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Increased Respiratory Restriction during Phosphate-Limited Growth in Transgenic Tobacco Cells Lacking Alternative Oxidase[1]

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We found that mitochondrial alternative oxidase (AOX) protein and the capacity for CN-resistant respiration are dramatically increased in wild-type tobacco (*Nicotiana tabacum*) suspension-cultured cells in response to growth under P limitation, and antisense (AS8) tobacco cells unable to induce AOX under these conditions have altered growth and metabolism. Specifically, we found that the respiration of AS8 cells was restricted during P-limited growth, when the potential for severe adenylate control of respiration (at the level of C supply to the mitochondrion and/or at the level of oxidative phosphorylation) is high due to the low cellular levels of ADP and/or inorganic P. As a result of this respiratory restriction, AS8 cells had altered growth, morphology, cellular composition, and patterns of respiratory C flow to amino acid synthesis compared with wild-type cells with abundant AOX protein. Also, AS8 cells under P limitation displayed high in vivo rates of generation of active oxygen species compared with wild-type cells. This difference could be abolished by an uncoupler of mitochondrial oxidative phosphorylation. Our results suggest that induction of non-phosphorylating AOX respiration (like induction of adenylate and inorganic P-independent pathways in glycolysis) is an important plant metabolic adaptation to P limitation. By preventing severe respiratory restriction, AOX acts to prevent both redirections in C metabolism and the excessive generation of harmful active oxygen species in the mitochondrion.

C oxidation in glycolysis and the tricarboxylic acid cycle is coupled to the reduction of pyridine nucleotides, which may then be oxidized by the mitochondrial electron transport chain (Douce, 1985). The usual cytochrome (Cyt) pathway of electron transport is coupled (through the generation at three sites of a proton motive force) to the synthesis of ATP from ADP and inorganic P (Pi) by the process of oxidative phosphorylation. The terminal oxidase of this pathway, reducing oxygen to water, is the CN-sensitive Cyt oxidase. Plant mitochondria are unique in having several additional electron transport chain components, distinguished in that they bypass some site(s) of energy conservation normally associated with respiration (Douce and Neuburger, 1989). The non-phosphorylative pathways include several NAD(P)H dehydrogenases mediating rotenone-insensitive oxidation of matrix or cytosolic NAD(P)H and alternative oxidase (AOX). The rotenone-insensitive dehydrogenases are poorly characterized at the molecular level, but functionally they bypass complex I, the usual first site of energy conservation in electron transport (Soole and Menz, 1995). The CN-resistant AOX catalyzes the oxidation of ubiquinol and the reduction of oxygen to water, bypassing the last two sites of energy conservation normally associated with the Cyt pathway (Day et al., 1995; Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997).

The presence of non-energy-conserving pathways of electron transport has important implications for the regulation of respiration. Since both key glycolytic reactions and oxidative phosphorylation require ADP and/or Pi as a substrate, the absolute concentration of these compounds in the cytosol and mitochondrion is usually considered a critical factor controlling flux through respiratory pathways (the so-called adenylate control of respiration) (Dry et al., 1987). Nonetheless, the presence of non-phosphorylative pathways of electron transport could provide a mechanism whereby respiratory flux is maintained under conditions in which the availability of ADP and/or Pi might otherwise be restrictive.

P is a macronutrient that commonly limits the growth of plants. Adaptive responses to P limitation include changes in growth pattern, metabolism, and Pi transport (Lynch and Beebe, 1995; Theodorou and Plaxton, 1995; Rao, 1997; Schachtman et al., 1998). A metabolic consequence of P limitation that appears to be common to plants, plant suspension cells, and green algae is a significant reduction in the cellular level of adenylates and Pi (Miginiac-Maslow et al., 1986; Thorsteinsson and Tillberg, 1987; Duff et al., 1989b; Dancer et al., 1990; Freeden et al., 1990; Theodorou et al., 1991; Rychter et al., 1992; Jacob and Lawlor, 1993; Hoefnagel et al., 1994). For example, P-limited chemostat-grown cells of *Selenastrum minutum* (at a growth rate 36% that of P-sufficient cells) maintained 81% lower levels of Pi, 70% lower levels of ATP, and 91% lower levels of ADP than P-sufficient cells (Theodorou et al., 1991). Resupply of P to P-limited plants results in a rapid recovery of adenylate and Pi levels (Dancer et al., 1990; Hoefnagel et al., 1994; Rao and Terry, 1995; Gauthier and Turpin, 1997).

It has been hypothesized that the reduced pool of adenylates and Pi during P limitation could restrict the activity of glycolytic enzymes requiring these compounds as sub-

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strate. However, extensive studies indicate that plants respond adaptively to P limitation by inducing alternate pathways in glycolysis that effectively bypass each of the adenylate and/or Pi-dependent steps (Theodorou and Plaxton, 1993, 1995). For example, the ADP-dependent conversion of phospho*enol*pyruvate to pyruvate by pyruvate kinase is thought to be functionally eliminated from cellular metabolism during P limitation as a result of the depressed levels of ADP. However, under these conditions, enzymes that catalyze two alternate routes for the conversion of phospho*enol*pyruvate to pyruvate are induced. One route is via a phospho*enol*pyruvate phosphatase (Duff et al., 1989a), while the second involves the combined action of phospho*enol*pyruvate carboxylase, malate dehydrogenase, and NAD-malic enzyme (Duff et al., 1989b; Theodorou et al., 1991; Nagano et al., 1994).

Considering the adaptive response of glycolysis to P limitation, it has been hypothesized that the mitochondrial electron transport chain might display similar adaptive responses (Rychter and Mikulska, 1990; Theodorou and Plaxton, 1993). Induction of the non-phosphorylating, rotenone-insensitive NAD(P)H dehydrogenase(s) and/or AOX could allow continued NAD(P)H oxidation and respiratory C flow without these processes becoming severely restricted by the availability of ADP and/or Pi. A possible link between AOX respiration and P nutrition has been investigated in *Phaseolus vulgaris* (Rychter and Mikulska, 1990; Rychter et al., 1992; Mikulska et al., 1998), in suspension cells of *Catharanthus roseus* (Hoefnagel et al., 1993,1994), and in the green alga *Chlamydomonas reinhardtii* (Weger and Dasgupta, 1993; Weger, 1996). In *P. vulgaris* and *C. reinhardtii*, P-limited growth resulted in an increase in the capacity for CN-resistant respiration (Rychter and Mikulska, 1990; Weger and Dasgupta, 1993), implying that the level of AOX protein may have increased. In *P. vulgaris* and *C. roseus*, it was concluded that P limitation increased the involvement of AOX in O_2 uptake by the cells (Rychter and Mikulska, 1990; Hoefnagel et al., 1993, 1994). However, such data should be interpreted with caution, since the use of AOX inhibitors to establish the actual involvement of AOX in respiration has since been seriously questioned (Day et al., 1996).

