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Mitochondrial Electron Transport Regulation of Nuclear Gene Expression[1]

Studies with the Alternative Oxidase Gene of Tobacco

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We have isolated a cDNA representing the tobacco (*Nicotiana tabacum* L. cv Bright Yellow) nuclear gene *Aox1*, which encodes the alternative oxidase of plant mitochondria. The clone contains the complete coding region (1059 base pairs) of a precursor protein of 353 amino acids with a calculated molecular mass of 39.8 kD. A putative transit peptide contains common signals believed to be important for import and processing of mitochondrially localized proteins. We have studied changes in *Aox1* gene expression in tobacco in response to changes in cytochrome pathway activity. Inhibition of the cytochrome pathway by antimycin A resulted in a rapid and dramatic accumulation of *Aox1* mRNA, whereas the level of mRNAs encoding two proteins of the cytochrome pathway did not change appreciably. This was accompanied by a dramatic increase in alternative pathway capacity and engagement in whole cells. Respiration under these conditions was unaffected by the uncoupler *p*-trifluoromethoxycarbonylcyanide (FCCP). When inhibition of the cytochrome pathway was relieved, levels of *Aox1* mRNA returned to control levels, alternative pathway capacity and engagement declined, and respiration could once again be stimulated by FCCP. The results show that a mechanism involving changes in *Aox1* gene expression exists whereby the capacity of the alternative pathway can be adjusted in response to changes in the activity of the cytochrome pathway.

Plants have two pathways of mET from ubiquinone to O_2 (Day et al., 1980). Electron transfer through the CP is coupled to the synthesis of ATP and the terminal oxidase (Cyt oxidase) is inhibited by CN^-. Electron flow through the AP is not coupled to ATP production. The terminal oxidase is called the AOX and is sensitive to SHAM (Lance et al., 1985; Moore and Siedow, 1991).

The AOX in plants is encoded by the nuclear gene *Aox1* (Rhoads and McIntosh, 1991; Kumar and Soll, 1992), which is present as a single copy in the genome of the thermogenic plant *Sauromatum guttatum* (Rhoads and McIntosh, 1993). Monoclonal antibodies to the *S. guttatum* AOX (Elthon et al., 1989a) have identified the AOX in the mitochondrion of several plant species. The presence of multiple (two to three) AOX proteins of similar size in purified mitochondria from *S. guttatum* (Elthon et al., 1989b) and nonthermogenic plants (Obenland et al., 1990; Kearns et al., 1992; Vanlerberghe and McIntosh, 1992b) suggests that posttranslational processing and/or modification of the AOX occurs. Studies also indicate that the amount of AOX protein in the mitochondrion correlates closely with the capacity of the AP to support O_2 consumption (Hiser and McIntosh, 1990; Obenland et al., 1990; Kearns et al., 1992; Rhoads and McIntosh, 1992; Vanlerberghe and McIntosh, 1992a, 1992b).

The partitioning of electrons between the CP and the AP appears to be dependent primarily on the redox poise of the Q pool (Dry et al., 1989; Siedow and Moore, 1993). Whereas CP activity varies linearly with the reduction state of the Q pool, the AP is not active until the Q pool reduction level reaches approximately 35 to 40% (Dry et al., 1989). This is consistent with numerous studies on both isolated mitochondria and whole tissue that suggest that the AP is engaged in O_2 consumption only when the CP becomes limiting or restricted (Theologis and Laties, 1978; Day and Lambers, 1983). It should be noted that partitioning between the two pathways may also be dependent on the substrates being oxidized by the mitochondria (Day et al., 1991; Wagner et al., 1992a), the level of potential allosteric activators of the AOX (Lidén and Åkerlund, 1993; Millar et al., 1993), and possibly the oxidation state of an AOX sulfhydryl residue (Umbach and Siedow, 1993). More work is required to determine the significance of these regulatory mechanisms.

