

Lower Growth Temperature Increases Alternative Pathway Capacity and Alternative Oxidase Protein in Tobacco¹

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ABSTRACT

Suspension cells of NT1 tobacco (*Nicotiana tabacum* L. cv bright yellow) have been used to study the effect of growth temperature on the CN-resistant, salicylhydroxamic acid-sensitive alternative pathway of respiration. Mitochondria isolated from cells maintained at 30°C had a low capacity to oxidize succinate via the alternative pathway, whereas mitochondria isolated from cells 24 h after transfer to 18°C displayed, on average, a 5-fold increase in this capacity (from 7 to 32 nanoatoms oxygen per milligram protein per minute). This represented an increase in alternative pathway capacity from 18 to 45% of the total capacity of electron transport. This increased capacity was lost upon transfer of cells back to 30°C. A monoclonal antibody to the terminal oxidase of the alternative pathway (the alternative oxidase) from *Sauromatum guttatum* (T.E. Elthon, R.L. Nickels, L. McIntosh [1989] *Plant Physiology* 89: 1311–1317) recognized a 35-kilodalton mitochondrial protein in tobacco. There was an excellent correlation between the capacity of the alternative path in isolated tobacco mitochondria and the levels of this 35-kilodalton alternative oxidase protein. Cycloheximide could inhibit both the increased level of the 35-kilodalton alternative oxidase protein and the increased alternative pathway capacity normally seen upon transfer to 18°C. We conclude that transfer of tobacco cells to the lower temperature increases the capacity of the alternative pathway due, at least in part, to de novo synthesis of the 35-kilodalton alternative oxidase protein.

Plants have two pathways of mET³ to oxygen (4). Electron flow via the CP is coupled to ATP production and its terminal oxidase (Cyt oxidase) is sensitive to KCN. Electron flow via the AP is not coupled to ATP production and its terminal oxidase (AO) is sensitive to SHAM (14, 15).

Several studies suggest that when plants grow at lower temperatures, the AP is a more important component of total respiration. This has been investigated in whole tissues and/or isolated mitochondria from maize (7, 29, 30), wheat (20), potato (10), *Brassica* (27), soybean (16), cucumber (24), *Plan-*

tago (28), and other species. Although it appears there is an increased capacity (or potential) for AP respiration in these tissues, the degree of engagement of this respiratory pathway at lower temperatures is not clear because of the inherent problems in using KCN and SHAM to measure engagement (22). Therefore, a conclusive answer concerning the degree of engagement of the pathway awaits measurements based upon oxygen-isotope discrimination (9, 31).

The role of AP respiration in growth at lower temperatures is not known. Growth at lower temperature can result in a substantial redirection of respiratory metabolism, possibly due to the differential effect of temperature on enzymes of metabolism (1, 8). Possibly, this altered metabolism places demands upon mET that bring about increased AP respiration. It has been shown that transfer of plants to lower temperatures can result in the accumulation of soluble sugars (8, 25, 27). In several species, high sugar levels correlate with increased AP respiration (2, 14), which is consistent with the hypothesis that AP respiration acts to remove excess carbohydrate (the "energy overflow" hypothesis, ref. 14). Changes in AP capacity at different temperatures might also be related to the sensitivity of plant mET pathways to temperature extremes. It has been suggested that the CP is more cold-labile than the AP (16, 21), and also that the AP is more heat-labile (3, 18).

It is not known what components of the mET chain are responsible for increased AP capacity at low temperature. In maize, plants grown at lower temperatures had more AO protein in some tissues (30), suggesting that increased AP capacity may have been brought about by an increase in the amount of the terminal oxidase of the pathway. We have now investigated this further in tobacco (*Nicotiana tabacum* L.), a species in which the effect of temperature on AP respiration has not yet been thoroughly investigated. In this paper, we show that when suspension cells of tobacco are transferred to a lower temperature there is a rapid increase in the capacity for AP respiration in whole cells and in isolated mitochondria due, at least in part, to de novo synthesis of the AO.

