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Rapid Communication

Molecular Genetic Evidence of the Ability of Alternative Oxidase to Support Respiratory Carbon Metabolism[1]

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With the cytochrome pathway inhibited, AOX was able to support considerable growth of cultured tobacco (*Nicotiana tabacum* cv Petit Havana SR1) cells but the efficiency of carbon utilization decreased dramatically. Antisense cells with decreased AOX protein did not grow, whereas sense cells with elevated AOX protein had higher growth and respiration rates than the wild type. In antisense cells a large accumulation of pyruvate resulted in aerobic ethanolic fermentation.

Higher plants have a branched mitochondrial electron transport chain so that electrons can flow through the usual Cyt pathway or to AOX (Siedow and Umbach, 1995). Electron transfer through the Cyt pathway is coupled at three sites to the generation of an electrochemical gradient used to produce ATP, whereas electron transfer to AOX bypasses two of these sites of energy conservation. This greatly lowers the ATP yield from carbon oxidation. The kinetic properties of soybean AOX measured in isolated mitochondria are dramatically altered by the redox state of a proposed AOX intermolecular disulfide bond (Umbach and Siedow, 1993; Umbach et al., 1994) and by certain cellular metabolites, particularly pyruvate (Millar et al., 1993; Day et al., 1994; Umbach et al., 1994).

Previously, we generated transgenic tobacco (*Nicotiana tabacum* cv Petit Havana SR1) plants and suspension cells with increased and decreased levels of mitochondrial AOX protein, providing specific "mutants" of AOX respiration in a higher plant (Vanlerberghe et al., 1994). Isolated transgenic leaf mitochondria with high levels of AOX protein were subsequently used to study the biochemical regulation of tobacco AOX (Vanlerberghe et al., 1995). We found that both the generation of intramitochondrial reducing power and the presence of pyruvate were necessary to obtain high rates of AOX activity when the Cyt pathway was inhibited. These results indicate that AOX activity in vivo may be regulated in a feed-forward fashion by upstream respiratory carbon metabolism. In this way, electron transport to AOX might help support high rates of carbon flow when electron flux through the Cyt pathway is saturated or limited by the availability of ADP (Vanlerberghe et al., 1995).

In this study transgenic suspension cells were used to provide genetic evidence of the ability of the mitochondrial AOX protein to support respiratory carbon metabolism. This was done by showing that the presence of the AOX protein can support the growth of cultured cells. In addition, the results provide genetic evidence that AOX may function to modulate the intracellular concentration of the key respiratory intermediate pyruvate, because this metabolite is shown to accumulate when there is an imbalance between carbon metabolism and electron transport.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Mitochondrial AOX is encoded by the nuclear gene *Aox1* (McIntosh, 1994). Transgenic tobacco (*Nicotiana tabacum* cv Petit Havana SR1) suspension cells containing *Aox1* in either sense or antisense orientation under the transcriptional control of the cauliflower mosaic virus 35S promoter were previously characterized and were grown as before (Vanlerberghe et al., 1994). When used, AA (70 mM stock in 2-propanol) was added to suspension cells at a concentration of 25 μM. Control cells had equal amounts of 2-propanol without AA.

RNA and Protein Analysis

RNA isolation and northern analysis were done as before using a purified *Eco*RI insert from pAONTI (a cDNA clone of tobacco *Aox1*) as a hybridization probe (Vanlerberghe and McIntosh, 1994). Mitochondrial proteins were separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX as before (Vanlerberghe and McIntosh, 1992).

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Abbreviations: AA, antimycin A; AEC, adenylate energy charge; AOX, alternative oxidase; CUE, carbon use efficiency; TCA cycle, tricarboxylic acid cycle.