Since plants have two pathways of mET to O_2, it seems imperative that the capacity of these pathways to support O_2 consumption be coordinately regulated to meet the metabolic demands of the cell. In appendix tissue of *S. guttatum*, a dramatic increase in the capacity of the AP during thermogenesis is accompanied by an almost complete loss of CP

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Abbreviations: AA, antimycin A; AOX, alternative oxidase; AP, alternative pathway; CP, cytochrome pathway; FCCP, *p*-trifluoromethoxycarbonylcyanide; mET, mitochondrial electron transport; Q, ubiquinone; ρ, fraction (0–1) of the AP capacity that is engaged in O_2 consumption; SHAM, salicylhydroxamic acid.

capacity (Elthon et al., 1989b; Skubatz et al., 1991). This forces electron flow through the AP, producing heat and thus effecting volatilization of amines to attract insects. In this specialized thermogenic tissue, these changes are brought about, at least in part, by increased levels of *Aox1* mRNA (Rhoads and McIntosh, 1993) and a decreased level of mRNA encoding CP proteins (Elthon et al., 1989b). In tobacco (*Nicotiana tabacum* L.), experiments using the CP inhibitor AA have shown that this nonthermogenic plant is capable of gradual alteration of AP capacity in response to gradual changes in the CP (Vanlerberghe and McIntosh, 1992b). This enables the cells to maintain high respiration rates, even when the CP is severely inhibited. An important mechanism involved in the coordinate regulation of the pathways appeared to be the level of the AOX protein. In the present report, we have isolated the nuclear gene *Aox1* from tobacco and have used this clone to investigate coordinate regulation of the mET paths in tobacco at the level of gene expression.

MATERIALS AND METHODS

Organism and Growth Conditions

Suspension cells of NT1 tobacco (*Nicotiana tabacum* L. cv *Bright Yellow*) were grown in batch culture under heterotrophic conditions as described (Vanlerberghe and McIntosh, 1992b).

Isolation of *Aox1* cDNA Clone

Poly(A)$^+$ RNA for library construction was isolated from tobacco suspension cells grown at 30°C and then transferred to 18°C for 8 h. This temperature shift has been shown to increase the level of AOX protein in these cells (Vanlerberghe and McIntosh, 1992a). Cells were separated from their growth medium by centrifugation (1800*g*, 3 min) and homogenized with a mortar and pestle in the ENT buffer described by McIntosh and Cattolico (1978) except that it contained 15 mM EDTA. Total RNA was extracted from this homogenate as described (McIntosh and Cattolico, 1978). The poly(A)$^+$ fraction was then isolated by poly(U)-Sepharose (Sigma) chromatography (Cashmore, 1982), and a cDNA expression library was constructed in the *Eco*RI site of phage λZAP II (Alting-Mess et al., 1992) by Stratagene (La Jolla, CA) using an oligo(dT) primer.

Library screening with a ^{32}P-labeled DNA probe and plaque purification were done according to standard procedures (Sambrook et al., 1989). The DNA probe (purified and radiolabeled as described below) used for library screening was a 1.25-kb cDNA representing the *Aox1* gene of potato (C. Hiser and L. McIntosh, unpublished data). Inserts from phage isolates that hybridized to this probe were subcloned into the phagemid vector pBluescript SK$^-$ by an in vivo excision procedure (Short and Sorge, 1992). Two positive clones were initially isolated, and it was shown by restriction enzyme analysis and agarose gel electrophoresis that the *Eco*RI inserts of these two clones were of similar size and with identical restriction maps. Hence, only one clone (named pAONT1) was further analyzed.