MATERIALS AND METHODS

Organism and Culture Conditions

Suspension cells of NT1 tobacco (*Nicotiana tabacum* L. cv bright yellow) were grown in batch culture under heterotrophic conditions in the medium previously described (19). This medium contains 3% (w/v) sucrose as a carbon source.

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³ Abbreviations: mET, mitochondrial electron transport; AO, alternative oxidase; AP, alternative pathway; CH, chloramphenicol; CP, cytochrome pathway; CX, cycloheximide; SHAM, salicylhydroxamic acid.

The culture was routinely grown at 30°C on a rotary shaker and when indicated was transferred to a rotary shaker at 18°C. In all of the experiments to be described, the transfer of cells from 30 to 18°C was done at 3 d after subculture, when the cells were at their maximum growth rate and culture density was approximately 15 to 20% packed cell volume. Cells at 30°C were subcultured every 6 to 7 d to a density of 4% packed cell volume.

AP Capacity: Whole Cells

Cells (approximately 20–50 mg fresh weight) in their culture medium were placed in a Rank Brothers oxygen electrode cuvette. O₂ uptake was measured at the same temperature at which the cells had been growing. For example, for cells shifted to 18°C, O₂ uptake was measured at 18°C. AP capacity was taken to be that portion of the O₂ consumption inhibited by 2 mM SHAM in the presence of 1 mM KCN. CP capacity was taken to be that portion of the O₂ consumption inhibited by 1 mM KCN in the presence of 2 mM SHAM. These concentrations of inhibitors were shown to produce maximal inhibition in whole cells. In cases where SHAM in the absence of KCN stimulated O₂ uptake, this stimulation was subtracted from the CP capacity. Total capacity is the sum of the CP and AP capacities. Residual respiration (in the presence of KCN and SHAM) was insignificant.

AP Capacity: Isolated Mitochondria

Washed mitochondria were isolated (all manipulations done at 4°C) from approximately 20 g fresh weight of cells. Cells were washed two times in fresh culture medium and then disrupted (in a commercial blender, 2 × 3 s) in 400 mL of isolation buffer (350 mM mannitol, 30 mM Mops, 1 mM EDTA, 4 mM cysteine, 0.2% BSA, 0.6% polyvinylpyrrolidone [pH 7.5]). After filtration through four layers of cheesecloth, the homogenate was centrifuged (6,000g, 2 min). The resulting supernatant was centrifuged (25,000g, 5 min) to

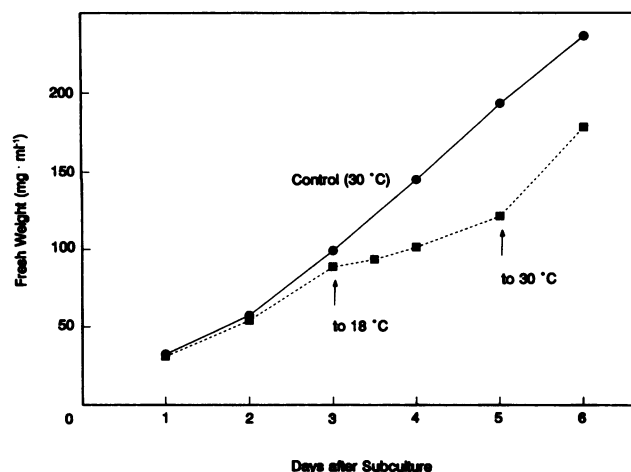


Figure 1. Fresh weight of a cell suspension culture of tobacco as affected by transfer from 30 to 18°C at 3 d after subculture and transfer back to 30°C at 5 d after subculture.