In this study, we have taken a molecular genetic approach to investigate whether AOX respiration is an important metabolic adaptation to nutritional P limitation. We show that AOX protein increases in wild-type tobacco cells in response to P limitation and that transgenic tobacco cells unable to induce AOX have altered metabolism and growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The suspension cells used were established in 1992 and have been previously described (Vanlerberghe et al., 1994, 1997). One line was derived from wild-type leaves of the tobacco (*Nicotiana tabacum*) cv Petit Havana SR1, while the other (AS8) was derived from leaves of a transgenic plant containing an antisense construct of the nuclear gene *Aox1*, which encodes a tobacco AOX.

Cells were grown in the dark in axenic batch cultures (200 mL) on a rotary shaker (140 rpm and 28°C) and were routinely subcultured every 7 d by 14-fold dilution of the cells in fresh growth medium. The growth medium was as previously described (Linsmaier and Skoog, 1965) and contains 3% (w/v) Suc as C source. The complete medium contained 2.5 mM KH_2PO_4 as the sole source of P, while the low-P medium contained 0.25 mM KH_2PO_4. In both the complete and low-P medium, K was also being supplied as 19 mM KNO_3. Therefore, the total K was only 12% lower in the low-P medium than in the complete medium. In some cases, low-P-grown cells were supplemented with P during the culture period by the addition of a sterile solution of 2.5 mM KH_2PO_4. In all cases, the various P manipulations of the medium did not affect the pH of the culture over the experimental period.

Cell Respiratory Characteristics

Suspension cells (adjusted to a density of 0.5–1.5 mg dry weight mL^{-1} in their culture medium) were placed in a Clark-type oxygen electrode cuvette (Hansatech, King's Lynn, UK) at 28°C. Steady rates of respiratory O_2 uptake were determined after about 2 to 5 min. An uncoupler of oxidative phosphorylation, *p*-trifluoromethoxycarbonylcyanide (FCCP, 1 μM), and inhibitors of Cyt oxidase (1 mM KCN) and AOX (2 mM salicylhydroxamic acid [SHAM]) were then added as described in the text and figure legends. We have previously described the use of the inhibitors to measure respiratory characteristics of these cells (Vanlerberghe et al., 1994, 1997). While FCCP is being used to target the mitochondrion in these cells, it will also dissipate other proton gradients. The O_2 concentration in air-saturated water at 28°C was assumed to be 253 μM.

AOX Protein Analysis

Washed mitochondria were isolated from suspension cells as previously described (Vanlerberghe et al., 1994). Reducing SDS-PAGE and immunoblot analysis of the AOX protein in isolated mitochondria was performed as previously described (Vanlerberghe et al., 1998) using a monoclonal antibody raised against the *Sauromatum guttatum* AOX (Elthon et al., 1989).

Amino Acid Analysis

An aliquot of cells (4–9 mg dry weight) was rapidly harvested from a culture growing under standard conditions (140 rpm, 28°C), immediately mixed with ice-cold $HClO_4$ (10% final concentration), and frozen in liquid N_2. The sample was then allowed to thaw on ice for 1 h and was then centrifuged at 16,000*g* for 5 min at 4°C. The pellet was washed twice in distilled water, frozen, and lyophilized to determine the cell dry weight. The supernatant was neutralized with 5 N KOH, weighed to determine the extract volume, and centrifuged (16,000*g*, 5 min, and 4°C) to remove the $KClO_4$ precipitate. The supernatant was then

stored at −80°C prior to derivatization and HPLC analysis of the amino acids at the Amino Acid Analysis Facility (Biotechnology Service Centre, Department of Lab Medicine and Pathobiology, University of Toronto). In each experiment, triplicate samples were taken for each treatment and the experiment was repeated three times with different subcultures of cells.

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′-dichlorodihydrofluorescin diacetate (DCFH-DA) (Molecular Probes, Eugene, OR). Upon cell entry, deacetylation of DCFH-DA by nonspecifc esterases generates 2′,7′-dichlorofluorescin, which, in the presence of H_2O_2 and cellular peroxidases is oxidized to yield a highly fluorescent product, 2′,7′-dichlorofluorescein (Cathcart et al., 1983). This probe has been used to monitor the generation of active oxygen species (AOS) in both plant and animal cells (Royall and Ischiropoulos, 1993; Vowells et al., 1995; Allan and Fluhr, 1997; Gus-Mayer et al., 1998; Vanden Hoek et al., 1998).

For these experiments, cells 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 approximately 4 mg dry weight mL^{-1}. Cells were then incubated under standard growth conditions (140 rpm and 28°C) for 20 min, followed by the addition of 20 μM DCFH-DA (from a 20 mM stock in 100% [w/v] ethanol). In some cases, 1 μM FCCP (from a 1 mM stock in 95% [w/v] ethanol) was added 1 min prior to the addition of the DCFH-DA. An aliquot of cells (0.5 mL) was then taken periodically, immediately mixed with KCN (5 mM final concentration) to inhibit peroxidase activity, and rapidly frozen in liquid N_2. Whether these samples were further processed immediately or stored for up to 24 h in liquid N_2 was found to have no significant effect on the fluorescence subsequently measured (data not shown). For further processing, the samples were thawed, centrifuged at 16,000 rpm for 2 min, and the supernatant diluted 10-fold. Fluorescence of the diluted sample was then measured immmediately with a spectrofluorometer (model F-4000, Hitachi, Tokyo) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. In each experiment, duplicate samples were taken at each time point.

Using the above method, we found in all cases that cell samples taken immediately after the addition of DCFH-DA yielded negligible fluorescence, that fluorescence yield from cells increased linearly with time after the addition of DCFH-DA, and that no fluorescence over time was generated in the absence of either the cells or the DCFH-DA.

