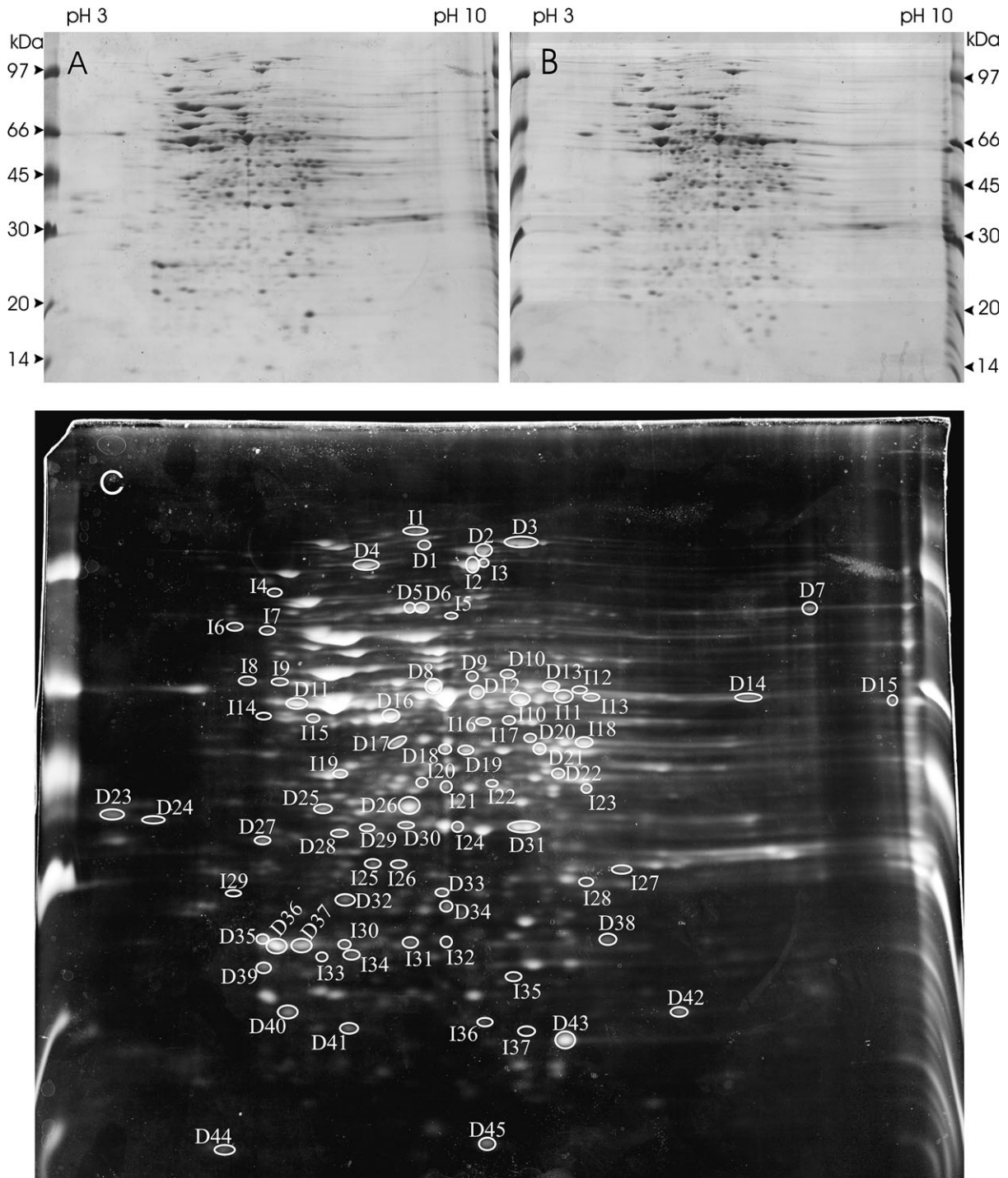
was investigated by comparing wt cells to transgenic (AS8) cells, in which AOX expression was suppressed under all conditions. The most dramatic finding of the present study was that AS8 cells lacking AOX display dramatically more growth under nutrient-limited conditions than do wt cells. These results indicate that induction of the non-energy-conserving AOX pathway in wt cells during nutrient limitation provided an important means to reduce growth, presumably since AOX respiration can reduce the energy yield supporting growth. Without AOX, energy yield is not reduced and growth continues largely unabated by the nutrient deficiency.

AOX respiration reduces the energy yield associated with carbohydrate oxidation and, since this energy supports new growth, it is expected that increased AOX respiration will decrease CUE, the efficiency with which carbohydrate consumption is coupled with cell biomass gain. Indeed, it was found that the CUE of wt cells declined abruptly during growth under nutrient limitation while no such decline occurred in AS8. It was also found that the CUE of low P-grown wt cells was significantly less than that of low N-grown wt cells ( $P < 0.01$ ), suggesting that low P growth was more dramatically uncoupling carbohydrate consumption from growth. In this respect, it is interesting that low P growth was associated with higher levels of AOX capacity than low N growth, as well as higher levels of the non-energy conserving internal and external rotenone-resistant dehydrogenases.

Studies examining the relationship between plant respiration and growth have often yielded conflicting results (Amthor, 2000; Hansen *et al.*, 2002). A potentially complicating factor in these analyses is that the growth to respiration relationship will depend upon the degree to which AOX (and other potential non-energy-conserving respiratory pathways) is contributing to respiration. The present results illustrate this point. In low P or low N-grown wt cells (when AOX is very high), the ratio of respiration to growth is much higher than in complete medium (where AOX is very low). By contrast, in AS8 (where AOX is always absent) the relationship between respiration and growth is relatively constant across growth conditions.

