Plant Physiol. (1994) 106: 1503–1510

Molecular Genetic Alteration of Plant Respiration[1]

Silencing and Overexpression of Alternative Oxidase in Transgenic Tobacco

Greg C. Vanlerberghe, Alison E. Vanlerberghe, and Lee McIntosh*

Michigan State University/Department of Energy Plant Research Laboratory and Biochemistry Department, Michigan State University, East Lansing, Michigan 48824

The alternative oxidase (AOX) of plant mitochondria is encoded by the nuclear gene *Aox1*. Sense and antisense DNA constructs of *Nicotiana tabacum Aox1* were introduced into tobacco, and transgenic plants with both increased and decreased levels of mitochondrial AOX protein were identified. Suspension cells derived from wild-type and transgenic plants were grown in heterotrophic batch culture. Transgenic cells with increased AOX protein had an increased capacity for cyanide-resistant, salicylhydroxamic acid-sensitive respiration compared to wild-type cells, whereas transgenic cells with decreased AOX protein had a decreased capacity for such respiration. Thus, genetic alteration of the level of AOX protein was sufficient to alter the capacity for electron transport through the alternative pathway. Under our standard growth conditions, "antisense" cells with dramatically reduced levels of AOX protein had growth and respiration rates similar to the wild type. However, whereas wild-type cells were able to grow under conditions that severely suppressed cytochrome pathway activity, antisense cells could not survive this treatment. This suggests that a critical function of AOX may be to support respiration when the cytochrome pathway is impaired. The much higher level of AOX protein in "sense" cells compared to the wild type did not appreciably alter the steady-state partitioning of electrons between the cytochrome path and the alternative pathway in vivo, suggesting that this partitioning may be subject to additional regulatory factors.

Higher plants have two paths of mET from UQ to O_2 (Day et al., 1980; Moore and Siedow, 1991). Electron transfer through the CP is coupled to ATP synthesis. The terminal oxidase (Cyt oxidase) is inhibited by CN. Electron flow from UQ through the AP is not coupled to ATP production. The terminal oxidase is called AOX and is inhibited by SHAM. Despite a great research effort, the functional role of the AP in nonthermogenic respiratory metabolism has not been clearly defined.

Studies have shown that both the capacity of the AP to support O_2 consumption and the activity of the AP in consuming O_2 are subject to complex regulation. AP capacity appears to be influenced by environmental (Bingham and Farrar, 1989; Vanlerberghe and McIntosh, 1992a), developmental (Elthon et al., 1989b; Bryce et al., 1990; Obenland et al., 1990), chemical (Kapulnik et al., 1992; Rhoads and McIntosh, 1992), tissue-specific (Kearns et al., 1992; Johns et al., 1993; Conley and Hanson, 1994), and other regulatory signals. The signal mechanisms responsible for altering AP capacity are beginning to be elucidated (Minagawa et al., 1992; Vanlerberghe and McIntosh, 1994). Whereas the capacity of the AP will determine the maximum partitioning of electron flow to AOX, it is known that AP activity is often considerably less than maximal. This may be due primarily to the differential response of the CP and the AP to the redox poise of the UQ pool. Although CP activity varies linearly with the reduction state of the UQ pool, the AP is not active until the UQ pool reduction level reaches approximately 35 to 40% (Dry et al., 1989; Siedow and Moore, 1993). This suggests that the AP becomes active once the CP is limiting electron flux, a suggestion consistent with numerous studies on isolated mitochondria and whole tissue (e.g. Day and Lambers, 1983). At least two types of limitation of the CP may occur (Day and Lambers, 1983; Hoefnagel et al., 1994). It may be that the carrier capacity of one of the electron transfer components of the CP is limiting or that the CP becomes restricted by ADP and/or Pi supply. Other mechanisms that may also contribute to the regulation of AP activity include the substrates being oxidized by the mitochondria (Day et al., 1991), the level of potential allosteric activators of AOX (Millar et al., 1993), and the oxidation state of an AOX sulfhydryl residue (Umbach and Siedow, 1993).

The amount of AOX protein in the mitochondria of higher plants correlates with the capacity of the AP to support O_2 consumption (Obenland et al., 1990; Kearns et al., 1992; Rhoads and McIntosh, 1992; Vanlerberghe and McIntosh, 1992a, 1992b). Also, it has been shown that expression of *Arabidopsis* AOX in *Escherichia coli* is sufficient to confer CN-resistant, SHAM-sensitive respiration to this organism (Kumar and Soll, 1992). These studies suggest that AOX is an

[1] This work was supported in part by Department of Energy grant DE-FG02–91ER20021 (to L.M.), National Science Foundation grant D.C.B.8904518 (to L.M.), and U.S. Department of Agriculture grant MICLO-8089 (to L.M.). G.C.V. acknowledges support from a Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship.

* Corresponding author; fax 1–517–353–9168.

Abbreviations: AA, antimycin A; AOX, alternative oxidase; AP, alternative pathway; CaMV, cauliflower mosaic virus; CP, cytochrome pathway; FCCP, p-trifluoromethoxycarbonylcyanide; Kn, kanamycin; mET, mitochondrial electron transport; SHAM, salicylhydroxamic acid; UQ, ubiquinone; wt, wild-type.

important target protein in any attempt toward molecular genetic manipulation of AP respiration in plants.

The AOX in plants is encoded by the nuclear gene *Aox1* (McIntosh, 1994). Here we report using sense and antisense DNA constructs of tobacco (*Nicotiana tabacum*) *Aox1* to generate transgenic plants with both increased and decreased levels of AOX. To our knowledge, it is the first example of antisense inhibition of the AP in any organism. Transgenic plants will be a useful system in which to study the mechanisms by which the capacity and activity of the AP are regulated as well as the function of AOX in plant metabolism.