Metabolite Analysis

Suspension cells (2 d after subculture) were concentrated by centrifugation (2000*g*, 2 min), resuspended to approximately 14 mg mL^{-1} dry weight in their culture medium, and incubated for 30 min under normal growth conditions before sampling for metabolites and adding AA. Cells (0.8 mL) were then rapidly killed by injection into $HClO_4$ (final concentration 10%) and frozen in liquid N_2. Samples were thawed, kept on ice for 1 h, and centrifuged (17,000*g*, 5 min). The pellet was washed twice with H_2O and lyophilized to determine cell dry weight. The supernatant was neutralized with 5 N KOH/1 M triethanolamine, stored in liquid N_2, and used for metabolite analyses. Metabolites were measured by coupled enzymatic assays (Stitt et al., 1989) using a HP8452A diode array spectrophotometer (Hewlett-Packard).

Cell Respiration

Suspension cells (1–3 mg mL^{-1} dry weight) in their culture medium were placed in a Clark-type O_2 electrode cuvette (Rank Brothers, Cambridge, UK) at 30°C to measure respiratory O_2 uptake. Potassium cyanide (0.5 mM) and salicylhydroxamic acid (1 mM) were used to inhibit Cyt pathway and AOX respiration, respectively (Vanlerberghe et al., 1994).

Growth and Carbon Use

Suspension cells (4 mL) were taken from culture daily and centrifuged (2000*g*, 5 min). The pellet was used to determine cell dry weight and cellular carbohydrate stores, and the supernatant was used to determine medium sugar and protein content. For cell dry weight, the above pellet was washed twice with H_2O, frozen, and lyophilized. For cellular carbohydrate stores (the sum of Suc, Fru, Glc, and Glc equivalents in starch), the lyophilized cells were resuspended in H_2O and incubated at 95°C for 2 h. Starch was then degraded to Glc as described (Jones, 1981), and Suc, Fru, and Glc were measured by coupled enzymatic assays (Stitt et al., 1989) as described above. Protein in the medium was measured by a modified Lowry assay (Larson et al., 1986). To determine total sugar in the medium, it was necessary to measure the total of Suc, Glc, and Fru, because Suc supplied to the cells in their culture medium was rapidly converted by the cells to Glc and Fru in the medium.

The specific growth rate (d^{-1}) was the slope of the linear portion of a semilogarithmic plot of corrected cell dry weight (corrected as described below) versus time (see Table I legend). The CUE (g dry weight g^{-1} sugar) was the slope of the linear portion of a plot of sugar depletion from the medium versus cell dry weight gain (see Table I legend; Schnapp et al., 1990). However, over the course of a subculture period, the level of cellular carbohydrate stores (sugars and starch) varied and there was a release of some protein into the culture medium. We corrected medium sugar and cell dry weight values to account for these factors: protein in the medium was added to the cell dry weight, whereas cellular carbohydrate stores were added to the medium sugar and subtracted from the cell dry weight.

RESULTS AND DISCUSSION

Previously, we generated transgenic tobacco suspension cells with altered levels of mitochondrial AOX protein (Vanlerberghe et al., 1994). Cell line S11 contains multiple sense constructs of *Aox1* and, under normal growth conditions (−AA), has high levels of *Aox1* mRNA and AOX protein (Fig. 1). Cell line AS8 contains an antisense *Aox1* gene that results in undetectable levels of AOX protein under normal growth conditions, whereas wild-type cells have low levels of *Aox1* mRNA and AOX protein (Fig. 1). All three cell lines show similar respiration and growth rates under normal growth conditions (Table I).

Studies of a range of organisms have addressed the question of whether AOX respiration can support growth (Slayman, 1978; Benichou et al., 1988; Wagner et al., 1992). Generally, interpretation of results was hindered by the

Table I. *Respiration rate, growth rate, and CUE of wild-type and transgenic (S11, AS8) tobacco suspension cells in the presence and absence of 25 μM AA*