Other Methods

To extract total cell protein, freeze-dried cells were placed in a solution containing 2% (w/v) Na_2CO_3, 0.1 N NaOH, and 0.01% (v/v) Triton X-100. After a 6-h incubation on ice (with occasional vortexing), the solution was cleared by centrifugation, and protein in the supernatant was quantified by a modified Lowry assay (Larson et al., 1986). Cell dry weight was determined as previously described (Vanlerberghe et al., 1994). The total P and the Pi content of freeze-dried cells were determined according to the method of Ames (1966). Cell dimensions (length and width) were measured using a light microscope with a calibrated micrometer in the ocular lens. Cells for measurement were chosen randomly by selecting only those cells nearest the center of particular fields. For cells found in files, cell length is defined as the dimension perpendicular

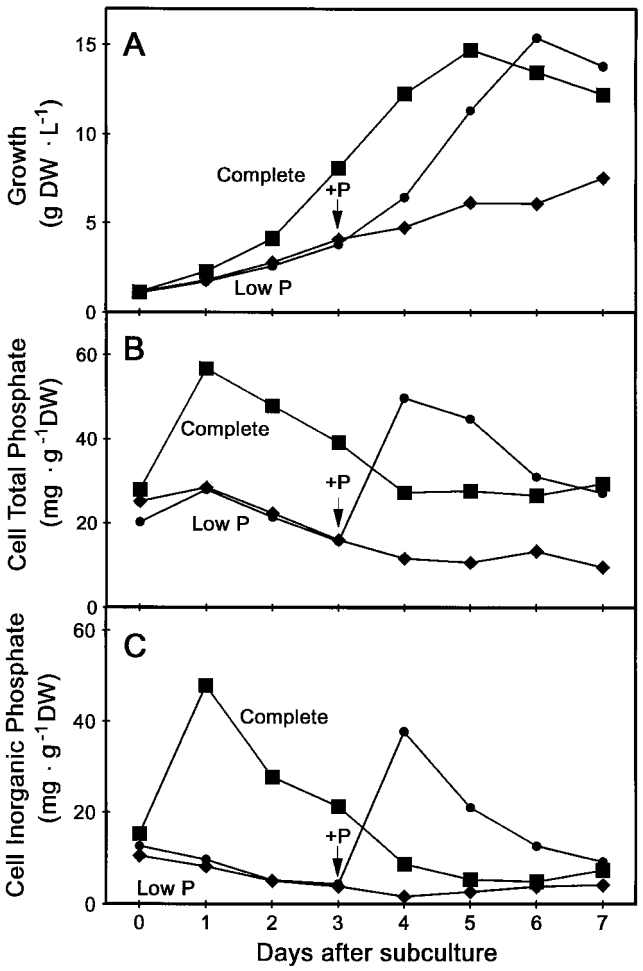

Figure 1. Growth and P content of wild-type tobacco suspension cells. At d 0, a 7-d-old culture of cells grown in complete medium was used to inoculate either fresh complete medium (■) containing 2.5 mM KH_2PO_4, or fresh low-P medium (●, ♦) containing 0.25 mM KH_2PO_4. At d 3 after subculture, one low-P culture (●) was supplemented with 2.5 mM KH_2PO_4, as indicated by the arrow. A, Growth of tobacco cells in complete or low-P medium for 7 d. B, Total P content (organic plus Pi) of tobacco cells grown in complete or low-P medium for 7 d. C, Pi content of tobacco cells grown in complete or low-P medium for 7 d. The data in B and C are the average from three independent experiments on different subcultures of cells, each of which showed similar results. The data in A are representative of experiments that were repeated several times, but not always with the same time points.

to the plane of cell division. For isolated cells, cell length is defined as the longest dimension.

RESULTS

Growth under P Limitation Increases the Level of Mitochondrial AOX Protein in Tobacco Suspension Cells

Figure 1A shows that when wild-type tobacco suspension cells were transferred to low-P medium, growth of the cells over the following culture period was significantly depressed compared with cells transferred to complete medium. Associated with this depressed growth were lower levels of both total P (Fig. 1B) and Pi (Fig. 1C) in the cells. When cells grown in low-P medium for 3 d were supplemented with P, growth recovered quickly, as did the levels of both total P and Pi in the cells (Fig. 1).

Figure 2 shows that the level of mitochondrial AOX protein was low (near the limit of detection) in wild-type cells grown in complete medium at d 3 after transfer and slightly higher by d 5. In low-P-grown wild-type cells, AOX protein was abundant at d 3 and appeared to increase further over the next 24-h (d 4) to 48-h (d 5) period. When cells grown in low-P medium for 3 d were supplemented with P, the level of AOX protein declined significantly over the next 24-h (d 4) to 48-h (d 5) period (Fig. 2).

Transgenic Tobacco Cells Unable to Induce AOX during P Limitation Have Altered Respiration, Growth, and Morphology

The respiratory characteristics, growth, and morphology of wild-type cells in complete and low-P medium were compared to that of a transgenic tobacco cell line (AS8) that contains an antisense construct of tobacco *Aox1* (Vanlerberghe et al., 1994).

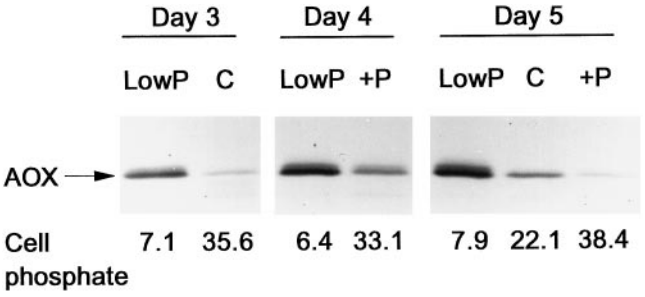

Figure 2. The level of AOX protein in wild-type tobacco suspension cells grown in complete (C) or low-P medium over a 3- to 5-d period. In some cases, a 3-d-old low-P culture was supplemented with P (+P) and the AOX protein level was examined 1 and 2 d later. For the determination of AOX protein level, washed mitochondria were isolated from cells and the mitochondrial proteins were separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX. Numbers along the bottom refer to the total (organic plus Pi) P content of the cells from which mitochondria were isolated. The experiment shown is representative of the trends in AOX protein level observed in several other independent experiments but not always with exactly the same time points being measured.

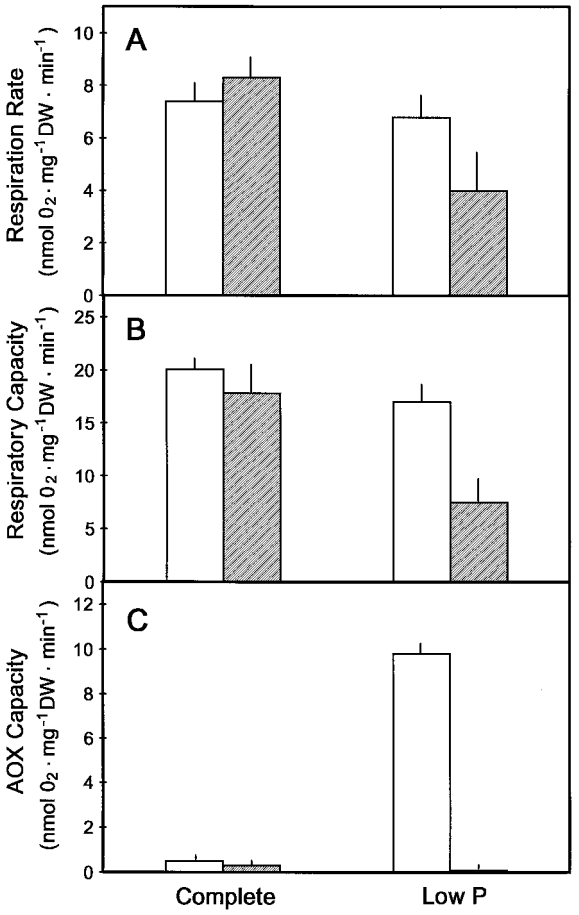

Figure 3. Respiratory characteristics (expressed on a cell dry weight basis) of wild-type (white bars) and AS8 antisense tobacco cells (shaded bars) grown in complete or low-P medium for 5 d. A, Respiration rate of cells, defined as the rate of O_2 uptake in the absence of any additions. B, Respiratory capacity of cells, defined as the rate of O_2 uptake by cells in the presence of 1 μM FCCP. C, AOX capacity of cells, defined as the rate of CN-resistant and SHAM-sensitive O_2 uptake by cells. Data are the averages ± SE from six independent experiments on different subcultures of cells.