### *Growth reduction mediated by AOX during nutrient limitation may represent a mechanism to help maintain tissue nutrient homeostasis*

It is proposed that the growth reduction mediated by AOX represents an important means to ensure an appropriate match between growth and nutrient availability such that the tissue concentration of the limiting nutrient does not fall below some critical threshold. Supporting this, it was found that the tissue nutrient status of AS8 cells was more severely reduced by the nutrient limitations than was that of wt cells. Low P-grown wt cells were able to maintain normal levels of cellular  $P_i$ , while the  $P_i$  content of AS8



**Fig. 8.** Two-dimensional isoelectric focusing/polyacrylamide gels showing total protein patterns of mitochondria from low P-grown wt (A, C) and AS8 (B) cells. Mitochondrial protein (1.0 mg) was first separated on linear pH 3–10 immobilized pH-gradient gels and then on denaturing SDS–PAGE in the second dimension. The pH-gradient and apparent molecular masses (kDa) are indicated on the gels (A, B). Protein patterns of wt and AS8 mitochondria were compared to identify protein spots with altered abundance. Spots numbered on the negative close-up of the wt gel (C) were identified by LC-MS/MS (Tables 1, 2). In this experiment, spots I1–I37 were increased while spots numbered D1–D45 were decreased in abundance in AS8 relative to wt. Pairwise gel analyses were performed on three separate mitochondrial preparations. A representative pair of gels (A, B) is shown.

cells was severely reduced. Low P-grown AS8 cells also had significantly lower levels of total cell P than the wt. Similarly, low N-grown AS8 cells had a significantly lower N:C ratio than did low N-grown wt. It remains to be

determined whether the reduced nutrient status of AS8 cells in these experiments has generated more severe nutrient deficiency symptoms in these cells compared with the wt.

**Table 1.** Proteins of decreased abundance in mitochondria of low P-grown AS8 cells compared with mitochondria of low P-grown wt cells

Only proteins displaying at least a 2-fold decrease in abundance in at least two of three independent experiments are shown. Mitochondrial proteins were separated and quantified by 2-D gel electrophoresis and identified by mass spectrometry. Acc. no. is the Genbank accession number of the predicted protein sequence. Sp. refers to the plant species containing the predicted protein sequence: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Bp, *Betula pendula*; Br, *Brassica rapa*; Bv, *Beta vulgaris*; Ca, *Capsicum annuum*; Cl, *Canavalia lineata*; Cm, *Cucurbita maxima*; Cs, *Cucumis sativus*; Dg, *Datisca glomerata*; Ee, *Euphorbia esula*; Fa, *Fragaria x ananassa*; Hv, *Hordeum vulgare*; La, *Lupinus albus*; Le, *Lycopersicon esculentum*; Ll, *Lilium longiflorum*; Np, *Nicotiana plumbaginifolia*; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ps, *Pisum sativum*; So, *Saccharum officinarum*; Sm, *Solanum melongena*; St, *Solanum tuberosum*; Ta, *Triticum aestivum*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Mass (kDa) refers to the mass of the predicted protein. Spot refers to the protein spot on the 2-D gels (Figs 8, 9). Fold decrease refers to the fold decrease in AS8 compared with the wt. The different values (separated by commas) refer to the fold decrease in each of the three independent experiments. Cov. (%) refers to the percentage of the predicted protein sequence, including the presequence covered by matched peptides. No. of pept. refers to the number of peptides identified that matched the predicted protein. Score refers to the Mascot score. Scores above 38 for individual peptides indicate strong homology or identity. Only proteins with at least one peptide with a score of at least 38 are included in the list. Localization refers to the known location of the predicted protein. In those cases in which the localization is marked with an asterisk (\*), the location is that predicted by TargetP (<http://www.cbs.dtu.dk/services/TargetP/>). If no clear prediction was made by TargetP, then the localization is indicated as Unclear\*.