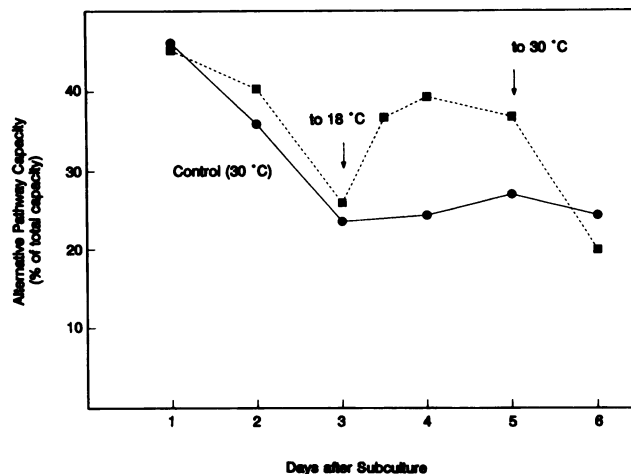


Figure 2. The effect of growth temperature on AP capacity (as a percentage of total capacity) measured in suspension cells of tobacco. Cells were transferred from 30 to 18°C at 3 d after subculture and then transferred back to 30°C at 5 d after subculture.

pellet the mitochondria, which were then resuspended in 160 mL of wash buffer (300 mM mannitol, 20 mM Mops, 1 mM EDTA, 0.2% BSA [pH 7.2]) and centrifuged (5,000g, 2 min). The supernatant was centrifuged (18,000g, 5 min) to pellet the mitochondria, which were then resuspended in 200 μ L of assay buffer (250 mM sucrose, 30 mM Mops [pH 6.8]). Samples (approximately 0.3–0.4 mg of protein) were then placed in a Rank Brothers oxygen electrode cuvette at 25°C in a total volume of 1 mL of assay buffer. ATP (200 μ M) was then added 2 min prior to addition of substrate. The exogenous substrate was 10 mM succinate with 500 μ M ADP. AP capacity was taken to be that O₂ consumption inhibited by 1 mM SHAM in the presence of 1 mM KCN. CP capacity was taken to be that O₂ consumption inhibited by 1 mM KCN in the presence of 1 mM SHAM. The sum of the two capacities was taken to be the total capacity.

AO Protein

Samples of washed mitochondria (100 μ g of protein) were added to SDS sample buffer (2% [w/v] SDS, 2% [v/v] 2-mercaptoethanol, 10% [v/v] glycerol, 42 mM Tris-HCl [pH 6.8]) and boiled for 2 min prior to addition of 0.08% (w/v) bromophenol blue tracking dye. Electrophoresis of these samples was carried out with the buffer system of Laemmli (13) using a 5% (w/v) stacking gel and a 10 to 17.5% (w/v) polyacrylamide gradient resolving gel. The resolved proteins were then blotted to nitrocellulose and probed with a 1:500 dilution of a monoclonal antibody against the *Sauromatum guttatum* AO (5) essentially as previously described (11).

Other Methods

Stocks of KCN and SHAM were prepared fresh weekly. For respiration assays on whole cells, SHAM was dissolved in 95% ethanol. For respiration assays on isolated mitochondria, SHAM was dissolved in DMSO.

Protein was determined by a modified Lowry method (11).

Table I. The Capacity to Oxidize Succinate via the AP and the CP in Mitochondria Isolated from Cells Either Maintained at 30°C (Mitochondria Isolated at 4 d after Subculture) or Transferred from 30 to 18°C for 24 h (Mitochondria Isolated at 4 d after Subculture)

The last column is the percentage of total capacity attributed to the AP. Data are the average \pm SD from three independent experiments.