DNA Sequencing and Sequence Analysis

Double-stranded DNA of pAONT1 (in *Escherichia coli* strain XL-1 Blue) was isolated using the Magic minipreps system (Promega, Madison, WI). Both strands of the *Eco*RI insert of pAONT1 were sequenced at the Department of Energy/Plant Research Laboratory Biochemistry Facility, Michigan State University. Standard primers (−21 M13, −21 M13 reverse) and synthetic oligonucleotides (synthesized at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University) were used as sequencing primers. Dideoxy cycle sequencing reactions were done on an ABI Robotic Catalyst (Applied Biosystems, Foster City, CA) using either the Taq Dye Primer Cycle Sequencing kit (when using standard primers) or the Taq Dyedeoxy Terminator Cycle Sequencing kit (when using nonstandard primers). Fluorescent-labeled DNA was then analyzed on a 373A DNA Sequencer (Applied Biosystems). Protein sequence was deduced from the nucleotide sequence using the Editbase Sequence Analysis Package (N. Nielson, Purdue University, West Lafayette, IN). Protein sequence comparisons were performed using the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin. Protein secondary structure predictions were performed using the programs of SEQANAL (A.R. Crofts, University of Illinois, Chicago, IL), which combine standard Chou-Fasman predictions with Rao-Argos modifications to accommodate membrane-spanning proteins. The hydrophobicity scale of Kyte and Doolittle was used.

Other Clones

Two other clones were also used in this work. The first was a cDNA representing the nuclear gene *Cc1*, which encodes the apoprotein of Cyt *c* (Kemmerer et al., 1991). This clone was isolated from the tobacco library described above (B. Dreschler-Thielmann and L. McIntosh, unpublished data). The second clone was a cDNA representing the mitochondrial gene *cox1* from maize, which encodes Cyt oxidase subunit I. It was a gift from C.S. Levings III (North Carolina State University, Raleigh, NC).

Plasmid Insert Isolation and Radiolabeling

All DNA fragments used as hybridization probes were purified by PAGE as described (Sambrook et al., 1989) using the elution buffer described (Rhoads and McIntosh, 1992). The purified fragment was used to make DNA radiolabeled with [α-^{32}P]dATP (Amersham) by the random primer method (Feinberg and Vogelstein, 1983).

Northern Analysis

RNA was isolated by a miniprep procedure (Verwoerd et al., 1989), separated on agarose gels containing formaldehyde (Ausubel et al., 1987), and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) as described (Sambrook et al., 1989). Molecular sizes were estimated by comparison with an ethidium bromide-stained lane of a 9.5- to 0.24-kb RNA ladder (Gibco-BRL). Northern blots were hybridized (1× Denhardt's solution, 750 mM NaCl, 75 mM sodium citrate,

30 mM Tris, pH 7.5, 1.0 mM EDTA, 50% [v/v] formamide, 0.1% [w/v] SDS, 0.25% [w/v] nonfat dry milk, 25 μg/mL salmon sperm DNA) at 42°C with radiolabeled DNA made using purified *Eco*RI insert from pAONT1 or purified insert from the other clones used (see above). Washes were done in 1× SSC with 0.1% SDS. Two washes (15 min each) were done at room temperature followed by two washes (15 min each) at 42°C. Blots were analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and by autoradiography using Kodak diagnostic film XAR-5 at −80°C with an intensifying screen. Experiments were repeated two or three times, and representative results are shown.

Respiration Measurements

Experiments using AA (Sigma No. A-8674), the measure of CP and AP capacities in whole cells by the use of KCN and SHAM, the measure of respiratory O_2 consumption in the presence and absence of FCCP, and the determination of engagement of the AP were all done as described (Vanlerberghe and McIntosh, 1992b). Briefly, CP capacity was taken to be that portion of the O_2 consumption inhibited by 1 mM KCN in the presence of 2 mM SHAM, and AP capacity was taken to be that portion of the O_2 consumption inhibited by 2 mM SHAM in the presence of 1 mM KCN. The engagement of the AP (ρ) was determined as previously described (Moller et al., 1988; Vanlerberghe and McIntosh, 1992b). Residual respiration (in the presence of KCN and SHAM) was subtracted from all measures of total respiration. This residual component represented 9 ± 3% (SD, n = 20) of total O_2 consumption. The effect of FCCP (1 μM) on respiration rate was determined in the absence of KCN and SHAM. Experiments were repeated two or three times, and representative results are shown.