MATERIALS AND METHODS

Plasmid Constructs and Plant Transformation

Standard techniques were used to construct recombinant DNA plasmids in *Escherichia coli* strain MC1000 (Sambrook et al., 1989). The cDNA clone pAONT1 (*Eco*RI fragment) contains the complete coding region of tobacco *Aox1* (1059 bp) along with an additional 64 nucleotides at the 5′ end and 273 nucleotides at the 3′ end (Vanlerberghe and McIntosh, 1994). This cDNA was ligated in the *Eco*RI site of the binary expression vector pGA748 (An et al., 1988) in both sense and antisense orientation to the 35S promoter of CaMV (Fig. 1). Transformation of *Agrobacterium* strain LBA4404 (carrying Ti plasmid pAL4404) was done by a direct DNA uptake method (An et al., 1988). Tobacco (*Nicotiana tabacum* cv Petit Havana SR1) was transformed by the leaf disc method and Kn-resistant plants were regenerated as described (Horsch et al., 1988).

Analysis of Transgenic Plants

Primary transformants were propagated in Magenta boxes on a modified Murashige and Skoog (MSO) medium (Horsch et al., 1988) supplemented with 100 μg mL^{-1} Kn, and under 24 h of fluorescent light. Percoll-gradient-purified mitochondria were isolated from 1 g fresh weight of leaf or root by a

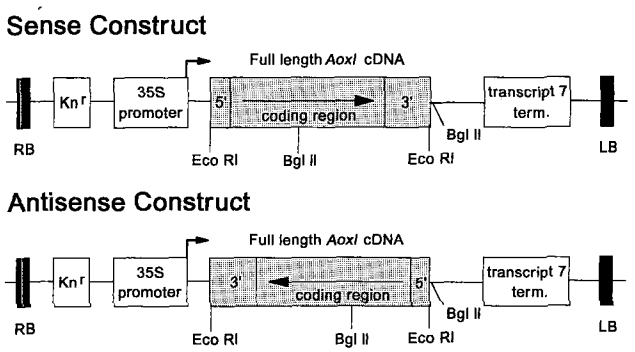

Figure 1. Sense and antisense constructs used to generate transgenic tobacco. The full-length cDNA pAONT1 representing the *Aox1* gene of tobacco (Vanlerberghe and McIntosh, 1994) was ligated in the *Eco*RI site of the binary expression vector pGA748 in both sense and antisense orientation to the 35S promoter of CaMV. This figure shows the position of *Bgl*II restriction sites used to confirm the integration and orientation of the introduced gene in transgenic tobacco. LB, Left border; RB, right border.

miniprep procedure (Boutry et al., 1984). Proteins (75 μg) from purified mitochondria were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX as described (Vanlerberghe and McIntosh, 1992a). In some cases, blots were also probed with a monoclonal antibody to Cyt oxidase subunit II (coxII), which was obtained from Dr. A. Tzagaloff (Columbia University, New York, NY).

Transgenic Suspension Cells

Fresh leaf tissue from wt (nontransformed) and some primary transformants was placed on a modified Murashige and Skoog (MSO) medium (Horsch et al., 1988) supplemented with 0.2 μg mL^{-1} 6-benzylaminopurine, 1.9 μg mL^{-1} naphthaleneacetic acid, and, in the case of transgenic lines, 100 μg mL^{-1} Kn. The tissue was incubated in the dark (28°C) to generate callus, which was then transferred to a liquid medium previously described (Linsmaier and Skoog, 1965). This medium contains 3% (w/v) Suc as a carbon source. After several months of growth in liquid medium, a fine suspension of heterotrophic cells was obtained. Cultures (200 mL) were then routinely grown in the dark on a rotary shaker (150 rpm, 30°C) and were subcultured every 9 d by 11-fold dilution of the cells in fresh medium. Transgenic lines were grown in medium supplemented with 100 μg mL^{-1} Kn, a concentration at which the wt could not grow.

RNA was isolated from suspension cells as previously described (Vanlerberghe and McIntosh, 1994). Once the RNA was removed by LiCl precipitation, the remaining supernatant was used to isolate DNA. To do this, the DNA was precipitated with 2 volumes of ethanol and centrifuged. The pellet was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) and then extracted with an equal volume of phenol:chloroform (1:1) followed by an equal volume of chloroform. Then, 0.1 volume of 3 M sodium acetate (pH 5.2) was added and the DNA was precipitated with 2 volumes of ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in TE.

Northern analysis of total RNA (50 μg) was done as before (Vanlerberghe and McIntosh, 1994). DNA (20 μg) was cut with *Bgl*II and used for Southern analysis using routine procedures (Sambrook et al., 1989). Both northern and Southern blots were probed with radiolabeled DNA made using a purified insert of pAONT1. Plasmid insert isolation and radiolabeling were done as before (Vanlerberghe and McIntosh, 1994). Northern and Southern blots were analyzed by autoradiography as previously described (Vanlerberghe and McIntosh, 1994).

Washed mitochondria were isolated from suspension cells as previously described (Vanlerberghe and McIntosh, 1992a). Proteins (75 μg) from washed mitochondria were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX as described (Vanlerberghe and McIntosh, 1992a).