Protein prediction	Acc. no.	Sp.	Mass (kDa)	Spot	Fold decrease	Cov. (%)	No. of pept.	Score	Localization
<b>Electron transport chain</b>									
NADH-ubiquinone oxidoreductase	gi 18423437	At	19.3	D39	2.2, 3.4, 3.4	14	2	113	Mitochondrion*
NADH2 dehydrogenase (ubiquinone)	gi 1076668	St	54.3	D14	15.9, 6.0, 6.0	10	4	162	Mitochondrion
NADH-ubiquinone oxidoreductase	gi 3122572	St	80.8	D5	3.9, 1.9, 2.9	15	8	435	Mitochondrion
NADH-ubiquinone oxidoreductase	gi 3122572	St	80.8	D6	2.6, 2.1, 4.6	17	9	488	Mitochondrion
Ubiquinol-cytochrome <i>c</i> reductase I	gi 477280	St	60.1	D8	2.4, 2.1, 2.0	20	7	445	Mitochondrion
Ubiquinol-cytochrome <i>c</i> reductase II	gi 266567	St	54.8	D16	4.3, 4.7, 5.4	16	6	334	Mitochondrion
Cytochrome <i>c</i> oxidase subunit 6b-1	gi 4586449	Os	19.1	D23	4.3, 39.1, 2.6	15	3	119	Mitochondrion
Cytochrome <i>c</i> oxidase subunit 6b-1	gi 4586449	Os	19.1	D24	2.9, 11.8, 2.2	11	2	69	Mitochondrion
Cytochrome <i>c</i> oxidase polypeptide II	gi 1169038	Bv	29.7	D34	3.2, 8.9, 6.2	3	1	83	Mitochondrion
<b>TCA cycle</b>									
Pyruvate dehydrogenase E1 $\beta$ subunit	gi 1709454	Ps	39.0	D25	5.9, 4.8, 5.2	7	2	126	Mitochondrion
Aconitase	gi 11066033	Nt	98.6	D3	2.6, 2.0, 17.9	28	20	944	Mitochondrion
Citrate synthase	gi 1556429	Nt	52.7	D20	2.5, 15.2, 4.7	15	6	263	Mitochondrion
2-oxoglutarate dehydrogenase E2	gi 4210332	At	5.1	D18	2.3, 5.9, 1.3	1	1	45	Mitochondrion*
NAD-malate dehydrogenase	gi 7769871	At	37.0	D30	4.3, 5.4, 2.4	9	3	163	Mitochondrion
Malate dehydrogenase	gi 7798706	Vv	37.1	D31	2.0, 2.2, 3.2	33	9	646	Mitochondrion
<b>Potential stress-related proteins</b>									
LMW heat shock protein	gi 7159338	Ee	23.0	D35	9.8, 1.4, 26.5	5	1	75	Mitochondrion
Heat shock protein MTHSP	gi 7441310	Le	23.8	D36	13.4, 4.3, 132.4	15	4	197	Mitochondrion
Chaperone GrpE type 1	gi 3851638	Nt	33.7	D27	4.1, 2.0, 14.8	27	8	314	Mitochondrion*
Aldehyde dehydrogenase BIS1	gi 18449337	Hv	61.5	D13	8.5, 4.8, 1.4	8	4	137	Mitochondrion*
Ascorbate peroxidase thylakoid-bound	gi 4996602	Nt	47.4	D30	4.3, 5.4, 2.4	9	3	171	Plastid
Monodehydroascorbate reductase	gi 7449850	Le	47.1	D17	4.9, 6.6, 8.2	11	4	178	Cytosol
ClpB (HSP100)	gi 9651530	Pl	109.6	D2	3.6, 3.8, 17.9	11	9	361	Plastid
<b>Other proteins</b>									
$\beta$ -Cyanoalanine synthase	gi 11559260	St	38.2	D26	26.9, 5.7, 20.5	21	6	254	Mitochondrion*
Glycine dehydrogenase (P-protein)	gi 15225249	At	114.7	D2	3.6, 3.8, 17.9	5	5	252	Mitochondrion
Glutamate dehydrogenase	gi 7431768	Np	44.9	D21	4.2, 89.1, 2.9	15	5	220	Mitochondrion*
Nucleoside diphosphate kinase 3	gi 8272416	Bn	21.5	D43	3.5, 3.5, 17.3	31	7	227	Mitochondrion*
Putative enoyl-CoA hydratase	gi 14140135	Os	32.6	D33	3.4, 10.0, 65.0	3	1	54	Mitochondrion*
Hypothetical protein	gi 7485073	At	40.8	D38	4.8, 4.2, 3.0	6	2	104	Mitochondrion*
Translation elongation factor EF-TuM	gi 11181616	Zm	48.8	D19	2.0, 4.9, 7.2	25	8	290	Mitochondrion
CBS-domain containing protein	gi 15238284	At	22.8	D42	2.0, 7.3, 1.7	33	6	168	Mitochondrion*
Putative aminopeptidase	gi 15222531	At	108.6	D4	2.9, 15.5, 5.0	7	8	347	Mitochondrion/plast*
Prolyl tRNA synthetase	gi 15237183	At	60.9	D9	2.4, 3.1, 1.9	6	2	107	Mitochondrion/plast*
Catechol oxidase	gi 7432982	Nt	67.4	D10	25.7, 2.3, 6.7	9	5	137	Plastid
Protein MFP-b	gi 551444	Cs	69.9	D7	7.7, 31.0, 2.7	5	3	143	Glyoxysome
H <sup>+</sup> -ATPase $\beta$ subunit	gi 6715512	Nt	54.0	D11	3.5, 4.0, 2.1	15	6	323	Vacuole
Nucleoside diphosphate kinase I	gi 2498077	So	16.6	D41	2.6, 3.0, 2.5	10	1	41	Cytosol*
Histone H4	gi 70772	Ta	11.3	D45	20.6, 6.5, 2.2	17	2	88	Nucleus
Isopentenyl diphosphate isomerase 1	gi 13603406	Nt	33.5	D32	9.7, 3.3, 2.5	31	9	393	Plastid*
Fructokinase	gi 585973	St	34.0	D28	2.3, 2.7, 6.0	12	3	167	ER/Golgi*
ATP synthase delta chain	gi 416681	Nt	26.8	D40	2.6, $\infty$ , 2.2	5	1	52	Plastid

Interestingly, the N:C ratio of wt cells increased under low P (in comparison to complete medium) while this did not occur in AS8. This result is consistent with previous results showing that the protein:DW ratio of wt cells increased significantly under low P while this did not occur

in AS8 (Parsons *et al.*, 1999). These results suggest a reduced capacity of low P-grown AS8 cells to effectively couple C and N metabolism, perhaps related to the level of cellular stress being experienced by these cells (see below).

**Table 2.** Proteins of increased abundance in mitochondria of low P-grown AS8 cells compared with mitochondria of low P-grown wt cells

Only proteins displaying at least a 2-fold increase in abundance in at least two of three independent experiments are shown. Mitochondrial proteins were separated and quantified by 2-D gel electrophoresis and identified by mass spectrometry. Fold increase refers to the fold increase in AS8 compared with the wt. The different values (separated by commas) refer to the fold increase in each of the three independent experiments. All other details are as given for Table 1.