Treatment	Electron Transport Capacity			Percentage Alternative
	AP	CP	Total	
	<i>natoms O · mg⁻¹ protein · min⁻¹</i>			
30°C	7 \pm 3	30 \pm 1	37 \pm 2	18 \pm 8
18°C	32 \pm 7	40 \pm 8	72 \pm 10	45 \pm 6

RESULTS

Growth

Transfer of cells from 30 to 18°C resulted in a lower growth rate of the culture (Fig. 1). However, if after 48 h cells were transferred back to 30°C, growth returned to control rates, indicating that the lower temperature had not brought about any apparent lethal effects on the culture (Fig. 1). We have also shown that cells can be maintained at 18°C for many subcultures, indicating that the cells remain viable at this temperature (data not shown).

AP Capacity: Whole Cells

Transfer of cells from 30 to 18°C brought about a rapid increase in the percentage of total mET that could be mediated via the AP. Within 24 h of transfer to 18°C, the capacity of the AP increased from 25.9 to 39.3% of the total

capacity of electron transport under these conditions, whereas there was no change in cells maintained at 30°C (Fig. 2). When cells at 18°C were transferred back to 30°C, the capacity of the AP returned to control levels (Fig. 2), indicating that this effect of temperature was reversible.

AP Capacity: Isolated Mitochondria

Mitochondria isolated from cells maintained at 30°C had a low capacity to oxidize succinate via the AP (Table I). Approximately 18% of total mET capacity during succinate oxidation was attributed to the AP (Table I). However, mitochondria isolated from cells 24 h after transfer to 18°C displayed almost a 5-fold increase in the rate of succinate oxidation via the AP (from 7–32 *natoms O · mg⁻¹ protein · min⁻¹*). There was only a small increase in CP capacity over this same time period (Table I). Therefore, AP capacity increased from 18 to 45% of the total capacity of mET (Table I). Mitochondria isolated from cells transferred from 18 back to 30°C for 24 h had AP capacities similar to those of control mitochondria, indicating that this effect of temperature was reversible (Fig. 3A).

AO Protein

A monoclonal antibody against the AO from *S. guttatum* also recognizes an AO protein in tobacco. The antibody reacts to a mitochondrial polypeptide of an apparent molecular mass of 35 kD (similar to the *S. guttatum* AO, [5]). Mitochondria isolated from cells maintained at 30°C had low levels of this AO protein (Fig. 3B). However, mitochondria from cells transferred to 18°C for 24 h had high levels of AO protein. This level was maintained until cells were transferred back