CN and SHAM were used as inhibitors of Cyt oxidase and AOX, respectively. In Figure 3, respiratory characteristics are expressed on a cell dry-weight basis, while in Figure 4, they are expressed on a cell protein basis. After transfer to low-P medium for 5 d, wild-type cells displayed a high rate of CN-resistant, SHAM-sensitive O_2 uptake (termed AOX capacity) compared with wild-type cells grown in complete medium (Figs. 3C and 4C). These results concur with the levels of AOX protein seen in these cells (Fig. 2). AS8 cells did not display high AOX capacity when grown in low-P medium (Figs. 3C and 4C). Also, we were never able to detect any mitochondrial AOX protein in AS8 cells after 5 d of growth in either medium (data not shown).

In both wild-type and AS8 cells, O_2 uptake could be stimulated to some extent by the uncoupler of oxidative phosphorylation, FCCP. This stimulation occurred regardless of whether cells were grown in complete or low-P

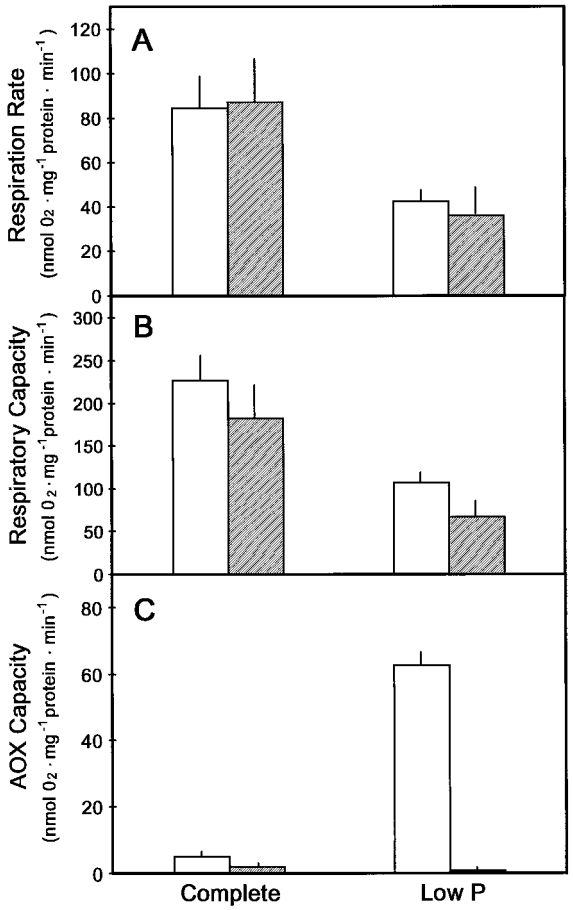

Figure 4. Respiratory characteristics (expressed on a cell protein basis) of wild-type (white bars) and AS8 antisense tobacco cells (shaded bars) grown in complete or low-P medium for 5 d. A, Respiration rate of cells, defined as the rate of O_2 uptake in the absence of any additions. B, Respiratory capacity of cells, defined as the rate of O_2 uptake by cells in the presence of 1 μM FCCP. C, AOX capacity of cells, defined as the rate of CN-resistant and SHAM-sensitive O_2 uptake by cells. Data are the averages ± SE from six independent experiments on different subcultures of cells.

medium for 5 d and ranged from 1.9- to 2.7-fold. The FCCP-stimulated rate of O_2 uptake (the respiratory capacity) did not differ between wild-type and AS8 cells when grown in complete medium (Figs. 3B and 4B). Under low P, the wild-type cells had a significantly higher respiratory capacity than AS8 (Student's unpaired *t* test, $P = 0.01$) when expressed on a dry-weight basis (Fig. 3B). The difference in respiratory capacity between wild-type and AS8 cells under low P could be accounted for by the lack of induction of AOX capacity in AS8 (compare Fig. 3, B and C). While wild-type, low-P-grown cells maintained a respiratory capacity similar ($P = 0.15$) to wild-type cells grown in complete medium (Fig. 3B), the AS8 cells under low P had a much lower respiratory capacity than complete medium grown AS8 cells ($P = 0.01$). However, when expressed on a protein basis, the difference in respiratory capacity between wild-type and AS8 cells grown under low P was not as apparent ($P = 0.11$) (Fig. 4B).

After 5 d growth in complete medium, the rate of respiration (in the absence of any additions) of wild-type cells did not differ from that of AS8 cells, whether expressed on a dry-weight (Fig. 3A) or protein (Fig. 4A) basis. After 5 d growth in low-P medium, the respiration rate of wild-type cells did not differ from that of wild-type cells grown in complete medium ($P = 0.54$) when expressed on a dry-weight basis (Fig. 3A). However, the respiration rate of AS8 cells in low-P medium was significantly lower ($P = 0.02$) than that of AS8 cells grown in complete medium when expressed on a dry-weight basis (Fig. 3A). When respiration is expressed on a protein basis, both wild-type and AS8 cells had significantly lower respiration rates when grown in low-P medium than when grown in complete medium (Fig. 4A).