Protein prediction	Acc. no.	Sp.	Mass (kDa)	Spot	Fold increase	Cov. (%)	No. of pept.	Score	Localization
<b>TCA cycle</b>									
Dihydrolipoamide dehydrogenase	gi 10444388	St	53.7	I10	3.8, 4.3, 4.7	9	4	198	Mitochondrion
Aconitate hydratase	gi 1351856	Cm	98.6	I2	2.2, 2.1, 1.9	16	11	423	Mitochondrion
Aconitate hydratase	gi 3121731	St	67.5 <sup>a</sup>	I3	3.8, 2.1, 1.9	10	6	255	Mitochondrion
Succinyl-CoA ligase $\beta$ subunit	gi 15225353	At	45.6	I19	2.1, 3.7, 2.3	15	6	282	Mitochondrion*
<b>Potential stress-related proteins</b>									
Aldehyde dehydrogenase (NAD) 2A	gi 7431444	Nt	59.7	I11	2.2, 5.0, 5.5	7	5	221	Mitochondrion
Aldehyde dehydrogenase (NAD) 2A	gi 7431444	Nt	59.7	I10	3.8, 4.3, 4.7	11	7	349	Mitochondrion
NifS-related aminotransferase	gi 15239125	At	50.6	I17	3.5, 2.7, 1.9	14	4	137	Mitochondrion*
Probable glutathione S-transferase	gi 416651	Nt	25.8	I32	17.5, 2.3, 6.0	36	9	420	Unclear*
Hsp70	gi 123620	Le	71.0	I6	18.2, 6.2, 2.9	21	12	548	Unclear*
Hsp70	gi 445605	Ps	75.5	I7	4.9, 2.7, 2.9	11	6	390	Plastid
Hypersensitive-induced response protein	gi 15241939	At	31.7	I25	6.7, 3.4, 2.1	20	6	276	Cytosol*
Hypersensitive-induced response protein	gi 15241939	At	31.7	I26	18.2, 4.3, 14.0	18	5	386	Cytosol*
Ascorbate peroxidase	gi 17066705	Ca	31.8	I27	6.0, 10.1, 1.6	21	5	267	Cytosol*
Catalase	gi 481958	Sm	58.4	I12	7.5, 7.6, 18.1	5	2	147	Peroxisome
Catalase isozyme 1 (SABP)	gi 1705613	Nt	57.3	I13	6.4, 2.4, 1.3	32	13	574	Peroxisome
Hypothetical protein	gi 11278559	At	17.9	I37	8.3, 8.4, 63.8	17	3	122	Cytosol*
Glyceraldehyde 3-phosphate dehydrogenase 1	gi 16304127	Fa	45.0	I23	11.5, 4.8, 4.8	8	3	159	Plastid*
Putative heat shock protein	gi 15228059	At	89.0	I4	5.5, 1.4, 9.8	9	6	276	Plastid*
Chaperonin 21 precursor	gi 7331143	Le	26.6	I34	4.3, 2.5, 14.8	32	6	362	Plastid*
Chaperonin 21 precursor	gi 7331143	Le	26.6	I33	5.5, 4.6, 1.2	24	6	241	Plastid*
Chaperonin 60K $\alpha$ chain	gi 99801	Bn	57.7	I8	10.4, 5.6, 3.3	3	1	48	Plastid
Protein disulphide isomerase precursor	gi 11133818	Dg	57.4	I8	10.4, 5.6, 3.3	3	1	47	ER/Golgi
Chaperonin 60 $\alpha$ subunit	gi 3790441	Cl	61.5	I9	333, 11.9, 12.8	5	2	144	Plastid*
<b>Other proteins</b>									
ADP-ribosylation factor	gi 1351974	Zm	20.7	I36	13.9, 5.0, 2.4	41	7	318	Mitochondrion*
Putative quinone reductase	gi 15239652	At	21.8	I35	21.3, 3.2, 13.4	23	4	240	Unclear*
Reversibly glycosylatable polypeptide 1	gi 7488840	Ps	42.1	I21	3.1, 2.7, 1.9	12	3	159	Unclear*
Reversibly glycosylatable polypeptide (RGP1)	gi 3646373	Os	40.1	I22	4.0, 14.5, 6.3	7	2	103	Unclear*
14-3-3 protein isoform g	gi 15419924	Nt	28.7	I29	5.3, 2.3, 3.5	35	7	371	Cytosol*
Carbamoylphosphate synthetase-related	gi 18397283	At	131.0	I1	2.6, 3.1, 1.3	1	1	40	Plastid*
Transketolase TKT1 precursor	gi 7433617	Ca	80.4	I5	9.3, 41.2, 2.1	6	3	148	Plastid
Tubulin $\beta$ -2 chain	gi 8928412	La	51.0	I14	6.1, 3.3, 1.0	10	3	83	Cytosol
$\alpha$ -Tubulin	gi 6723478	Bp	50.2	I15	3.8, 3.8, 1.0	17	5	275	Cytosol*

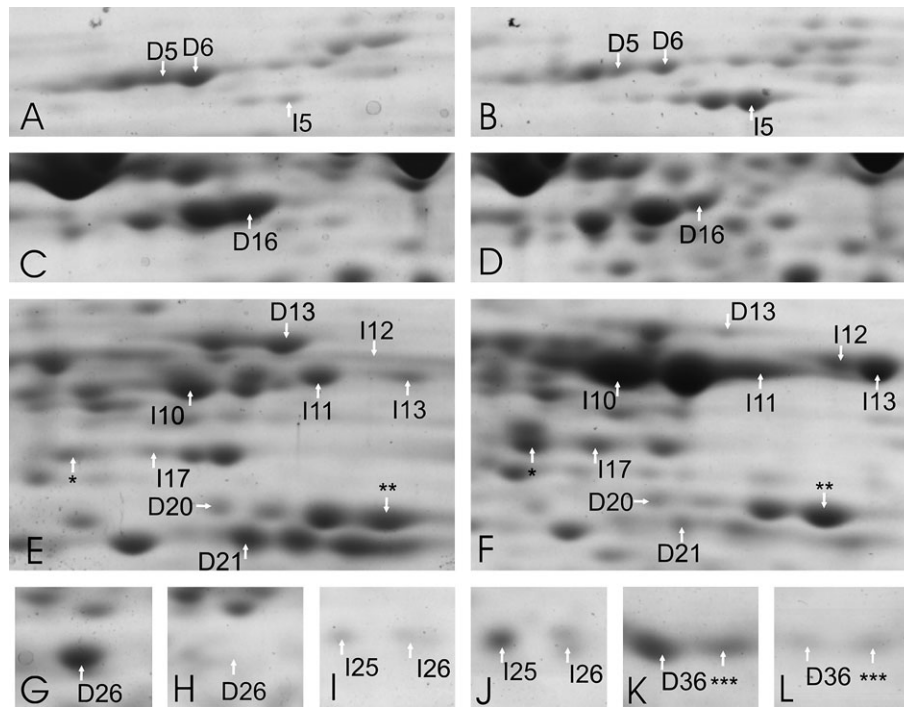
<sup>a</sup> The entry is a partial sequence covering the C-terminus.