Respiratory Characteristics and Growth

Suspension cells (about 1–3 mg dry weight) in their culture medium were placed in an oxygen electrode cuvette (Rank

Brothers, Cambridge, UK) at 30°C. Steady rates of respiratory O_2 uptake were determined after about 1 to 4 min under these conditions. Inhibitors (KCN, SHAM) and an uncoupler (FCCP) were then used as described in the text and figure legends. Stocks of KCN (1 M, pH 8.5) and SHAM (0.5 M in DMSO) were stored frozen and prepared fresh weekly. DMSO alone had no effect on rates of O_2 uptake. Cell dry weight was determined as described below.

To measure growth, an aliquot of suspension cells (4 mL) was removed from culture daily and centrifuged. The pellet was washed twice with H_2O, frozen, and freeze-dried to determine cell dry weight. Under normal growth conditions, a semilogarithmic plot of cell dry weight versus time was linear from d 1 to 6 after subculture, indicating exponential growth over this period. The slope of the plot over this period is the specific growth rate (d^{-1}). In cells treated with AA (AA added at d 2 after subculture), growth rate was calculated in the same manner but from d 2 to 8 after subculture. AA (70 mM stock dissolved in 95% ethanol) was added to cells at a final concentration of 25 μM.

RESULTS

Transgenic Plants with Altered Levels of AOX Protein

A cDNA representing tobacco *Aox1* was put in both sense and antisense orientation under the transcriptional control of the CaMV 35S promoter and introduced into tobacco (Fig. 1). Transgenic plants were regenerated and the integration and orientation of the introduced *Aox1* gene was confirmed by Southern analysis (data not shown).

Transgenic plants containing the sense construct were analyzed for levels of AOX protein in purified mitochondria using a monoclonal antibody to AOX (Elthon et al., 1989a). Of the 16 independent transformants analyzed, 15 had dramatically increased levels of AOX protein. Figure 2 is an example of one such line, S24. The dramatic increase in AOX protein was seen in both leaves and roots and was sometimes accompanied by accumulation of an immunoreactive protein of a slightly higher molecular mass (Fig. 2). This protein of an "apparent" higher molecular mass is seen more dramatically after overexpression of AOX in potato and may represent an incompletely processed and/or modified form of the protein (C. Hiser and L. McIntosh, unpublished data). At present, the size/activity relationship of the AOX protein is not known. One transgenic line (S11), which appeared by Southern analysis to contain multiple copies of the sense construct, had no detectable leaf AOX protein, even after a 24-h treatment of plants at 10°C (data not shown, see below).

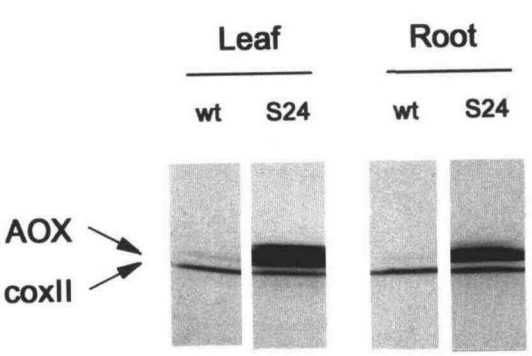

Figure 2. The level of 35-kD AOX protein in purified mitochondria from leaf and root of SR1 tobacco plants. wt, Wild-type plant (nontransformed); S24, transgenic plant containing *Aox1* in sense orientation under the transcriptional control of the 35S promoter of CaMV. Mitochondrial proteins (75 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX. The blot was then probed with a monoclonal antibody to Cyt oxidase subunit II (coxII) to confirm that each lane had approximately equal amounts of total mitochondrial protein.

The wt tobacco grown at 28°C had levels of AOX protein in leaves and roots that were near the detection limit of our analysis (Fig. 2). This made it difficult to identify transgenic plants in which an introduced antisense *Aox1* gene was effectively reducing levels of AOX protein below wt levels. To overcome this problem, a more effective screening procedure was developed. We found that when wt plants grown at 28°C were transferred to 10°C for 24 h, there was a dramatic increase in AOX protein in isolated mitochondria. This is similar to what is observed in suspension cells of tobacco when shifted to a lower growth temperature (Vanlerberghe and McIntosh, 1992a). Therefore, to screen antisense plants for reduced levels of AOX, we compared wt and transgenic plants after 24 h at 10°C. Twenty-seven independent transformants containing the antisense construct were analyzed in this fashion and most had levels of AOX protein similar to wt (Fig. 3). However, one line (AS8) consistently had no detectable AOX protein, either at 28°C (data not shown) or after transfer to 10°C for 24 h (Fig. 3).

When the pAONT1 clone was hybridized to a northern blot of total RNA from wt tobacco leaf, it recognized a single 1.6-kb *Aox1* transcript (data not shown). The transcript level increased approximately 7-fold within 12 h of transfer of wt plants to 10°C (data not shown), consistent with the dramatic increase in AOX protein seen after 24 h (Fig. 3). In AS8, we did not see a distinct band for the *Aox1* transcript, but rather a smeared signal of smaller-sized hybridizing RNAs. After transfer of AS8 plants to 10°C for 12 h, the intensity of this smeared signal increased (data not shown).

Transgenic Suspension Cells: Molecular Analysis

Suspension cells were derived from wt and three transgenic lines (S24, S11, AS8). Southern analysis confirmed that each of the transgenic suspension cultures had retained the introduced *Aox1* gene (Fig. 4). The analysis also confirmed the orientation of the *Aox1* gene to its CaMV promoter (Fig. 1). An introduced *Aox1* gene in sense orientation yielded a unique *Bgl*II fragment of about 920 bp (cell lines S24 and S11; Fig. 4), whereas an antisense orientation gave a unique fragment of about 490 bp (cell line AS8; see legend to Fig. 4). The strong hybridization signal seen by Southern analysis in the S11 suspension cells indicates that this line may contain multiple copies of the introduced *Aox1* gene (Fig. 4).