The amount of culture growth (whether measured as total cell dry weight or total cell protein) did not differ between wild-type and AS8 cells after 5 d in complete medium, as shown in Figure 5, A and B. In low-P medium, both wild-type and AS8 cells displayed significantly less

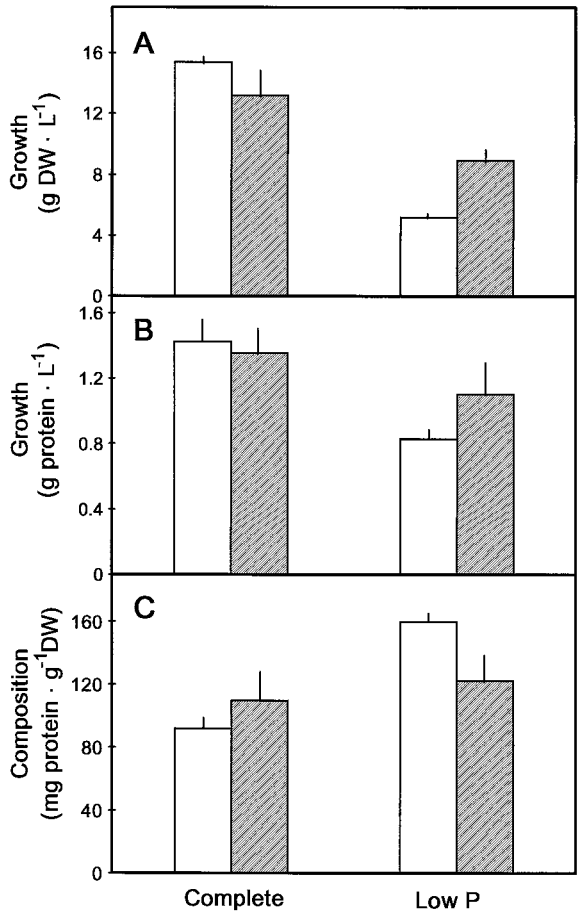

Figure 5. Growth and cellular composition of wild-type (white bars) and AS8 antisense tobacco cells (shaded bars) grown in complete or low-P medium for 5 d. A, Growth as measured by the total dry weight of cells. B, Growth as measured by the total protein of cells. C, Composition of cells, expressed as protein/dry weight. Data are the averages ± SE from six independent experiments on different subcultures of cells.

Table I. *Dimensions of wild-type and AS8 antisense tobacco cells grown in complete or low-P medium for 3 and 5 d*

Data are the average ± SE (number in brackets) from three independent experiments on different subcultures of cells (n = 100).

Growth Medium	Cell Line	d	Dimensions		
			Width	Length	Length/width
			μm		*ratio*
Complete	Wild type	3	37.8 (0.9)	44.9 (2.6)	1.19
		5	34.4 (0.6)	50.8 (2.3)	1.48
	AS8	3	22.0 (2.6)	55.0 (0.4)	2.50
		5	22.6 (2.1)	59.8 (2.4)	2.65
Low-P	Wild type	3	38.0 (0.8)	47.3 (1.8)	1.24
		5	38.4 (0.9)	58.9 (3.5)	1.53
	AS8	3	22.5 (0.5)	74.6 (3.0)	3.32
		5	23.7 (1.2)	116.2 (4.8)	4.90

growth than in complete medium when expressed on a dry weight basis (Fig. 5A). However, AS8 cells displayed significantly more growth than wild-type cells (P = 0.00) under these conditions. When expressed on a protein basis, the difference in growth between wild-type and AS8 cells in low-P medium was not as apparent (P = 0.22).

The above data indicate that interpretation of the respiration and growth responses of wild-type and AS8 cells to the low-P treatment is dependent, to some extent, upon whether data is expressed on a dry-weight or protein basis. This appears to be due to a differential change in cellular composition (protein/dry weight) of wild-type and AS8 cells during growth under low P. For wild-type cells, the protein/dry weight increases significantly during growth under low P (P = 0.00) while in AS8, no significant change in this composition occurs (P = 0.62) (Fig. 5C).

Table I shows dimensions (length and width) of wild-type and AS8 cells after 3 and 5 d of growth in either complete or low-P medium. There were striking differences between the dimensions of wild-type and AS8 cells grown in complete medium, as well as in the response of wild-type and AS8 cells to low-P medium. These differences can be illustrated most simply by examining the length to width ratio of the cells (Table I). In the wild-type, the length to width ratio of cells increased marginally between d 3 and 5 after subculture, regardless of the growth medium. Furthermore, the length to width ratio of the wild type in low-P medium was only slightly higher than in complete medium on either day. AS8 cells grown in complete medium had a much higher length to width ratio than wild-type cells grown in complete medium. Like wild-type cells, this ratio in AS8 cells in complete medium increased only marginally between d 3 and 5. Unlike wild-type cells, the length to width ratio of AS8 cells was dramatically affected by growth in low P. On d 3, the length to width ratio was much higher in low-P medium than in complete medium, and this effect became even more pronounced by d 5.

Transgenic Tobacco Cells Unable to Induce AOX during P Limitation Have Altered Levels of Free Amino Acids

The level of individual free amino acids (the 20-protein amino acids) was determined in wild-type and AS8 cells after 5 d of growth in complete or low-P medium. In Figure 6, the amino acids have been subsequently grouped into five families based on the primary respiratory C intermediate required for their synthesis (2-oxoglutarate, oxaloacetate, pyruvate, phospho*enol*pyruvate, or phosphoglycerate), while in Table II the level of individual amino acids is shown.

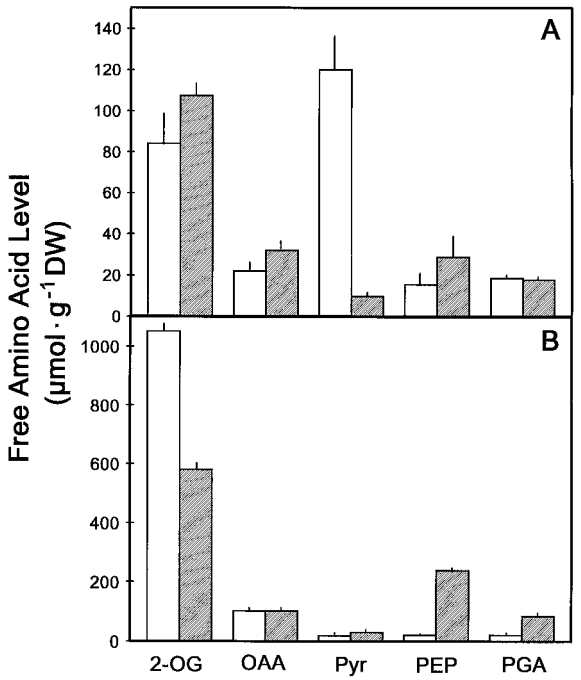

Figure 6. Level of free amino acids in wild-type (white bars) and AS8 antisense tobacco cells (shaded bars) grown in complete (A) or low P (B) medium for 5 d. Note the different scale in A and B. The amino acids have been grouped into five families (excluding His) based on the primary respiratory C intermediate required for their synthesis. These families are: 2-oxoglutarate (2-OG), Arg, Gln, Glu, Pro; oxaloacetate (OAA), Asn, Asp, Ile, Lys, Met, Thr; pyruvate (Pyr), Ala, Leu, Val; phospho*enol*pyruvate (PEP), Phe, Trp, Tyr; and phosphoglycerate (PGA), Cys, Gly, Ser. Also, the level of some select individual amino acids from these data is presented in Table II. Data are the averages ± SE from three independent experiments on different subcultures of cells.