### Possible underlying causes of AOX induction in WT cells during nutrient limitation

The cyt path capacity of wt cells was significantly reduced under low N. Since AOX expression is known to be responsive to changes in cyt pathway capacity (Vanlerberghe and McIntosh, 1996), it is possible that AOX induction under these growth conditions is occurring in response to the cyt pathway decline. Interestingly, reduced levels of cyt c (relative to cox II) were consistently seen in both wt and AS8 cells under low N, perhaps hinting at a causal relationship between cyt c level and cyt pathway capacity under these growth conditions.

The induction of AOX in wt cells under low P may relate more to regulatory aspects of respiration since here no evidence was found that cyt pathway capacity had declined (Fig. 2A). One consequence of P limitation is a significant

reduction in the level of adenylates and  $P_i$  (Theodorou and Plaxton, 1995). Since both key glycolytic reactions and oxidative phosphorylation require ADP and/or  $P_i$  as substrate, their concentration in the cytosol and mitochondrion is considered a critical factor controlling flux through respiratory pathways (the so-called adenylate control of respiration). However, plant glycolysis responds to P limitation by inducing alternate pathways that bypass adenylate and/or  $P_i$ -dependent steps, thus allowing glycolysis to continue without being subject to severe adenylate control (Theodorou and Plaxton, 1995). On the other hand, carbon flow beyond glycolysis in the TCA cycle depends upon continued ETC activity, which itself could be subject to tight adenylate control. This limitation could be partly alleviated by AOX, hence providing a biochemical rationale for AOX induction under low P.



**Fig. 9.** Close-ups of 2-D-gel protein patterns of mitochondria from low P-grown wt and AS8 cells. Comparisons of selected gel areas from wt (A, C, E, G, I, K) and AS8 (B, D, F, H, J, L). The identity of spots which decreased at least 2-fold in abundance in AS8 relative to the wt (D-spots) are listed in Table 1 while the identity of spots which increased at least 2-fold in abundance in AS8 relative to the wt (I spots) are listed in Table 2. Spots marked with asterisks were additionally identified but are not included in Tables 1 and 2 because they did not consistently show the same change in abundance in AS8 compared with the wt. In (E) and (F), spot \* (corresponding to I16 in Fig. 8C) was identified as a Nif-S related aminotransferase (identical to I17) and spot \*\* (I18 in Fig. 8C) was identified as a citrate synthase identical to spot D20. In (K) and (L) spot \*\*\* (D37 in Fig. 8C) was identified as a 27 kDa NADH-ubiquinone oxidoreductase (gi 6226880). For a detailed discussion of the spots see the Discussion.

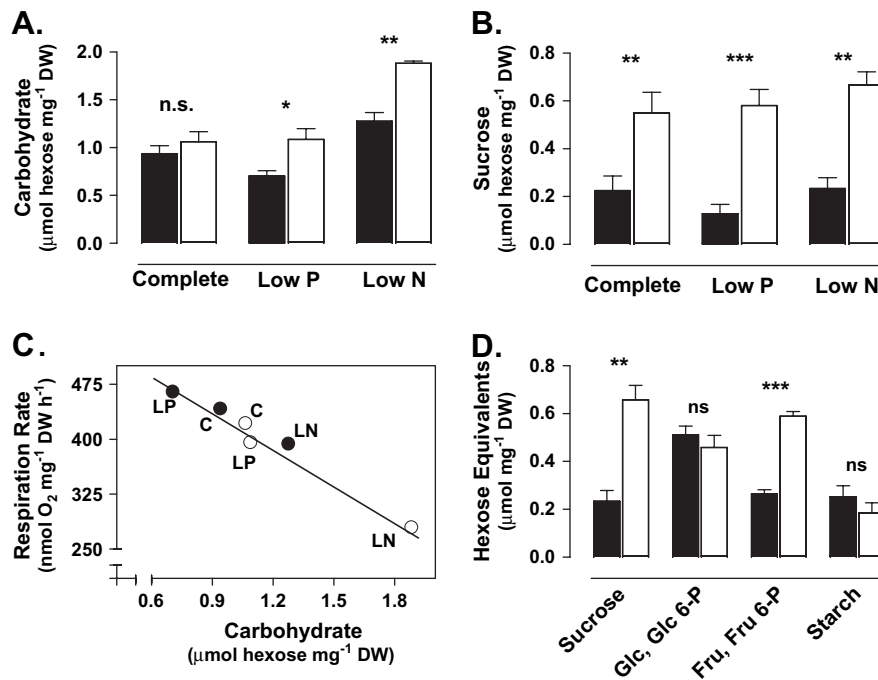
In summary, AOX induction under low N may serve to increase total ETC capacity at a time when cyt path *capacity* has been curtailed. By contrast, AOX induction under low P may act primarily to supplement a cyt path whose *activity* is being restricted by severe adenylate control. The physiological significance of AOX respiration under these two contrasting metabolic conditions can be obtained by again comparing wt cells with AS8 (see below).

#### *AOX has nutrient-specific roles in maintaining cellular redox and carbon balance*

If AOX respiration under low P acts to alleviate adenylate control at the ETC level, then it is expected that AS8 will experience a severe adenylate control under these growth conditions. One consequence of severe adenylate control at the ETC may be an increased generation of ROS due to the increased reduction state of ETC components under such conditions (Møller, 2001). Indeed, it has been shown previously that complete medium-grown AS8 cells had moderately higher ROS generation than complete medium-grown wt cells, and that this difference between cell lines became much more pronounced under low P (Parsons *et al.*, 1999).

Here, further evidence that AOX acts to dampen the generation of ROS is provided and that this role of AOX is indeed of particular importance during low P growth. Cat and GPx are two ROS-detoxifying enzymes (Mittler, 2002), while PR-1a is a protein whose expression is sensitive to ROS levels (Green and Fluhr, 1995). Low P-grown AS8 cells maintained much higher levels of all these transcripts than did low P-grown wt cells, suggesting a chronically higher rate of generation of ROS in AS8 under these growth conditions. By contrast, low N-grown wt and AS8 cells maintained similar levels of transcript, suggesting that the lack of AOX in AS8 under these conditions was inconsequential in terms of ROS generation. Severe adenylate control at the ETC level is not expected to be an inherent feature of N limitation. In complete medium, AS8 maintained marginally higher levels of Cat and GPx transcript than did wt cells, consistent with the marginally higher rates of generation of ROS by AS8 as measured previously (Parsons *et al.*, 1999).