Northern analysis showed that the sense lines (S24, S11) maintained higher steady-state levels of the 1.6-kb *Aox1* mRNA than the wt (Fig. 5). This was particularly pronounced in S11, consistent with it maintaining the highest levels of AOX protein (see below). In the antisense line AS8, we saw

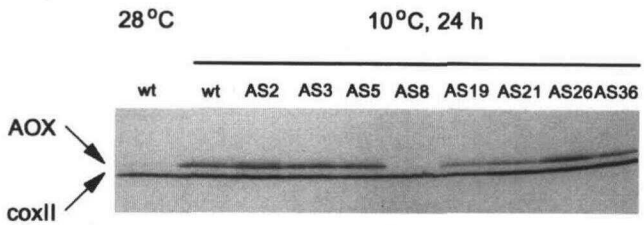

Figure 3. The level of 35-kD AOX protein in purified mitochondria from leaf of SR1 tobacco plants. Levels of AOX protein were determined in plants either (a) grown at 28°C or (b) grown at 28°C and then transferred to 10°C for 24 h. wt, Wild-type plant (non-transformed); AS2, AS3, AS5, etc., transgenic plants containing *Aox1* in antisense orientation under the transcriptional control of the 35S promoter of CaMV. Mitochondrial proteins (75 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX. The blot was then probed with a monoclonal antibody to Cyt oxidase subunit II (coxII) to confirm that each lane had approximately equal amounts of total mitochondrial protein.

a hybridizing band that consistently appeared to be of a slightly higher molecular mass than that of the wt endogenous *Aox1* mRNA (Fig. 5). This RNA probably represents the antisense *Aox1* transcript, although this was not confirmed with a strand-specific nucleotide probe. We also consistently saw a smear of smaller-sized hybridizing RNAs, which may represent degradation products resulting from some interaction between the endogenous *Aox1* transcript and the antisense transcript (see "Discussion").

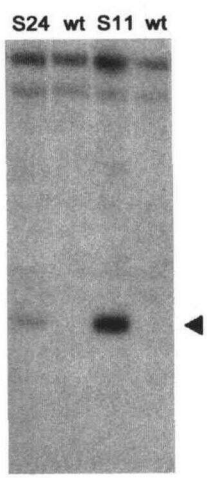

Figure 4. Southern analysis of genomic DNA from wt and transgenic (S24, S11) suspension cells of tobacco. Genomic DNA (20 μg) was cut with *Bgl*II, separated by agarose gel electrophoresis, transferred to nitrocellulose, hybridized to radiolabeled pAONT1 DNA, and analyzed by autoradiography. Plasmid DNA of the sense construct of *Aox1* (Fig. 1) was also cut with *Bgl*II and analyzed on this gel to show the expected size of the *Bgl*II fragment. The expected size is indicated by the arrowhead. A similar analysis of genomic DNA from AS8 suspension cells confirmed that this line contained the antisense construct, but because of the small size of this *Bgl*II fragment, the hybridization signal was too faint to be reproduced for publication.

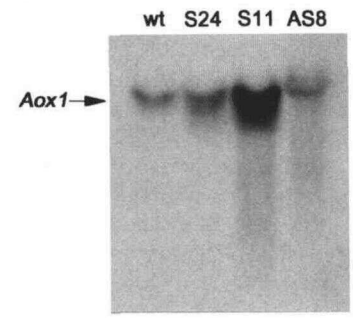

Figure 5. Northern analysis of total RNA from wt and transgenic (S24, S11, AS8) suspension cells of tobacco at 5 d after subculture. Total RNA (50 μg) was separated on agarose gels containing formaldehyde, transferred to nitrocellulose, hybridized to radiolabeled pAONT1 DNA, and analyzed by autoradiography.

Figure 6 compares levels of AOX protein in mitochondria isolated from the different suspension cells at two different times during the subculture period. At 5 d after subculture (exponential growth phase), the level of AOX protein in mitochondria from wt cells was near the limit of detection, whereas the sense lines (S24 and S11) had dramatically elevated levels. At 9 d after subculture (stationary phase), more AOX protein was visible in the wt but levels were still much below those seen in S24 and S11. The very high level of AOX protein seen in S11 suspension cells is consistent with the presence of multiple introduced copies of the *Aox1* gene (Fig. 4) and the high level of *Aox1* mRNA (Fig. 5). Nonetheless, this result was surprising considering that S11 plants had no detectable leaf AOX protein (see above). This will be discussed further below (see "Discussion"). No AOX protein was visible in mitochondria from suspension cells of the antisense line AS8 (Fig. 6). Even after prolonged development of western blots (data not shown), we did not detect any AOX protein in this cell line.

Transgenic Suspension Cells: Respiratory Characteristics and Growth

Respiratory characteristics of the suspension cultures were determined using CN and SHAM as specific inhibitors of the CP and the AP, respectively. To use these inhibitors in vivo requires that they do not have any substantial side effects

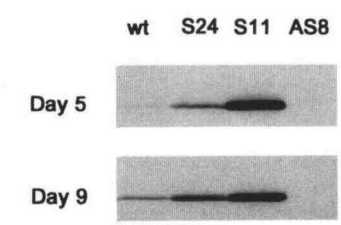

Figure 6. The level of 35-kD AOX protein in mitochondria from wt and transgenic (S24, S11, AS8) suspension cells of tobacco at 5 and 9 d after subculture. Mitochondrial proteins (75 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody to AOX.