Respiration rate (−AA) is the average (± SD) of rates obtained at 1, 3, 5, 7, and 9 d after subculture, and results are from three experiments. Residual O_2 uptake (measured after addition of 0.5 mM KCN and 1 mM SHAM) was subtracted from all rates. This residual component (averaged over all days measured) was: wild type, 49; S11, 50; and AS8, 51. Respiration rate (+AA) is the average (± SD) of rates obtained at 1, 3, and 5 d after AA addition (AA added at 2 d after subculture), and results are from four experiments. Residual O_2 uptake (measured after addition of 1 mM SHAM) was subtracted from all rates. This residual component (averaged over all days measured) was: wild type, 33; S11, 41; and AS8, 31. Growth rate and CUE results are from three to five experiments (avergae ± SE). These were calculated from d 1 to 6 (−AA) or d 2 to 8 (+AA) after subculture (AA added at 2 d after subculture). CUE could not be determined for AS8 cells in the presence of AA, since these cells did not grow.

Cell Line	Respiration Rate		Growth Rate		CUE	
	−AA	+AA	−AA	+AA	−AA	+AA
	nmol O_2 mg^{-1} dry wt h^{-1}		*d^{-1}*		*g dry wt g^{-1} sugar*	
Wild type	510 ± 141	677 ± 84	0.39 ± 0.02	0.13 ± 0.01	0.44 ± 0.02	0.23 ± 0.02
S11	468 ± 129	914 ± 106	0.40 ± 0.03	0.18 ± 0.02	0.47 ± 0.03	0.24 ± 0.02
AS8	508 ± 115	165 ± 70	0.37 ± 0.03	0.0	0.44 ± 0.01	—[a]

[a] —, 0.0.

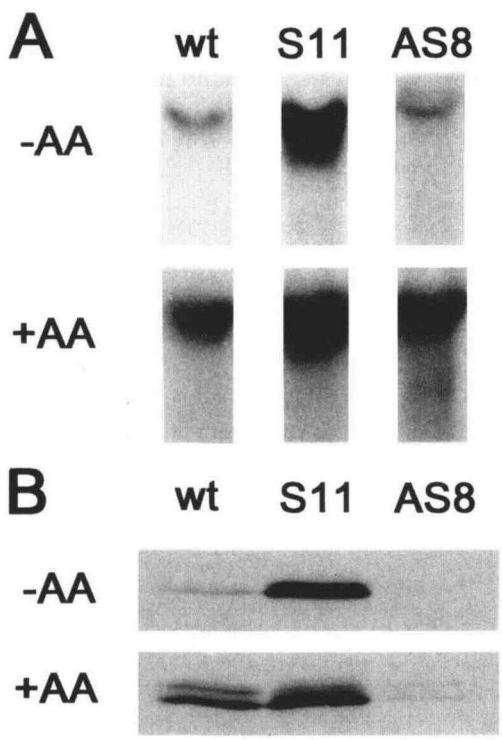

Figure 1. A, The level of *Aox1* mRNA in wild-type (wt) and transgenic (*S11*, *AS8*) tobacco suspension cells under normal growth conditions (2 d after subculture) and after 8 h with 25 μM AA. Total RNA (50 μg) was separated on agarose gels containing formaldehyde, transferred to nitrocellulose, hybridized to radiolabeled pA-ONT1 DNA, and analyzed by autoradiography. B, The level of AOX protein in isolated mitochondria from wild-type and transgenic (S11, AS8) tobacco suspension cells under normal growth conditions (2 d after subculture) and after 24 h with 25 μM AA. Mitochondrial proteins (75 μg) were separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX.

presence of some Cyt pathway activity and/or by the lack of mutants with altered AOX expression. Here we have used AA to ensure complete inhibition of the Cyt pathway and have utilized "mutants" with increased and decreased levels of AOX protein to show that AOX respiration can support growth. In wild-type cells suppression of Cyt pathway activity by AA increased *Aox1* mRNA and AOX protein (Fig. 1), enabling the cells to maintain high respiration rates using AOX (Table I). It is known that inhibition of the Cyt pathway induces the synthesis of AOX in these cells (Vanlerberghe and McIntosh, 1994). These wild-type cells were able to maintain substantial growth with AA (Table I). AS8 cells, with compromised AOX protein levels compared with wild type, did not grow with AA, showing that induction of AOX was critical to maintain growth under these conditions (Fig. 1; Table I). Alternatively, S11 cells, with elevated AOX protein levels compared with wild type, respired and grew about 35% faster than wild-type cells with AA (Table I).