As indicated earlier, the cyt path capacity of wt cells was significantly reduced under low N. Here, it is hypothesized that AOX induction, by providing ETC capacity, may be an important means to balance respiration rate with the external supply of carbohydrate. Interestingly, a good negative



**Fig. 10.** The level of total carbohydrate (A) and sucrose (B) in wt (filled columns) and AS8 (open columns) cells during growth in different nutrient media. Total carbohydrate is defined here as the hexose equivalents in the sum of sucrose, Glc, Glc 6-P, Fru, Fru 6-P, and starch. All data are the average  $\pm$  standard error from three to five independent experiments. For each experiment, metabolite levels were determined by taking the average of the level after 1, 2, 3, 4, 5, and 6 d of growth. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant. (C) A plot of respiration rate versus carbohydrate level for wt (filled circles) and AS8 (open circles) cells grown in complete (C), low P (LP), or low N (LN) nutrient media. Respiration and carbohydrate data are from Fig. 4A and 10A, respectively. (D) Metabolite levels in wt (filled columns) and AS8 (open columns) cells during growth in low N media. All data are the average  $\pm$  standard error from three independent experiments. For each experiment, metabolite levels were determined by taking the average of the level after 1, 2, 3, 4, 5, and 6 d of growth. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant.

correlation was found between cell respiration rate and the internal pool of carbohydrate in cells (Fig. 10C). This analysis showed that low N-grown AS8 cells maintained both a lower respiration rate and a higher pool of internal carbohydrate than all other wt or AS8 cells. A large part of the difference in carbohydrate level between low N-grown wt and AS8 cells was due to a much higher level of Fru+Fru 6-P in AS8 (Fig. 10D). This suggests a bottleneck in carbon flow from the upper half of glycolysis. These results support the idea that AOX induction during N limitation acted to maintain a balance between respiratory capacity and carbohydrate supply, thereby preventing the build-up of an excessive internal carbohydrate pool.

#### *Changes in mitochondrial protein pattern during P limitation highlight metabolic stress responses*

Two-dimensional gel electrophoresis showed that the overall pattern of mitochondrial proteins was very similar between low P-grown wt and AS8 cells, but that many proteins reproducibly differed in abundance between the cell lines. Of the 72 proteins differing in abundance, nine were ETC proteins, all of which were of decreased abundance in AS8 (Table 1; Fig. 8C, spots D8, D14, D23, D24, D34, and D39; Fig. 9A–D, K, L, spots D5, D6,

and D16). This finding is consistent with the finding that low P-grown AS8 cells had a significantly lower cyt path capacity than low P-grown wt cells.

Interestingly, the level of reduction of cyt path capacity in AS8 versus the wt under different growth conditions correlates with the degree of increased oxidative stress in AS8 versus the wt under different growth conditions. That is, the difference is most pronounced under low P, less pronounced in complete medium and perhaps absent in low N. One possibility therefore is that ROS generation in AS8 mitochondria is reducing cyt path capacity by generating oxidative damage to ETC components. Related to this suggestion, Sweetlove *et al.* (2002) compared the mitochondrial protein profiles of *Arabidopsis* suspension cells that were untreated or treated with H<sub>2</sub>O<sub>2</sub>. Of the 37 proteins investigated, 10 were ETC proteins and all of these were decreased in abundance (in some cases as breakdown products) following the oxidative stress.

Besides targeting ETC proteins, oxidative stress might be expected to affect matrix proteins. In this regard, it is interesting that five matrix proteins with decreased abundance in AS8 (D19, D21, D25, D30, and D31) are among the 20 matrix proteins identified in rice leaf mitochondria as being oxidized *in vivo* (Kristensen *et al.*, 2004). Since oxidized proteins are more susceptible to proteolytic

breakdown (Møller and Kristensen, 2004), one possible explanation for the decrease in the amount of certain matrix enzymes in AS8 is their susceptibility to oxidation. Two proteins were identified as a tobacco ALDH and were of increased abundance in AS8 (Table 2; Fig. 9E, F, spots I10 and I11). ALDHs are known to detoxify reactive aldehydes, the levels of which could increase under oxidative stress due to such processes as lipid peroxidation or defectively functioning TCA cycle enzymes (Bardel *et al.*, 2002).

Some of the proteins identified are known extra-mitochondrial proteins, while others were predicted by TargetP to be extra-mitochondrial or to have an unclear localization. Many of these (e.g. histone H4 and plastidic ATP synthase) are clearly contaminants. However, several of these potential contaminants are also seen in other studies of plant mitochondrial proteomes. For example, protein disulphide isomerases, GSTs, transketolase, and GAPDH have been identified (Sweetlove *et al.*, 2002; Giegé *et al.*, 2003; Heazlewood *et al.*, 2003). Recently, Giegé *et al.* (2003) provided evidence that GAPDH (as well as several other glycolytic enzymes) are functionally associated with the outer mitochondrial membrane. Hence, some apparent contaminants in mitochondrial protein profiles may actually represent true functional associations and it is intriguing to hypothesize that some of the 'stress proteins' discussed below might be acting in this manner.

GST was identified as a more abundant protein in AS8. GSTs are present in mammalian mitochondria where they defend against oxidative stress, in part due to their capacity to conjugate metabolites arising from oxidative damage (Raza *et al.*, 2002). Localization of GSTs to plant mitochondria is not unequivocally established, but when *Arabidopsis* cells were exposed to oxidative stress, a GST was identified in the mitochondrial fraction (Sweetlove *et al.*, 2002). The targeting prediction of the GST database entry matched in this study was unclear, suggesting either cytosolic or mitochondrial localization (Table 2, spot I32). Other GSTs that did not change in a reproducible way in all experiments were also identified on the gels (Fig. 8, spots I30 and I31). One of these (I30) was predicted to be mitochondrial and displayed 86% sequence similarity to I32.