(see Moller et al., 1988, for a discussion). We found that low concentrations of inhibitor were sufficient and rapid acting, that residual O_2 uptake in the presence of both inhibitors was low, and that there was no significant SHAM-stimulated peroxidase activity. Figure 7 is an example of data obtained using different concentrations of each inhibitor in the presence and absence of the second inhibitor, for two of the cultures (wt and S24). These analyses indicate that 0.5 mM KCN and 1 mM SHAM are sufficient concentrations to completely inhibit the CP and the AP, respectively, and that these low concentrations have no substantial side effects. Hence, the capacity of the AP in vivo (Fig. 8B) was taken to be that O_2 uptake inhibited by 1 mM SHAM in the presence of 0.5 mM KCN. The activity of the AP in vivo (Table I) is that O_2 uptake inhibited by 1 mM SHAM in the absence of KCN. The validity of this general experimental protocol to measure AP capacity and activity has been thoroughly tested (e.g. Day, 1992).

Respiratory characteristics of the cells varied over the course of each 9-d subculture period. Respiration rate peaked at d 3 after subculture in all lines and then declined slowly until d 9 (Fig. 8A). There was considerable variation in respiration rate from one subculture period to the next but no indication of any significant difference in respiration rates between the different cell lines. Residual respiration (in the presence of CN and SHAM) was minor and was the same in the different cell lines (see legend to Fig. 8).

The capacity of the AP in wt cells was highest at the beginning (lag phase) and end (stationary phase) of the subculture period (Fig. 8B). S24 showed a similar pattern but the absolute capacity in S24 was substantially higher than in the wt (Fig. 8B). When determining AP capacity in S11, we often found that addition of CN actually stimulated respiration rate (a phenomenon seen in tissues with very high AP capacity, see "Discussion") and that respiration in the presence of CN was almost completely SHAM sensitive. Hence, AP capacity in S11 often exceeded the control respiration rates observed (Fig. 8B). In the antisense line AS8, we observed only a low basal level of CN-resistant, SHAM-sensitive O_2 uptake that did not appear to vary over the subculture period (Fig. 8B).

The activity of the AP in vivo was investigated by determining the extent to which SHAM could inhibit respiration in the absence of CN. At 5 d after subculture, 1 mM SHAM had little or no effect on O_2 uptake in either the wt or S11 (Table I). This suggests that there was little AP activity, even in the cell line (S11) that had been genetically altered to have very high AOX protein (Fig. 6) and AP capacity (Fig. 8B). We also investigated whether the AP was active after stimulation of respiration by an uncoupler (Table I). FCCP stimulated respiration rate about 2-fold in both the wt and S11 at 5 d after subculture (Table I). The stimulated rates of respiration were inhibited to a small extent by SHAM, suggesting some AP activity under these conditions. However, the magnitude of this activity was similar in the wt and S11. Taken as a whole, the data suggest that the large increased AP capacity in S11 compared to the wt did not increase steady-state AP activity in vivo under these growth conditions.

In heterotrophic batch culture, wt and AS8 cells had similar growth rates, suggesting that under these conditions the AP was not critical to metabolism (Table II). We next tested whether AOX was critical under conditions in which the CP was suppressed. In the presence of AA in the culture medium to inhibit CP respiration, wt cells still grew, albeit at a lower rate, but AS8 cells did not grow under these conditions (Table II). Within 24 h of AA addition, the AS8 cells were browning and consuming little medium sugar (G.C. Vanlerberghe, A.E. Vanlerberghe, and L. McIntosh, unpublished data).

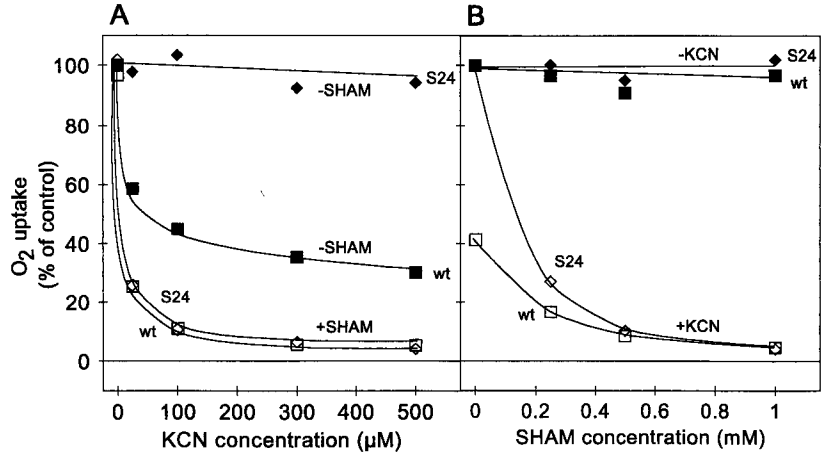

Figure 7. Effects of KCN and SHAM on respiratory O_2 uptake by wt (squares) and S24 (diamonds) transgenic suspension cells. Oxygen uptake was measured with an O_2 electrode. A, The effect of KCN concentration on O_2 uptake by wt and S24 suspension cells in the presence (open symbols) or absence (closed symbols) of 1 mM SHAM. SHAM was added about 2 min prior to KCN. B, The effect of SHAM concentration on O_2 uptake by wt and S24 suspension cells in the presence (open symbols) or absence (filled symbols) of 0.5 mM KCN. KCN was added about 2 min prior to SHAM. The results are the average of three separate experiments using cells from different subcultures. The rates of control O_2 uptake (in the absence of any inhibitors, expressed as nmol O_2 mg^{-1} dry weight h^{-1}) were: wt, 621 ± 120 (average ± SD, $n = 21$); S24, 658 ± 73 (average ± SD, $n = 21$).