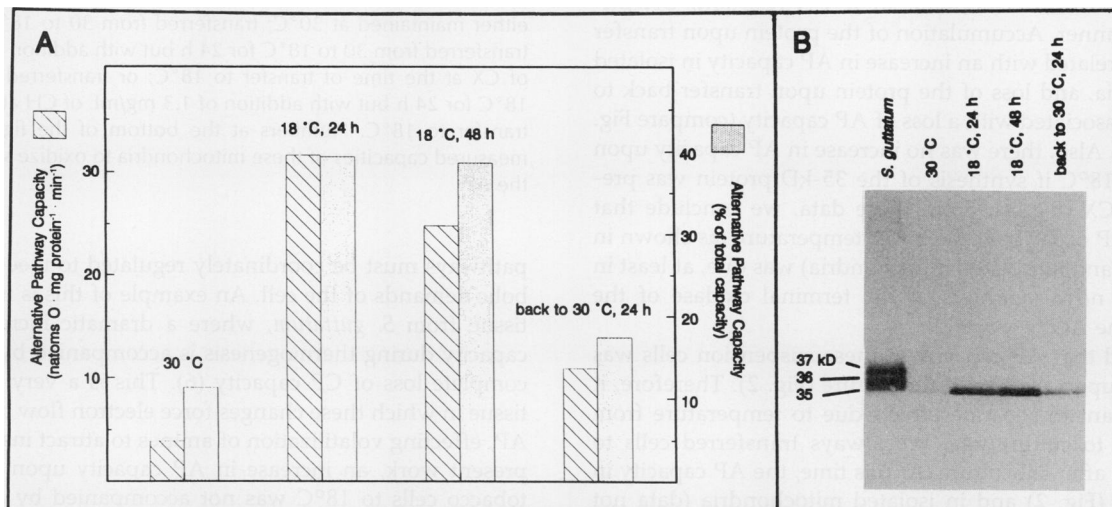


Figure 3. A, The capacity to oxidize succinate via the AP in mitochondria isolated from cells either maintained at 30°C (mitochondria isolated at 4 d after subculture); transferred from 30 to 18°C for 24 h (mitochondria isolated at 4 d after subculture); transferred from 30 to 18°C for 48 h (mitochondria isolated at 5 d after subculture); or transferred after 48 h at 18°C back to 30°C for 24 h (mitochondria isolated at 6 d after subculture). B, The amount of a 35-kD AO protein in isolated mitochondria. A western blot of total mitochondrial protein was probed with a monoclonal antibody to the AO of *S. guttatum*. Lane 1 is total mitochondrial protein from *S. guttatum* used as a control and shows the polypeptide profile previously reported (5). All other lanes are mitochondrial protein from tobacco (100 μ g protein/lane). The cells used were the same as described in A. The AP capacity of these same mitochondria are given in A.

to 30°C, at which time the protein declined back to near control levels (Fig. 3B).

CX (an inhibitor of cytoplasmic protein synthesis) inhibited the appearance of AO protein seen upon transfer of cells to 18°C (Fig. 4). It also prevented the increased capacity of isolated mitochondria to oxidize succinate via the AP (Fig. 4). On the other hand, CH (an inhibitor of mitochondrial protein synthesis) did not prevent the increase in AO protein nor the increased capacity of mitochondria to oxidize succinate via the AP (Fig. 4).

DISCUSSION

Suspension and callus cultures of tobacco have a CN-resistant, SHAM-sensitive component of respiration (12, 17). Here we show that a monoclonal antibody to the AO from *S. guttatum* recognizes an AO protein in suspension cells of tobacco. The 35-kD mitochondrial protein recognized is similar in size to that in other species (5, 23, 30). It was present at low abundance in cells at 30°C but accumulated to high levels within 24 h of transfer of cells to 18°C (Fig. 3B). This accumulation was prevented by an inhibitor of cytoplasmic protein synthesis (CX), suggesting de novo synthesis of this protein in response to the transfer to the lower temperature (Fig. 4). The inhibition by CX but not by CH (an inhibitor of mitochondrial protein synthesis) is consistent with the AO being the product of a nuclear-encoded gene (26) and translated in the cytosol. In some cases, we saw a second immunoreactive protein of a slightly higher molecular mass (approximately 35.5 kD) on western blots. This band was difficult to resolve but was less intense than the major 35-kD band. The second band may be a mitochondrial precursor or a posttranslationally modified form of the 35-kD protein.

In all of our experiments, the abundance of the 35-kD protein correlated closely with the capacity of isolated mitochondria to oxidize succinate in a CN-resistant, SHAM-sensitive manner. Accumulation of the protein upon transfer to 18°C correlated with an increase in AP capacity in isolated mitochondria, and loss of the protein upon transfer back to 30°C was associated with a loss of AP capacity (compare Fig. 3, A and B). Also, there was no increase in AP capacity upon transfer to 18°C if synthesis of the 35-kD protein was prevented by CX (Fig. 4). From these data, we conclude that increased AP capacity at the lower temperature (as shown in whole cells and in isolated mitochondria) was due, at least in part, to de novo synthesis of the terminal oxidase of the pathway, the AO.

We found that AP capacity in these suspension cells was dependent upon the age of the culture (Fig. 2). Therefore, it was important to separate effects due to temperature from effects due to culture age. We always transferred cells to 18°C at 3 d after subculture. At this time, the AP capacity in whole cells (Fig. 2) and in isolated mitochondria (data not shown) was low. Levels of AO protein were also low (data not shown). At 4 d after subculture, those cells maintained at 30°C still had low AP capacity (Figs. 2 and 3A, Table I) and AO protein (Figs. 3B and 4), whereas cells that were transferred to 18°C had high AP capacity (Figs. 2 and 3A, Table I) and AO protein (Figs. 3B and 4).