A protein strongly induced in AS8 is highly similar to prohibitins (Table 2, hypersensitive induced response protein; Fig. 9I, J, spots I25 and I26). In yeast and mammals, they function in the inner mitochondrial membrane as chaperones, protecting and stabilizing proteins of the respiratory chain complexes. While the protein identified here is predicted by TargetP to be cytosolic, such proteins are consistently found in mitochondrial proteomes (Kruft *et al.*, 2001; Millar *et al.*, 2001; Eubel *et al.*, 2003; Heazlewood *et al.*, 2003). Also, a rice prohibitin lacking any mitochondrial signal sequence was nonetheless confirmed to localize specifically to mitochondria (Takahashi *et al.*, 2003).

A protein increased in AS8 is highly similar to members of the universal stress protein A (UspA) family found in

bacteria, fungi, and plants (Table 2, hypothetical protein; Kvint *et al.*, 2003). In bacteria, their level is increased by a variety of stresses but their function is unknown (Kvint *et al.*, 2003).

Several ROS-detoxifying enzymes were found in increased expression and/or abundance in AS8. In particular, two Cat proteins (peroxisomal) and an ascorbate peroxidase (predicted to be cytosolic) were increased in abundance. ROS-scavenging enzymes of the mitochondrion include manganese superoxide dismutase (MnSOD), ascorbate peroxidase and peroxiredoxin (Møller, 2001) but no mitochondrial isoforms of these were identified amongst the proteins found to change at least 2-fold in AS8. The apparent lack of induction of such proteins in AS8 supports the idea that increased ROS being generated in these mitochondria can indeed accumulate, rather than being scavenged by increased levels of mitochondrial antioxidant enzymes. This could result in both oxidative stress in the mitochondrion and the movement of ROS to other cellular compartments. In both maize mitochondrial mutants and in AS8 cells, altered ETC functions increase ROS generation (Maxwell *et al.*, 1999; Karpova *et al.*, 2002), but in neither case did this alter the expression of MnSOD, despite increases in the levels of non-mitochondrial ROS-detoxifying enzymes. It is intriguing to consider that ETC-generated ROS may be a relatively weak inducer of genes encoding the mitochondrial ROS-detoxifying enzymes. Perhaps this is a necessary precondition if such ROS are to act as signalling molecules between the mitochondrion and the rest of the cell. In this case, increases in AOX expression (and possibly other non-energy-conserving ETC components) might represent the primary mitochondrial response when too much ROS is produced by the ETC.

In summary, growth of cells under low P, and in the absence of AOX (AS8), resulted in an altered abundance of numerous mitochondrial proteins in comparison to the wt. Most pronounced were the reduction of many ETC components and the increased abundance of many proteins commonly associated with stress. The results are consistent with AOX having a key role in alleviating oxidative stress under these growth conditions and highlight the metabolic stress responses of plant mitochondria.

#### *Growth modulation by AOX*

Lambers (1982) proposed that AOX in plant roots acts as an 'energy overflow' pathway that is active when there is an imbalance between the supply of carbohydrates to the roots and the demand of the roots for carbohydrate to support growth and associated processes. The present results support the idea that AOX can essentially act as an 'energy overflow' during nutrient limitation, at least in suspension cell cultures. In the experiments described here, a clear imbalance between the supply and demand for carbohydrate is introduced during P or N limitation since demand (for

growth) will be strongly curtailed by the deficiency in mineral nutrient. The major contribution of the present work, however, is establishing that in the absence of this 'energy overflow' mechanism during nutrient limitation, a strong imbalance between growth and nutrient availability develops. In other words, with the inability to correct the imbalance between carbohydrate supply and demand (by utilizing AOX), AS8 cells opt to maintain anabolism and growth (i.e. maintain demand) *despite* the mineral deficiency. The inevitable consequence of this is a drop in tissue nutrient status below that experienced by wt cells. It is concluded, therefore, that AOX respiration provides an important general mechanism by which plant cells can appropriately modulate their growth rate in response to nutrient availability. AOX respiration also had nutrient-specific roles, maintaining redox balance during P limitation and carbon balance during N limitation.