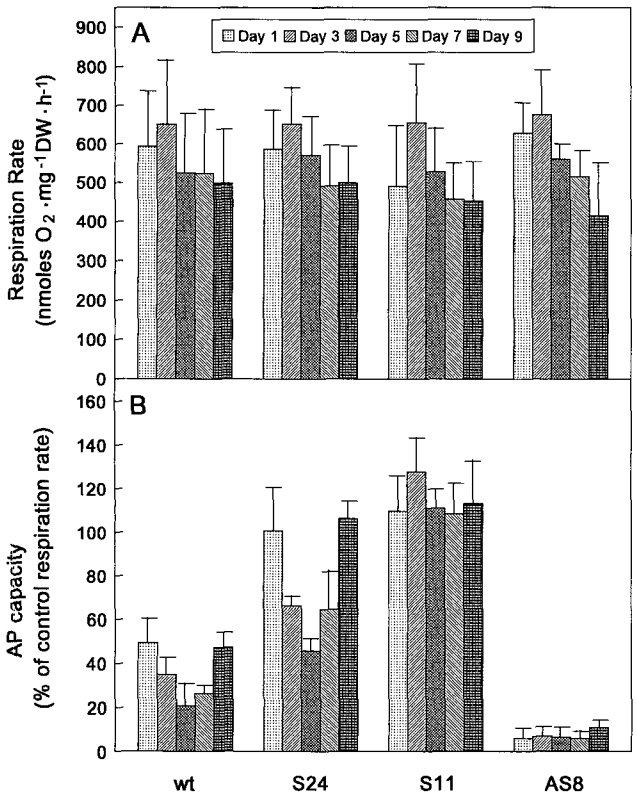

Figure 8. Respiratory characteristics of wt and transgenic (S24, S11, AS8) suspension cells of tobacco at different days after subculture. O_2 uptake was measured with an O_2 electrode. A, Control O_2 uptake in the absence of any inhibitor (average ± SD, $n = 9$). B, AP capacity, defined as that O_2 uptake resistant to 0.5 mM KCN and sensitive to 1 mM SHAM (average ± SD, $n = 3$). It is presented as a percentage of the control respiration rates shown in A. The results are the average of three separate experiments using cells from different subcultures. Residual O_2 uptake (in the presence of KCN and SHAM and averaged over all 5 d measured) was (as a percentage of control respiration rate): wt, 9 ± 5; S24, 10 ± 5; S11, 10 ± 5; AS8, 9 ± 3 (average ± SD, $n = 15$).

DISCUSSION

We have generated suspension cell lines of tobacco that, when growing under identical conditions, have dramatically different capacities to support O_2 consumption by the AP (Fig. 8B). This indicates that genetic alteration of the level of the 35-kD AOX protein is sufficient to alter AP capacity.

An increase in AOX protein in mitochondria of transgenic suspension cells (S24, S11) increased the capacity of the AP (Figs. 6 and 8B). In S11, AP capacity was high enough so that CN actually stimulated respiration rate (see "Results"). This has been seen in other tissues with high AP capacity (e.g. Weger et al., 1990) and may be due to a Pasteur effect in which addition of CN increases cellular ADP. Then, lack of adenylate control of respiratory pathways (such as glycolysis) results in increased O_2 uptake if AP capacity is large enough to support such an increase.

Under the growth conditions used in this study, the dramatically increased AP capacity in S11 compared to the wt did not appreciably affect the steady-state activity of the AP in vivo (Table I). Even in the presence of FCCP to dramatically increase respiration rate (and increase the supply of reducing equivalents to mET), AP activity was a minor component of total respiration in both the wt and S11, indicating that, for the most part, CP capacity was large enough to support the respiratory increase. The results are consistent with the view that partitioning of electrons to AOX may be primarily dependent on the redox state of the UQ pool and that electrons are only likely to enter the AP once the CP becomes limiting or restricted (see text). Since overexpression of AOX is not necessarily expected to affect operation of the CP or the redox state of the UQ pool, increased AOX protein in transgenics may be expected to have a marginal effect on the in vivo partitioning of electrons between the two paths. The amount of AOX protein may primarily determine the maximum potential partitioning of electrons to the AP, whereas other factors may determine the actual partitioning. The multiple mechanisms that may contribute to partitioning of electrons between the two paths (see text) is an important area for future research.

There are numerous examples in which the introduction of

Table I. *Respiratory characteristics of wt and S11 transgenic suspension cells of tobacco*

O_2 uptake was determined with an O_2 electrode. Additions were made at the following final concentrations: 1 mM SHAM, 0.5 mM KCN, 0.5 μM FCCP. Results (average ± SD) are expressed as a percentage of the control rate of O_2 uptake in the absence of any additions. Control rates are not significantly different between the two cell lines (Fig. 8A). Results are from three to six experiments using cells from different subcultures.

Additions (sequential)	O_2 uptake	
	wt	S11
	% of control	
Control	100	100
+SHAM	100 ± 10	96 ± 10
Control	100	100
+FCCP	197 ± 32	218 ± 26
+SHAM	184 ± 43	195 ± 20
+KCN	9 ± 3	10 ± 8

Table II. *Growth rate of wt and AS8 suspension cells of tobacco in the presence and absence of 25 μM AA*

Results are from three to five experiments using cells from different subcultures (average ± SD).