Table II. *The level of free amino acids in wild-type and AS8 antisense tobacco cells grown in complete or low-P medium for 5 d*

Data are the average ± SE (number in brackets) from three independent experiments on different subcultures of cells.

Amino Acid	Complete Medium		Low-P Medium	
	Wild type	AS8	Wild type	AS8
			μmol g⁻¹ dry wt	
Ala	116 (16)	7 (2)	6 (1)	25 (2)
Asn	9 (2)	8 (0.3)	68 (3)	68 (4)
Asp	3 (1)	10 (1)	8 (0.3)	12 (1)
Gln	57 (8)	67 (8)	1,018 (18)	546 (18)
Glu	22 (6)	38 (3)	21 (1)	31 (0.4)
Ser	6 (1)	13 (1)	6 (2)	67 (5)
Tyr	3 (0.3)	9 (1)	14 (0.2)	213 (8)
Total[a]	265 (40)	201 (5)	1,232 (31)	1,041 (20)

[a] This is the sum of the free amino acid levels of all 20 of the protein amino acids (see also Fig. 6).

In complete medium, wild-type and AS8 cells maintained similar pool sizes of all of the 20 amino acids, with one notable exception. The level of Ala was approximately 18-fold higher in wild-type cells than in AS8 (Table II). As a result, Ala represented 44% of the total amino acid pool in wild-type cells but only 3% of the total pool in AS8 cells. This difference in Ala level accounts for the large difference between wild-type and AS8 cells in the level of the pyruvate family of amino acids (Fig. 6A). Alternatively, wild-type and AS8 cells maintained similar pool sizes of amino acids derived from 2-oxoglutarate, oxaloacetate, phospho*enol*pyruvate, and phosphoglycerate (Fig. 6A).

In low-P medium, both wild-type and AS8 cells had a much higher total free amino acid pool (4.7-fold higher in wild-type; 5.2-fold higher in AS8) (Table II). In wild-type cells, this increase was the result of a massive (18-fold) accumulation of Gln, which now accounted for 83% of the total amino acid pool (Table II). In AS8, the increase was due to a large increase in Gln (8-fold), Tyr (24-fold), and Ser (5-fold) (Table II). While Tyr and Ser account for less than 2% of the total amino acid pool of low-P-grown wild-type cells, they account for 27% of the total amino acid pool of low-P-grown AS8 cells. As a result, AS8 cells maintained a lower pool of the 2-oxoglutarate family of amino acids than wild-type cells, but a much higher pool of the phospho*enol*-pyruvate and phosphoglycerate families of amino acids (Fig. 6B). The level of the pyruvate family of amino acids was similar between wild-type and AS8 cells when grown in low-P medium and was very low (Fig. 6B).

Transgenic Tobacco Cells Unable to Induce AOX during P Limitation Display High Rates of Intracellular H_2O_2 Generation

Rates of cellular H_2O_2 generation were determined for wild-type and AS8 cells grown in complete or low-P medium using the cell-permeable probe DCFH-DA (Cathcart et al., 1983). Figure 7A shows that after growth in complete medium, there was a tendency for AS8 cells to display higher rates of H_2O_2 production (measured as increasing fluorescence over time) than wild-type cells, although this difference was not significant. For both wild-type and AS8 cells, the rate of H_2O_2 production was significantly reduced by FCCP (Fig. 7A).

After growth in low-P medium, AS8 cells displayed dramatically higher rates of H_2O_2 generation than low-P-grown wild-type cells (Fig. 7B). This high rate of H_2O_2 generation in AS8 cells could be reduced to that of the wild-type cells by FCCP (Fig. 7B). FCCP addition had no effect on the rate of H_2O_2 production by low-P-grown wild-type cells.

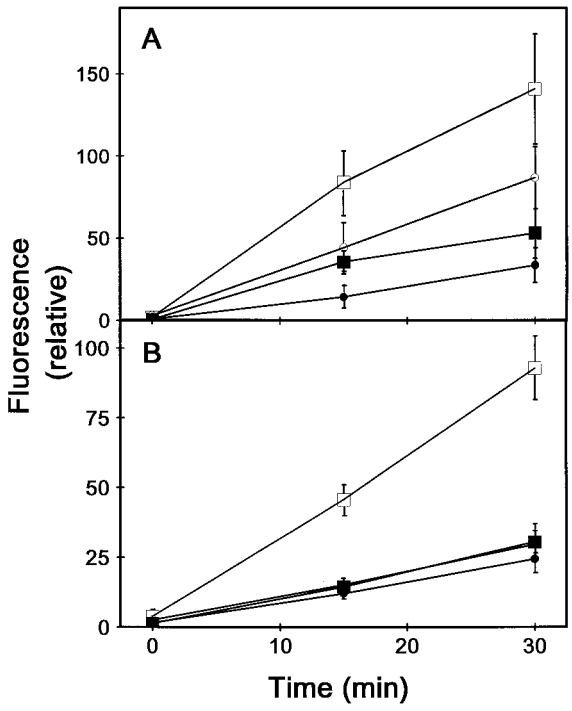

Figure 7. Generation of AOS by wild-type (circles) and AS8 antisense tobacco cells (squares) grown in complete (A) or low-P (B) medium. Cells were either untreated (white symbols) or treated with 1 μM FCCP (black symbols). Data represent the fluorescence yield from cells at a density of 4 mg mL^{-1}. Data are the averages ± SE from five independent experiments on different subcultures of cells.

DISCUSSION

When the growth of wild-type tobacco suspension cells was limited by P, there was a large induction of mitochondrial AOX protein (Fig. 2), resulting in a large increase in the capacity for CN-resistant, SHAM-sensitive O_2 uptake (AOX capacity) by the cells (Figs. 3C and 4C). It was previously reported that when bean plants were grown without P, there was an increased capacity for CN-resistant respiration, suggestive of an induction of AOX protein (Rychter and Mikulska, 1990). When we grew tobacco plants on agar in complete or low-P nutrient medium, the low-P-grown plants maintained much higher levels of AOX protein, the level of which declined upon P refeeding (data not shown). Our results, along with those of Rychter and coworkers (1992) and others (see introduction), indicate that plants and suspension cells of a range of species respond to P limitation by inducing AOX.

The uncoupler FCCP induced a large increase in O_2 uptake by wild-type cells whether grown in complete or low-P medium (Figs. 3 and 4). This indicates that respiration in both cases was limited by the availability of ADP (Dry et al., 1987), either at the level of substrate supply to the mitochondrion (adenylate control of glycolysis) and/or at the level of the mitochondrial electron transport chain (adenylate control of oxidative phosphorylation). Therefore, the large induction of AOX protein in low-P-grown wild-type cells does not release respiration in these cells from adenylate control. Active AOX respiration in these cells might nonetheless be functioning to relieve the degree of adenylate control, thus allowing the cells to maintain high respiration rates when the potential for severe adenylate restriction of respiration (due to low cellular levels of ADP and/or Pi) is high. If AOX does have such a function in wild-type cells, then AS8 cells unable to induce AOX (Figs. 3C and 4C) might be expected to display a more severe restriction of respiration than wild-type cells, particularly when grown at low P.