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## References

- Amthor JS.** 2000. The McCree–de Wit–Penning de Vries–Thornley respiration paradigms: 30 years later. *Annals of Botany* **86**, 1–20.
- Bardel J, Louwagie M, Jaquinod M, Jourdain A, Luche S, Rabilloud T, Macherel D, Garin J, Bourguignon J.** 2002. A survey of the plant mitochondrial proteome in relation to development. *Proteomics* **2**, 880–898.
- Church GM, Gilbert W.** 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences, USA* **81**, 1991–1995.
- Emanuelson O, Nielsen H, Brunak S, von Heijne G.** 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300**, 1005–1016.
- Eubel H, Jansch L, Braun H-P.** 2003. New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of Complex II. *Plant Physiology* **133**, 274–286.
- Giegé P, Heazlewood JL, Roessner-Tunali U, Millar AH, Fernie AR, Leaver CJ, Sweetlove LJ.** 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. *The Plant Cell* **15**, 2140–2151.
- Gonzalez-Meler MA, Giles L, Thomas RB, Siedow JN.** 2001. Metabolic regulation of leaf respiration and alternative pathway activity in response to phosphate supply. *Plant Cell, and Environment* **24**, 205–215.
- Green R, Fluhr R.** 1995. UV-B-induced PR-1 accumulation is mediated by active oxygen species. *The Plant Cell* **7**, 203–212.
- Hansen LD, Church JN, Matheson S, McCarlie VW, Thygersen T, Criddle RS, Smith BN.** 2002. Kinetics of plant growth and metabolism. *Thermochimica Acta* **388**, 415–425.
- Heazlewood JL, Howell KA, Whelan J, Millar AH.** 2003. Towards an analysis of the rice mitochondrial proteome. *Plant Physiology* **132**, 230–242.
- Hunt R.** 1990. *Basic growth analysis*. London: Unwin Hyman.
- Jones MGK.** 1981. Enzymatic assay for starch and glycogen. In: Kornberg H, Metcalfe J, Northcote D, Pogson C, Tipton K. eds. *Techniques in carbohydrate metabolism*. Amsterdam: Elsevier/North Holland, 1–13.
- Karpova OV, Kuzmin EV, Elthon TE, Newton KJ.** 2002. Differential expression of alternative oxidase genes in maize mitochondrial mutants. *The Plant Cell* **14**, 3271–3284.
- Kristensen BK, Askerlund P, Bykova NV, Egsgaard H, Møller IM.** 2004. Identification of oxidized proteins in the matrix of rice leaf mitochondria by immunoprecipitation and two-dimensional liquid chromatography-tandem mass spectrometry. *Phytochemistry* **65**, 1839–1851.
- Krufft V, Eubel H, Jansch L, Werhahn W, Braun H-P.** 2001. Proteomic approach to identify novel mitochondrial proteins in *Arabidopsis*. *Plant Physiology* **127**, 1694–1710.
- Kvint K, Nachin L, Diez A, Nyström T.** 2003. The bacterial universal stress protein: function and regulation. *Current Opinions in Microbiology* **6**, 140–145.
- Lambers H.** 1982. Cyanide-resistant respiration: a non-phosphorylating electron transport pathway acting as an energy overflow. *Physiologia Plantarum* **55**, 478–485.
- Lambers H, Posthumus F, Stulen I, Lanting L, van de Dijk SJ, Hofstra R.** 1981. Energy metabolism of *Plantago lanceolata* as dependent on the supply of mineral nutrients. *Physiologia Plantarum* **51**, 85–92.
- Larson E, Howlett B, Jagendork A.** 1986. Artificial reductant enhancement of the Lowry method for protein determination. *Analytical Biochemistry* **155**, 243–248.
- Maxwell DP, Wang Y, McIntosh L.** 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proceedings of National Academy of Sciences, USA* **96**, 8271–8276.
- Millar AH, Sweetlove LJ, Giegé P, Leaver CJ.** 2001. Analysis of the *Arabidopsis* mitochondrial proteome. *Plant Physiology* **127**, 1711–1727.
- Mittler R.** 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**, 405–410.
- Møller IM.** 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 561–591.
- Møller IM, Kristensen BK.** 2004. Protein oxidation in plant mitochondria as a stress indicator. *Photochemical and Photobiological Sciences* **3**, 1–7.
- Moore AL, Albury MS, Crichton PG, Affourtit C.** 2002. Function of the alternative oxidase: is it still a scavenger? *Trends in Plant Science* **7**, 478–481.
- Parsons HL, Yip JYH, Vanlerberghe GC.** 1999. Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. *Plant Physiology* **121**, 1309–1320.
- Raghothama KG.** 1999. Phosphate acquisition. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 665–693.
- Raza H, Robin M-A, Fang J, Avadhani NG.** 2002. Multiple isoforms of mitochondrial glutathione S-transferases and their differential induction under oxidative stress. *Biochemistry Journal* **366**, 45–55.
- Robson CA, Vanlerberghe GC.** 2002. Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. *Plant Physiology* **29**, 1908–1920.

- Rychter AM, Mikulska M.** 1990. The relationship between phosphate status and cyanide-resistant respiration in bean roots. *Physiologia Plantarum* **79**, 663–667.
- Schägger H, von Jagow G.** 1987. Tricine sodium dodecyl-sulfate polyacrylamide-gel electrophoresis for the separation of proteins in the range from 1-kDa to 100-kDa. *Analytical Biochemistry* **166**, 368–379.
- Siedow JN, Umbach AL.** 2000. The mitochondrial cyanide-resistant oxidase: structural conservation amid regulatory diversity. *Biochimica et Biophysica Acta* **1459**, 432–439.
- 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*. New York: Marcel Dekker, 265–286.
- Stitt M, Lilley RM, Gerhardt R, Heldt HW.** 1989. Metabolite levels in specific cells and subcellular compartments of plant leaves. *Methods in Enzymology* **174**, 518–552.
- Stitt M, Scheible WR.** 1998. Understanding allocation to shoot and root growth will require molecular information about which compounds act as signals for the plant nutrient status, and how meristem activity and cellular growth are regulated: opinion. *Plant and Soil* **201**, 259–263.
- Sweetlove LJ, Heazlewood JL, Herald V, Holtzapffel R, Day DA, Leaver CJ, Millar AH.** 2002. The impact of oxidative stress on *Arabidopsis* mitochondria. *The Plant Journal* **32**, 891–904.
- Takahashi A, Kawasaki T, Wong HL, Suharsono U, Hirano H, Shimamoto K.** 2003. Hyperphosphorylation of a mitochondrial protein, prohibitin, is induced by calyculin A in a rice lesion-mimic mutant *cdr1*. *Plant Physiology* **132**, 1861–1869.
- Theodorou ME, Plaxton WC.** 1995. Adaptations of plant respiratory metabolism to nutritional phosphate deprivation. In: Smirnoff N, ed. *Environment and plant metabolism: flexibility and acclimation*. Oxford: Bios Scientific Publishers, 79–109.
- Thomas H, Sadras VO.** 2001. The capture and gratuitous disposal of resources by plants. *Functional Ecology* **15**, 3–12.
- Vance CP, Uhde-Stone C, Allan DL.** 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytologist* **157**, 423–447.
- Vanlerberghe GC, McIntosh L.** 1996. Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. *Plant Physiology* **111**, 589–595.
- Vanlerberghe GC, Vanlerberghe AE, McIntosh L.** 1994. Molecular genetic alteration of plant respiration: silencing and over-expression of alternative oxidase in transgenic tobacco. *Plant Physiology* **106**, 1503–1510.
- Vanlerberghe GC, Vanlerberghe AE, McIntosh L.** 1997. Molecular genetic evidence of the ability of alternative oxidase to support respiratory carbon metabolism. *Plant Physiology* **113**, 657–661.
- Wang Y-H, Garvin DF, Kochian LV.** 2001. Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiology* **127**, 345–359.
- Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M.** 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466–469.