RESULTS

Isolation of an AOX cDNA Clone

By using a cDNA representing the *Aox1* gene of potato to screen an *N. tabacum* cDNA expression library, we have isolated a cDNA (pAONT1) representing the *Aox1* gene of tobacco. The insert of pAONT1 (1396 bp) contains the complete coding region (1059 bp) of a protein of 353 amino acids with a calculated molecular mass of 39.8 kD (Fig. 1). Protein secondary structure analysis predicts two extended regions

```
                                                                  ▼
                                                                  M  M  T  R  G  A  T
    1  gaattcggcacgagccaagtttctttccaaacttcctgcaactcttgaattttgatcggagttatcaaagatgatgacacgtggagcgac

         R  M  T  R  T  V  L  G  H  M  G  P  R  Y  F  S  T  A  I  F  R  N  D  A  G  T  G  V  M  S
   91  aaggatgacccgaactgtgttgggtcacatgggtccacgttacttctccactgcaatttttcgaaatgatgccggaaccggagttatgag

                                                                        •
         G  A  A  V  F  M  H  G  V  P  A  N  P  S  E  K  A  V  V  T  W  V  R  H  F  P  V  M  G  S
  181  tggcgccgccgttttcatgcatggggttccggcgaatccgtcggagaaagcagtggttacatgggttagacattttccggtgatgggttc
       •     †
         R  S  A  M  S  M  A  L  N  D  K  Q  H  D  K  K  A  E  N  G  S  A  A  A  T  G  G  G  D  G
  271  acgcagcgctatgtctatggctttgaatgataagcagcacgacaagaaagcggaaaatggcagcgccgccgcaaccggcggcggtgatgg

         G  D  E  K  S  V  V  S  Y  W  G  V  Q  P  S  K  V  T  K  E  D  G  T  E  W  K  W  N  C  F
  361  tggtgatgaaaaatcagtagtgagttattggggtgttcagccttcaaaggttactaaagaggatggtactgaatggaaatggaattgctt

         R  P  W  E  T  Y  K  A  D  L  S  I  D  L  T  K  H  H  A  P  T  T  F  L  D  K  F  A  Y  W
  451  taggccatgggagacgtacaaggctgatttatcaatagatctgacgaaacaccacgcgccgaccactttcttggacaaattcgcttattg

         T  V  K  S  L  R  Y  P  T  D  I  F  F  Q  R  R  Y  G  C  R  A  M  M  L  E  T  V  A  A  V
  541  gactgttaagtcccttcgatacccccactgatatatttttttcagaggagatatggttgtagagcaatgatgttggaaacagtggctgcagt

         P  G  M  V  G  G  M  L  L  H  C  K  S  L  R  R  F  E  Q  S  G  G  W  I  K  T  L  L  D  E
  631  gcctggaatggtaggaggaatgttgttgcactgcaaatcacttaggcgattcgaacagagtgggggatggatcaaaacactgttagatga

         A  E  N  E  R  M  H  L  M  T  F  M  E  V  A  K  P  N  W  Y  E  R  A  L  V  F  A  V  Q  G
  721  agctgaaaatgagaggatgcacctcatgactttcatggaagtagccaagcctaattggtacgagcgtgcacttgtctttgcagtgcaagg

         V  F  F  N  A  Y  F  V  T  Y  L  L  S  P  K  L  A  H  R  I  V  G  Y  L  E  E  E  A  I  H
  811  cgtcttcttcaacgcctactttgtcacttaccttctttctccaaagttggctcatcgtattgttggatacttggaagaagaggctattca

         S  Y  T  E  F  L  K  E  L  D  K  G  N  I  E  N  V  P  A  P  A  I  A  I  D  Y  C  R  L  P
  901  ttcctacaccgagttcctaaaggaattggacaagggtaacattgagaacgtccctgctcctgctattgccattgattactgccgtctccc

         K  D  S  T  L  L  D  V  V  L  V  V  R  A  D  E  A  H  H  R  D  V  N  H  F  A  S  D  I  H
  991  taaagactccactctcctggatgttgtcttggttgttagggctgacgaggctcatcaccgtgatgtcaaccactttgcatctgacattca

         Y  Q  G  Q  Q  L  K  D  S  P  A  P  I  G  Y  H  *
 1081  ttatcagggacaacagctgaaggactctccagcaccaattgggtatcactaagccaccagagattgatcaagttaaatgtcactattatc
 1171  ttctttttttgtactggtacacagctagctaatgtagtacctactactattttgaatgttgatgtacatatttttttctgtggatttggt
 1261  tggtttttactactgccttcatgaatttctgagatagcagtagagcacaatgagctctgtatgattggatcatataccttcttgataaa