Approaches were taken to investigate whether low-P growth conditions resulted in a more pronounced restriction of respiratory metabolism in AS8 than in wild-type cells. Figure 3A compares the rates of O_2 uptake (expressed on a dry-weight basis) by the wild type and AS8 in the two growth media. This analysis showed that low-P-grown wild-type cells had O_2 uptake rates similar to that of wild-type cells grown in complete medium, and that, when grown in complete medium, wild-type and AS8 cells had similar rates of O_2 consumption. However, when grown in low-P medium, AS8 cells had lower rates of O_2 uptake than AS8 cells grown in complete medium (Fig. 3A), indicating that AS8 responds differently than the wild type to growth under low P. Such a differential response was also seen when the composition (protein/dry weight) of wild-type and AS8 cells was compared. The composition of wild-type cells was dramatically affected by the low-P treatment, but this did not occur in AS8. While this is additional evidence that AS8 cells respond differently than wild-type cells to the low-P treatment, it also complicates the respiratory analysis. When O_2 uptake is expressed on a protein basis, no measurable difference in the response of wild-type and AS8 cells to low P was observed (Fig. 4A).

Given the above findings, other approaches were taken to investigate whether AS8 cells display more severe respiratory restriction than wild-type cells when grown under low P. One approach was to look for evidence of such restriction at the level of respiratory C metabolism. Our hypothesis was that a more severe restriction of C flow through glycolysis and/or the tricarboxylic acid cycle in AS8 cells than in the wild type might result in some redirection of C metabolism. To examine this possibility, we measured the level of free amino acids in the cells. This approach is useful in that the synthesis of specific amino acids is dependent upon the supply of specific C intermediates in glycolysis and the tricarboxylic acid cycle (Ireland, 1997). Therefore, the level of individual amino acids may, at least in part, indicate the relative availability of different respiratory intermediates.

Ala was the most abundant amino acid in wild-type cells grown in complete medium, representing 44% of the total amino acid pool (Table II). Ala is often a dominant amino acid in plant tissues (Ireland, 1997). In comparison, the Ala pool in AS8 cells grown in complete medium was severely depressed, representing only 3% of the total amino acid pool. This result is consistent with the idea that in complete medium, the lack of AOX in AS8 compared with wild-type cells restricts the availability of pyruvate for Ala synthesis.

Note that even in complete medium, wild-type cells had low levels of AOX protein, while AS8 cells had no detectable protein (Fig. 2; data not shown). The conversion of phospho*enol*pyruvate plus ADP to pyruvate plus ATP by cytosolic pyruvate kinase is considered a potentially critical point at which the rate of C flow in respiration is subject to biochemical controls of enzyme activity and regulation by the availability of ADP (ap Rees et al., 1977; Day and Lambers, 1983; Day et al., 1985; Douce, 1985; Copeland and Turner, 1987; Turpin et al., 1990; Plaxton, 1996).

Our results suggest that in wild-type cells grown in complete medium, a low level of AOX activity relieves the adenylate control of pyruvate kinase to some extent, allowing sufficient pyruvate synthesis to support respiration and the maintenance of a large Ala pool. However, in AS8 cells lacking AOX, pyruvate synthesis is subject to more severe adenylate control, allowing only enough pyruvate synthesis to support respiration and the maintenance of a small Ala pool. Ala was the only amino acid for which there was a substantial difference between the wild-type and AS8 cells, indicating that under P-sufficient growth conditions only the availability of the intermediate pyruvate for amino acid synthesis is likely to be dramatically impacted in AS8 by the lack of AOX. This is consistent with studies in a range of plant tissues and under a range of experimental conditions suggesting that pyruvate kinase is a critical step in the regulation of glycolysis and respiration (Plaxton, 1996).

When grown under P limitation, both wild-type and AS8 cells maintained a much larger total pool of amino acids than when grown in complete medium (Table II). In wild-type cells, this was due to a massive accumulation of Gln (Fig. 6B; Table II). A dramatically enlarged free amino acid

pool in response to P limitation was also seen in tobacco seedlings (Nielsen et al., 1998) and in suspension cells of *Catharanthus roseus* (Ukaji and Ashihara, 1987; Nagano and Ashihara, 1993) and *Brassica nigra* (Duff et al., 1989b). When the level of individual amino acids was reported (Ukaji and Ashihara, 1987; Nielsen et al., 1998), the increase was largely due to an increase in Gln, as we found. The large increase in the Gln to Glu ratio during P limitation (Table II) indicates that there is ample N available to the Gln synthetase-Glu synthase cycle, and that it is the availability of C (2-oxoglutarate) to the cycle that limits N assimilation under these growth conditions (Vanlerberghe et al., 1990). In AS8, Gln accumulated to a lesser extent than in the wild type and was accompanied instead by large accumulations of Ser and Tyr. Presumably, the lack of AOX in AS8 results in a greater restriction of C flow than in the wild type, such that there is less accumulation of Gln (an amino acid derived from a downstream C intermediate in respiration) but a significant accumulation of Ser and Tyr (amino acids derived from upstream C intermediates).

When grown under P limitation (unlike in complete medium), the pool of Ala (and the other pyruvate family amino acids) was similar and low in wild-type and AS8 cells (Fig. 6B). In fact, this was the only family of amino acids to actually decline in wild-type cells in response to P limitation. These results suggest that the availability of pyruvate for Ala synthesis is limited during P limitation, and that the presence or absence of AOX now has little impact on this availability. It may be that under P limitation, the adenylate control of pyruvate kinase in the cytosol is so severe that AOX activity cannot relieve it. Alternatively, it may indicate that pyruvate is now being supplied by another route, which is not directly subject to adenylate control and hence not impacted by the level of AOX activity. Such bypasses of pyruvate kinase (which have been reported to be induced during P limitation) include phospho*enol*pyruvate phosphatase (Duff et al., 1989a) or the combined action of phospho*enol*pyruvate carboxylase, malate dehydrogenase, and malic enzyme (Duff et al., 1989b; Theodorou et al., 1991; Nagano et al., 1994).