Treatment	Growth Rate	
	wt	AS8
	d^{-1}	
Control	0.42 ± 0.03	0.39 ± 0.02
+AA	0.14 ± 0.01	0.0[a]

[a] Actually some loss of dry weight over time.

sense transgenes homologous to endogenous plant genes has reduced or eliminated expression of both genes (reviewed by Matzke and Matzke, 1993; Flavell, 1994). The cellular mechanism(s) responsible for this "co-suppression" are not well understood but the phenomenon has, in many cases, been associated with multicopy integration of the transgene. Also, the suppression is reversible and influenced by developmental, physiological, and environmental factors. The fact that *Aox1* gene expression is suppressed in the leaves of S11 plants but highly expressed in S11 suspension cells may be due to such a reversible co-suppression event. Interestingly, we found that when S11 plants were regenerated from S11 suspension cells, *Aox1* gene expression was again suppressed (data not shown). Further analysis of this phenomenon is beyond the scope of this paper.

Antisense inhibition of *Aox1* gene expression has effectively reduced AOX protein in AS8 to undetectable levels (Fig. 6). To our knowledge, this is the first report of antisense inhibition of any component of mET in plants. More significant is the finding that the capacity for AP respiration in AS8 has been dramatically reduced, confirming again that this capacity is primarily determined by AOX protein levels (Fig. 8B). We have not characterized the specific mechanism by which the *Aox1* antisense transgene has silenced expression of the endogenous *Aox1* gene. Several levels of inhibition of a target gene by antisense RNA may be possible, including target mRNA transcription, processing, transport from the nucleus, and translation (Murray, 1992; Nellen and Lichtenstein, 1993).

Heterotrophic cells of AS8 growing in batch culture with ample carbon and nutrient supplies respired and grew at rates similar to wt cells, suggesting that under these growth conditions the AP was not critical to metabolism (Fig. 8A; Table II). This is consistent with the finding that there was minimal AP activity in wt cells under these conditions. Nonetheless, the AP is known to be active in vivo under a wide range of conditions (Azcon-Bieto et al., 1983; Jolivet et al., 1990; Palet et al., 1991; Robinson et al., 1992; Rychter et al., 1992; Hoefnagel et al., 1994). In wt tobacco suspension cells, a mechanism exists whereby the capacity and activity of the AP is adjusted in response to changes in the activity of the CP (Vanlerberghe and McIntosh, 1992b, 1994). When CP activity is suppressed, increased *Aox1* gene expression results in increased AP capacity, enabling the cells to maintain high respiration rates using the AP. Since *Aox1* gene expression is impaired in AS8 cells, we were able to test whether increased AP capacity is a critical adaptation when the CP is suppressed. We examined the ability of wt and AS8 cells to grow in the presence of AA, an effective and specific inhibitor of CP activity in tobacco suspension cells (Vanlerberghe and McIntosh, 1992b, 1994). Although wt cells were able to maintain some growth in the presence of AA, AS8 cells could not survive this treatment (Table II). This indicates that AOX can support the respiration of wt cells and that this may be a critical function if the CP is impaired. Conditions that may impair CP activity in plants include chilling temperatures (Prasad et al., 1994), nutrient deprivation (Bingham and Farrar, 1989; Hoefnagel et al., 1994), salt stress (Jolivet et al., 1990), high CO_2 (Palet et al., 1991), and Pi limitation (Rychter et al., 1992). Using transgenic plants in which endogenous *Aox1* gene expression has been specifically down-regulated by an antisense *Aox1* gene, we are now in a position to critically access the role of AOX under environmental conditions restricting CP activity.

Received July 5, 1994; accepted September 14, 1994.
Copyright Clearance Center: 0032-0889/94/106/1503/08.

LITERATURE CITED

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. *In* SB Gelvin, RA Schilperoort, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp A3, 1–19

Azcón-Bieto J, Lambers H, Day DA (1983) Effect of photosynthesis and carbohydrate status on respiratory rates and the involvement of the alternative pathway in leaf respiration. Plant Physiol **72:** 598–603

Bingham IJ, Farrar JF (1989) Activity and capacity of respiratory pathways in barley roots deprived of inorganic nutrients. Plant Physiol Biochem **27:** 847–854

Boutry M, Faber AM, Charbonnier M, Briquet M (1984) Microanalysis of plant mitochondrial protein synthesis products: detection of variant polypeptides associated with cytoplasmic male sterility. Plant Mol Biol **3:** 445–452

Bryce JH, Azcón-Bieto J, Wiskich JT, Day DA (1990) Adenylate control of respiration in plants: the contribution of rotenone-insensitive electron transport to ADP-limited oxygen consumption by soybean mitochondria. Physiol Plant **78:** 105–111

Conley CA, Hanson MR (1994) Tissue-specific protein expression in plant mitochondria. Plant Cell **6:** 85–91

Day DA (1992) Can inhibitors be used to estimate the contribution of the alternative oxidase to respiration in plants? *In* H Lambers, LHW van der Plas, eds, Molecular, Biochemical and Physiological Aspects of Plant Respiration. SPB Academic Publishing, The Hague, The Netherlands, pp 37–42

Day DA, Arron GP, Laties GG (1980) Nature and control of respiratory pathways in plants: the interaction of cyanide-resistant respiration with the cyanide-sensitive pathway. *In* DD Davies, ed, The Biochemistry of Plants, A Comprehensive Treatise, Vol 2, Metabolism and Respiration. Academic Press, New York, pp 581–611

Day DA, Dry IB, Soole KL, Wiskich JT, Moore AL (1991) Regulation of alternative pathway activity in plant mitochondria: deviations from Q-pool behavior during oxidation of NADH and quinols. Plant Physiol **95:** 948–953

Day DA, Lambers H (1983) The regulation of glycolysis and electron transport in roots. Physiol Plant **58:** 155–160