The above results illustrate that AOX can support respiratory carbon metabolism when the Cyt pathway is limiting to carbon flow. Metabolic and/or environmental conditions under which the Cyt pathway may limit carbon flux include high carbohydrate supply (Azcon-Bieto et al., 1983), chilling temperatures (Prasad et al., 1994), nutrient deprivation (Rychter et al., 1992), salt stress (Jolivet et al., 1990), and high CO_2 (Palet et al., 1991). In such situations, AOX respiration may function to provide additional carbon and energy for growth.

Under normal growth conditions (−AA), the wild-type and transgenic cells appear to respire predominantly using the Cyt pathway (Vanlerberghe et al., 1994), whereas with AA, respiration proceeds exclusively through AOX (Table I). Comparison of growth and carbon use in the absence and presence of AA allows a comparison of CUE (g cell dry weight gain g^{-1} sugar consumed) associated with respiration through the Cyt pathway and AOX, respectively. Normal growth conditions produced CUE values of approximately 0.45 g dry weight g^{-1} sugar for each of the cell lines (Table I), a value similar to previous results with wild-type tobacco cells (Schnapp et al., 1990). The CUE was significantly lower during growth using AOX (Table I), presumably because of the nonphosphorylative nature of this pathway. The drop in CUE represents a direct measure of the metabolic cost of AOX respiration.

In general, changes in the rate of ATP-generating processes (such as mitochondrial electron transport) are accommodated by adjustments in ATP-consuming processes (such as growth) so that the energy status of a cell remains relatively constant over a range of metabolic conditions (Atkinson, 1977). Complete inhibition of the Cyt pathway by AA in wild-type or S11 suspension cells resulted in relatively minor drops in AEC over 24 h (Fig. 2A). Maintenance of AEC may have been the result of both the reduction in growth rate and the high respiration rate using AOX (Table I). In AS8 AEC was also maintained at a relatively high value with AA (Fig. 2A), despite a low respiration rate (Table I) caused by low levels of AOX protein (Fig. 1). In this case, maintenance of AEC may have been a result of the complete cessation of growth (Table I) and of the high rate of ethanol production (Fig. 2C; see below).

The degree of accumulation of pyruvate in cells after AA addition was inversely related to the amount of AOX protein present in the mitochondrion to metabolize the pyruvate (Fig. 2B). In S11, which has constitutively high AOX protein (Fig. 1), there was only a minor increase in the pyruvate level. In wild type, which has low AOX protein before AA addition but which rapidly synthesizes AOX after AA addition (Fig. 1; Vanlerberghe and McIntosh, 1994), there was an intermediate increase in pyruvate. In AS8, which has undetectable AOX protein before AA addition and only low levels after AA addition (Fig. 1), pyruvate increased dramatically (Fig. 2B). These results provide genetic evidence that pyruvate will accumulate if there is an imbalance between respiratory carbon metabolism and electron transport. Furthermore, the results show that high levels of pyruvate can result in fermentation under aerobic conditions. With AA, AS8 cells produced large amounts of ethanol from the pyruvate pool (Fig. 2C). This did not occur in the wild type or in S11 (Fig. 2C). Interestingly, whereas S11 overexpresses AOX in suspension culture