Another approach to investigate whether AS8 cells display more severe respiratory restriction than wild-type cells when grown under low P was to look for evidence of such restriction at the level of the mitochondrial electron transport chain. It has been established from both in organello and in vivo studies that mitochondrial electron transport is a major source of generation of AOS in eukaryotic cells, including plant cells (Puntarulo et al., 1991; Shigenaga et al., 1994; Purvis et al., 1995; Longo et al., 1996). A major source of these AOS is at complex III, since a transient quinone radical (ubisemiquinone) is an intermediate in the chemiosmotic Q cycle but can also participate in a one-electron transfer to molecular oxygen to generate the superoxide anion (Boveris et al., 1976; Turrens et al., 1985). The superoxide anion may then be rapidly converted to H_2O_2 by mitochondrial superoxide dismutase (Bowler et al., 1989).

The rate of superoxide generation by complex III is highly dependent upon the proton motive force across the inner mitochondrial membrane, since increasing the proton motive force increases the half-life of ubisemiquinone. It has been shown that chemical inhibition of downstream electron transport chain components or an ADP or Pi limitation of oxidative phosphorylation strongly promotes AOS generation, while the addition of ADP/Pi or protonophorous uncouplers strongly inhibits such AOS generation (Budd et al., 1997; Korshunov et al., 1997). We hypothesized that restricted activity of the Cyt pathway as a result of severe adenylate control of oxidative phosphorylation during P-limited growth could promote an over-reduction of electron transport chain components and the associated generation of AOS. However, induction of non-phosphorylating AOX respiration under low P might function to prevent such over-reduction, thereby playing an important role in preventing the generation of deleterious AOS. To our knowledge, a potential function for AOX in preventing the generation of AOS was first proposed by Purvis and Shewfelt (1993), with regard to the damaging effects of chilling temperatures on mitochondrial electron transport. The hypothesis has since been expanded upon (Millar and Day, 1997; Wagner and Moore, 1997).

If the induction of AOX in wild-type cells during P-limited growth does indeed function to prevent over-reduction of electron transport chain components, then AS8 cells unable to induce AOX may be expected to have higher rates of generation of AOS, and this is indeed what we observed. Particularly under low-P conditions (but also to a lesser extent in complete medium), AS8 displayed higher rates of in vivo H_2O_2 generation than the wild type (Fig. 7). H_2O_2 generation could be largely abolished by an uncoupler of oxidative phosphorylation, FCCP. This is consistent with the H_2O_2 generation being dependent upon the proton motive force. Interestingly, FCCP significantly decreased H_2O_2 generation in all cells in which AOX protein was low or absent (wild-type, complete medium; AS8, complete medium; AS8, low-P medium), but had no significant impact on the low rate of H_2O_2 generation by the low-P-grown wild-type cells with abundant AOX protein (Fig. 7).

Recent studies in the literature support a role for AOX in preventing the generation of AOS. Studies with isolated plant mitochondria showed that chemical inhibition of AOX increased the generation of AOS (Popov et al., 1997; Purvis, 1997). Also, Purvis (1997) showed that activation of AOX activity by treatment of mitochondria with dithiothreitol slowed the generation of AOS, while inactivation of AOX by diamide treatment stimulated AOS production. Furthermore, chemical inhibition of complex III by antimycin A dramatically stimulated H_2O_2 production by cells lacking AOX compared with cells with abundant AOX (McIntosh et al., 1998). Each of the above studies, while hindered by the use of inhibitors and other compounds that may complicate interpretation of the results (for a discussion of this, see Purvis et al., 1995; Purvis, 1997), do support a role for AOX in preventing the generation of AOS.

Interestingly, H_2O_2 treatment of tobacco cells caused a rapid increase in *Aox1* mRNA and AOX capacity (Vanlerberghe and McIntosh, 1996). Therefore, the accumulation of AOS in the cell as a result of over-reduction of mitochondrial electron transport chain components may act as an important signal to induce the synthesis of additional AOX

protein so that it can perform its antioxidant function. An intermediate in the regulation of AOX gene expression by H_2O_2 may be citrate, since H_2O_2 treatment causes citrate accumulation, and citrate treatment of cells in itself can induce the synthesis of AOX (Vanlerberghe and McIntosh, 1996). Citrate accumulation is possibly the result of H_2O_2 inhibition of aconitase. In a wide range of organisms, including plants, aconitase appears to be a particularly sensitive mitochondrial target to oxidative inactivation (Gardner and Fridovich, 1992; Verniquet et al., 1991; Yan et al., 1997; Melov et al., 1999). Whether AOX plays a role in protecting plant mitochondrial aconitase against such inactivation is not yet known.

A striking difference between wild-type and AS8 cells is their morphology (Table I), but we do not yet understand the basis for this difference. The difference in morphology became most pronounced during prolonged growth in low-P medium, and may explain at least in part why the cellular composition (protein/dry weight) of wild-type and AS8 cells differs during growth under low P (Fig. 5C). However, there were no significant difference between wild-type and AS8 cells in cellular levels of starch, malate, or citrate after 5 d growth in low-P medium (H.L. Parsons, J.Y.H. Yip, and G.C. Vanlerberghe, unpublished data).

This study compared wild-type tobacco cells and antisense (AS8) cells that completely lack the non-phosphorylating AOX respiration pathway. In complete medium, wild-type cells maintained only low levels of AOX protein, but, even under these growth conditions, some clear differences were seen between the wild-type and AS8. In particular, Ala represented a much smaller percentage of the total amino acid pool in AS8 than in wild-type cells (Table II). This may indicate that a lack of AOX results in a more severe adenylate control of cytosolic pyruvate kinase, a glycolytic step often suspected to be subject to such control, but for which convincing data have been difficult to obtain. In low-P medium, wild-type cells induced large amounts of AOX protein, but this response was completely suppressed in AS8. As a result, while the respiration rate of wild-type cells under low P was very similar to that in complete medium, the respiration of AS8 cells was restricted (Fig. 3A). Despite this restricted respiration, growth of the AS8 culture under low P was either similar to (when measured on a protein basis; Fig. 5B) or greater than (when measured on a dry weight basis, Fig. 5A) that seen in the wild-type. In low-P medium, there were also differences in cellular composition, morphology, and respiratory C flow to amino acid synthesis between the wild-type and AS8. Most significantly, under low-P growth conditions, AS8 cells displayed dramatically higher rates of generation of AOS than wild-type cells, and this difference was abolished by FCCP (Fig. 7). The results suggest that induction of non-phosphorylating AOX respiration is an important plant metabolic adaptation to P limitation. By preventing severe respiratory restriction, AOX acts to prevent both redirections in C metabolism and the excessive generation of AOS in the mitochondrion.

Finally, Sakano (1998) presented a hypothesis in which AOX respiration functions as part of a biochemical pH-stat during periods of rapid cytosolic acidification, such as would occur during rapid P uptake by a proton cotransport system (Schachtman et al., 1998). Therefore, AOX respiration may play an important role during relief from P deficiency. AS8 cells should prove useful in testing this hypothesis.

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