Dry IB, Moore AL, Day DA, Wiskich JT (1989) Regulation of alternative pathway activity in plant mitochondria: nonlinear relationship between electron flux and the redox poise of the quinone pool. Arch Biochem Biophys **273:** 148–157

Elthon TE, Nickels RL, McIntosh L (1989a) Monoclonal antibodies to the alternative oxidase of higher plant mitocondria. Plant Physiol **89:** 1311–1317

Elthon TE, Nickels RL, McIntosh L (1989b) Mitochondrial events during the development of thermogenesis in *Sauromatum guttatum* (Schott). Planta **180:** 82–89

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc Natl Acad Sci USA **91:** 3490–3496

Hoefnagel MHN, Van Iren F, Libbenga KR, van der Plas LHW (1994) Possible role of adenylates in the engagement of the cyanide-resistant pathway in nutrient-starved *Catharanthus roseus* cells. Physiol Plant **90:** 269–278

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1988) Leaf disc transformation. *In* SB Gelvin, RA Schilperoort, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp A5, 1–9

Johns C, Nickels R, McIntosh L, Mackenzie S (1993) The expression of alternative oxidase and alternative respiratory capacity in cytoplasmic male sterile common bean. Sex Plant Reprod **6:** 257–265

Jolivet Y, Pireaux JC, Dizengremel P (1990) Changes in properties of barley leaf mitochondria isolated from NaCl-treated plants. Plant Physiol **94:** 641–646

Kapulnik Y, Yalpani N, Raskin I (1992) Salicylic acid induces cyanide-resistant respiration in tobacco cell-suspension cultures. Plant Physiol **100:** 1921–1926

Kearns A, Whelan J, Young S, Elthon TE, Day DA (1992) Tissue-specific expression of the alternative oxidase in soybean and siratro. Plant Physiol **99:** 712–717

Kumar AM, Soll D (1992) *Arabidopsis* alternative oxidase sustains *Escherichia coli* respiration. Proc Natl Acad Sci USA **89:** 10842–10846

Linsmaier EM, Skoog F (1965) Organic growth factor requirement of tobacco tissue cultures. Physiol Plant **18:** 100–127

Matzke M, Matzke AJM (1993) Genomic imprinting in plants: parental effects and trans-inactivation phenomena. Annu Rev Plant Physiol Plant Mol Biol **44:** 53–76

McIntosh L (1994) Molecular biology of the alternative oxidase. Plant Physiol **105:** 781–786

Millar AH, Wiskich JT, Whelan J, Day DA (1993) Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett **329:** 259–262

Minagawa N, Koga S, Nakano M, Sakajo S, Yoshimoto A (1992) Possible involvement of superoxide anion in the induction of cyanide-resistant respiration in *Hansenula anomala*. FEBS Lett **302:** 217–219

Moller IM, Bérczi A, van der Plas LHW, Lambers H (1988) Measurement of the activity and the capacity of the alternative pathway in intact tissues: identification of problems and possible solutions. Physiol Plant **72:** 642–649

Moore AL, Siedow JN (1991) The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. Biochim Biophys Acta **1059:** 121–140

Murray JAH (1992) Antisense RNA and DNA. Wiley-Liss, New York

Nellen W, Lichtenstein C (1993) What makes an mRNA anti-sense-itive? Trends Biochem Sci **18:** 419–423

Obenland D, Diethelm R, Shibles R, Stewart C (1990) Relationship of alternative respiratory capacity and alternative oxidase amount during soybean seedling growth. Plant Cell Physiol **31:** 897–901

Palet A, Ribas-Carbo M, Argilés JM, Azcón-Bieto J (1991) Short-term effects of carbon dioxide on carnation callus cell respiration. Plant Physiol **96:** 467–472

Prasad TK, Anderson MD, Stewart CR (1994) Acclimation, hydrogen peroxide, and abscisic acid protect mitochondria against irreversible chilling injury in maize seedlings. Plant Physiol **105:** 619–627

Rhoads DM, McIntosh L (1992) Salicylic acid regulation of respiration in higher plants: alternative oxidase expression. Plant Cell **4:** 1131–1139

Robinson SA, Yakir D, Ribas-Carbo M, Giles L, Osmond CB, Siedow JN, Berry JA (1992) Measurements of the engagement of cyanide-resistant respiration in the Crassulacean acid metabolism plant *Kalanchoë daigremontiana* with the use of on-line oxygen isotope discrimination. Plant Physiol **100:** 1087–1091

Rychter AM, Chauveau M, Bomsel JL, Lance C (1992) The effect of phosphate deficiency on mitochondrial activity and adenylate levels in bean roots. Physiol Plant **84:** 80–86

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Siedow JN, Moore AL (1993) A kinetic model for the regulation of electron transfer through the cyanide-resistant pathway in plant mitochondria. Biochim Biophys Acta **1142:** 165–174

Umbach AL, Siedow JN (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol **103:** 845–854

Vanlerberghe GC, McIntosh L (1992a) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. Plant Physiol **100:** 115–119

Vanlerberghe GC, McIntosh L (1992b) Coordinate regulation of cytochrome and alternative pathway respiration in tobacco. Plant Physiol **100:** 1846–1851

Vanlerberghe GC, McIntosh L (1994) Mitochondrial electron transport regulation of nuclear gene expression: studies with the alternative oxidase gene of tobacco. Plant Physiol **105:** 867–874

Weger HG, Guy RD, Turpin DH (1990) Cytochrome and alternative pathway respiration in green algae: measurements using inhibitors and $^{18}O_2$ discrimination. Plant Physiol **93:** 356–360