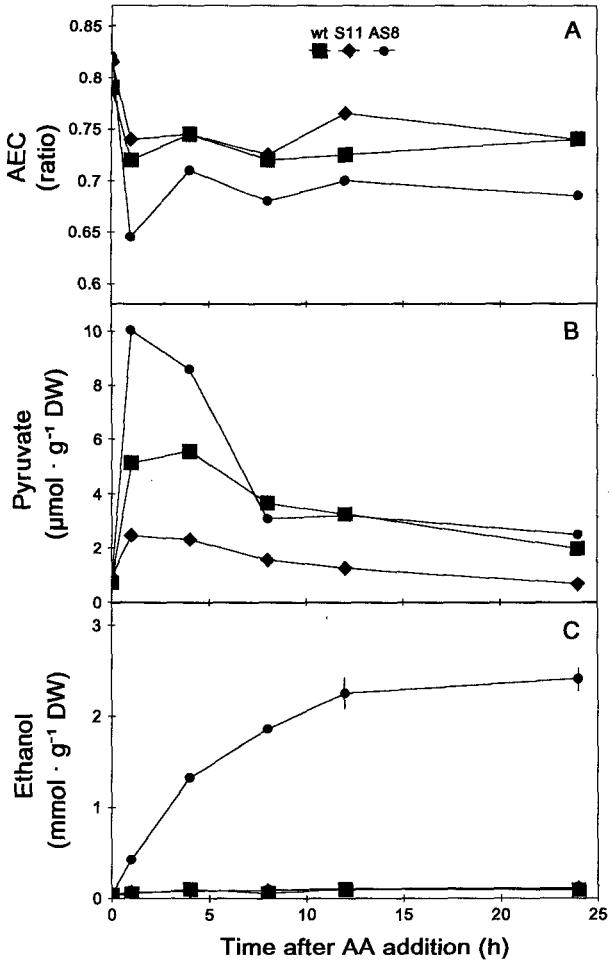

Figure 2. Metabolite changes in wild-type and transgenic (S11, AS8) tobacco suspension cells after addition of 25 μM AA at time 0. AEC was calculated as [ATP + 0.5 ADP]/[ATP + ADP + AMP] (Atkinson, 1977). Results are the mean from two (A and B) or three (C) experiments using cells from different subcultures. Each experiment had similar results. For simplicity, SD values in C are only shown for AS8. In some cases these values were smaller than the figure symbols. wt, Wild type; DW, dry weight.

cells, AOX expression is completely suppressed in leaves of this transgenic line, possibly because of the phenomenon of cosuppression (Vanlerberghe et al., 1994). Accordingly, we have found that whereas leaves of this transgenic line produce ethanol in the presence of AA (Vanlerberghe et al., 1995), suspension cells do not (Fig. 2).

Possibly, any condition that selectively stimulates glycolysis or slows the TCA cycle may increase pyruvate level. The biochemical regulatory properties of the AOX enzyme are consistent with the possibility that the presence of an AOX pathway in plant mitochondria is a mechanism to modulate pyruvate level by maintaining adequate rates of respiratory carbon flow. First, pyruvate is a strong activator of AOX in both soybean (Millar et al., 1993; Day et al., 1994) and tobacco (Vanlerberghe et al., 1995), acting presumably by an allosteric mechanism. Second, both soybean (Umbach and Siedow, 1993; Umbach et al., 1994) and tobacco (Vanlerberghe et al., 1995) AOX activity are regulated by the redox state of a proposed AOX intermolecular disulfide bond. Reduction of AOX to its high activity form is necessary for pyruvate activation (Umbach et al., 1994; Day et al., 1994). In tobacco it has been shown that AOX reduction is mediated by intramitochondrial reducing power generated by the oxidation of certain TCA cycle substrates, most notably isocitrate and malate (Vanlerberghe et al., 1995). Hence, metabolic conditions resulting in the accumulation of intramitochondrial reducing power and/or pyruvate have the potential to activate the AOX enzyme. For example, under conditions when the Cyt pathway is restricting the rate of TCA cycle carbon flow, the resulting increase in pyruvate and/or intramitochondrial reducing power will feed-forward activate AOX, resulting in increased electron transport, increased TCA cycle carbon flow, and a lowering of pyruvate level. In such a way, AOX could function to modulate the concentration of the key respiratory intermediate pyruvate and to supplement the Cyt pathway in providing respiratory carbon and energy for growth.

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