Because plants have two pathways of mET to O₂, these

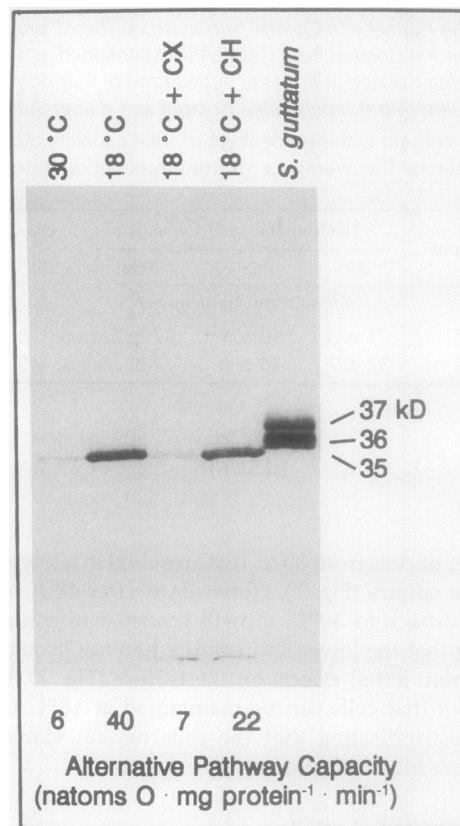


Figure 4. The amount of a 35-kD AO protein in isolated mitochondria. A western blot of total mitochondrial protein was probed with a monoclonal antibody to the AO of *S. guttatum*. Lane 5 is total mitochondrial protein from *S. guttatum* used as a control and shows the polypeptide profile previously reported (5). All other lanes are mitochondrial protein from tobacco (100 μ g protein/lane). The mitochondria (all isolated at 4 d after subculture) are from cells either maintained at 30°C; transferred from 30 to 18°C for 24 h; transferred from 30 to 18°C for 24 h but with addition of 50 μ g/mL of CX at the time of transfer to 18°C; or transferred from 30 to 18°C for 24 h but with addition of 1.3 mg/mL of CH at the time of transfer to 18°C. Numbers at the bottom of the figure are the measured capacities of these mitochondria to oxidize succinate via the AP.

pathways must be coordinately regulated to meet the metabolic demands of the cell. An example of this is in appendix tissue from *S. guttatum*, where a dramatic increase in AP capacity during thermogenesis is accompanied by an almost complete loss of CP capacity (6). This is a very specialized tissue in which these changes force electron flow through the AP, effecting volatilization of amines to attract insects. In the present work, an increase in AP capacity upon transfer of tobacco cells to 18°C was not accompanied by any loss in CP capacity (Table I). It is clear, therefore, that an increase in AP capacity need not be accompanied by a loss of CP capacity in all cases.

The study of coordinate regulation of CP and AP respiration is still in its infancy. Although it is clear that lower temperature increases AP capacity in tobacco as well as several other species (see introduction), it is not yet clear to

what extent the pathway is engaged at lower temperature. Also, the signals that regulate AP capacity in response to temperature and the functional role of the pathway at different temperatures are not known. It appears that this cell suspension of tobacco may be an excellent system in which to study AP respiration in response to temperature.

CONCLUSION

This study shows that transfer to a lower growth temperature rapidly increases the capacity of the CN-resistant, SHAM-sensitive component of respiration in both whole cells and isolated mitochondria of tobacco. These observations support previous work in other species of plants. We have extended previous work by demonstrating that this increase in AP capacity is due, at least in part, to *de novo* synthesis of the terminal oxidase of the pathway, a 35-kD AO protein.

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