 1351  atttaccatcttggtttttccaagaacatttgcttttaaaaaaactcgtgccgaattc
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Figure 1. Nucleotide and deduced amino acid sequence of the insert of pAONT1, representing the *Aox1* gene of tobacco. The inverted black triangle (▼) marks the assumed start codon and first amino acid of the precursor protein. The first in-frame stop codon is indicated by the star (*). The Ala residue denoted by a dagger (†, amino acid 70) is the potential N-terminal amino acid of the mature mitochondrial protein. This assumption is based on the position of common processing signals of mitochondrial transit peptides (the −2 Arg and −10 Arg denoted by bullets; ●). The terminal *Eco*RI linkers attached during cDNA library construction are underlined.

of hydrophobic α-helix (from Ala^{178} to Cys^{198} and from Ala^{240} to Arg^{266} in the tobacco sequence; Fig 2). These may represent membrane-spanning regions of the protein. The AOX from *S. guttatum* has been proposed to form two transmembrane α-helices within the same regions (Rhoads and McIntosh, 1991; Siedow et al., 1992).

Protein sequence comparison indicates that the C-terminal 250 amino acids of the tobacco AOX are 87% identical to those in *Arabidopsis thaliana*, 80% identical to those in *S. guttatum*, and 39% identical to those in the yeast *Hansenula anomala* (Fig. 2). Several amino acids that are completely conserved among all the sequences could potentially be involved in metal binding (Cys at position 126 and His at positions 144, 197, 224, 265, 326, and 331 in the tobacco sequence; Fig. 2). The completely conserved Cys^{126} is also a potential candidate for the residue involved in the disulfide linkage of AOX protein molecules, a linkage recently proposed by Umbach and Siedow (1993). There is little homology observed in the N-terminal portion of the protein sequences, particularly within the putative transit peptides (see below).

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tobacco  MMTRGATRMT RTVLGHMGPR YFSTAIFRN- -------DAG TGVMSGAAVF   42
Arabid.  ---------- ---------- ---------- ---------- ----------
Saurom.  ---------- -----M.SS. LVG..LC.QL SHVPVPQYLP .LRPTADT.S   35
Hansen.  ---------- ---------- ---------- ---------- ----------

tobacco  MHGVPANPSE KAVVTWV-RH FPVMGSRSAM SMALNDKQHD -------KKA   84
Arabid.  ---------- -----MDT.A PTIG.M.F.S TIT.GE.TPM KEEDANQ..T   35
Saurom.  SLLHGCSAAA P.QRAGLWPP SWFSPP.H.S TLS------A PAQDGGKE..   79
Hansen.  --------MI .TYQYRSILN SRNV.I.FLK TLS----PSP HSKDPNS.SI   38
                                                    •
tobacco  ENGSAAA-TG GGDGGDEKSV VSYWGVQPSK VTKEDGTEWK WNCFRPWETY  133
Arabid.  ..E.TGGDAA ..NNKGD.GI A.....E.N. I.....S... ..........   85
Saurom.  AGTAGKVPP. EDG.AEKEA. ....A.P... .S....S..R .T........  129
Hansen.  FDIGTKLIVN PPPQMADNQY .THPLFPHP. YSD..----- --.EAVHFV-   80
                 •
tobacco  KADLSIDLTK HHAPTTFLDK FAYWTVK--- -------SLR YPTDI-----  168
Arabid.  ...IT...K. ..V......R I.....--- -------... W...L-----  120
Saurom.  Q.......H. ..V...I... L.LR...--- -------A.. W...-----  164
Hansen.  ---------- .RE.K.IG.. I.DRG..FCR ASFDFVTGYK K.K.VNGMLK  120
                                            •
tobacco  --------FF QRRYGCRAMM LETVAAVPGM VGGMLLHCKS LRRFEQSGGW  210
Arabid.  --------.. .......... .......... .......... ..........  162
Saurom.  --------.. ....A..... .......... ...V...L.. .....H....  206
Hansen.  SWEGTRYEMT EEKWLT.CIF ..S..G.... .AAFIR.LH. ..LLKRDKA.  170
                 •
tobacco  IKTLLDEAEN ERMHLMTFME VAKPNWYERA LVFAVQGVFF NAYFVTYLLS  260
Arabid.  ..A..E.... .......... ....K..... ..IT...... ....LG..I.  212
Saurom.  .RA..E.... .......... ..Q.R..... ..L....... ....LG....  256
Hansen.  .E......Y. .....L..IK IGN.S.FT.F IIYMG....A .LP.LV..IK  220
          •
tobacco  PKLAHRIVGY LEEEAIHSYT EFLKELDK-- -GNIENVPAP AIAIDYC-RL  306
Arabid.  ..F...M... .......... ........-- -......... ......W-..  258
Saurom.  ..F...V... .......... ....DI.S-- -.A.QDC... ...L..W-..  302
Hansen.  .RYC..F... .....VST.. HLI.DI.SKR LPKFDD.NL. E.SWL.WTD.  270
                    •    •
tobacco  PKDSTLLDVV LVVRADEAHH RDVNH----- ---------FA S---DIHY-Q  339
Arabid.  .A.A..R... M......... .....----- --------.. .-------.-  291
Saurom.  .QG...R... T......... .....----- ---------- .----V..-.  335
Hansen.  NEK..FR.LI QRI....SK. .E...TLANL EQKKDRNP.. LKVE.VPKE.  320

tobacco  GQQ---LKDS PAPIGYH--- ---                               353
Arabid.  .RE----..EA .......--- ---                               305
Saurom.  DLE----..TT ...L...--- ---                               349
Hansen.  QPDEYS..T- .H.E.WNREQ MRL                                342
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Figure 2. Comparison of the deduced amino acid sequence of the tobacco AOX with those of *A. thaliana* (Kumar and Soll, 1992), the thermogenic plant *S. guttatum* (Rhoads and McIntosh, 1991), and the yeast *H. anomala* (Sakajo et al., 1991). Dots (·) denote amino acids identical to those in the tobacco sequence. Dashes (–) indicate breaks in the sequence inserted to maximize alignment. Amino acids that are underlined are the potential N-terminal amino acids of the mature mitochondrial protein as proposed by the respective authors. Conserved Cys and His residues are indicated by a bullet (●) above the amino acid. Two regions in the tobacco sequence (marked with a bar above the amino acids) are predicted by protein secondary structure analysis to be potential transmembrane α-helices.

The 353-amino acid AOX precursor protein may be further processed during import into the mitochondrion. In tobacco, if the mature mitochondrial protein began at Ala^{70}, then a putative 69-amino acid transit peptide would contain a −2 Arg and a −10 Arg (Fig. 1), both of which are common processing signals in mitochondrial targeting peptides (von Heijne et al., 1989). The −8 Phe (Fig. 1) is also a common feature (von Heijne et al., 1989). This processing site would be close to that proposed for *S. guttatum* (Fig. 2), *A. thaliana* (Fig. 2), and soybean (Whelan et al., 1993). Based on this assumption, the mature AOX protein in tobacco mitochondria would contain 284 amino acids with a molecular mass of 32.3 kD, a size similar to that estimated from SDS-PAGE gels (approximately 35 kD, Vanlerberghe and McIntosh, 1992a, 1992b).

Respiration and Gene Expression

When the pAONT1 clone was hybridized to a northern blot of total RNA from tobacco suspension cells, it recognized a single 1.6-kb transcript that encodes the AOX protein (Fig. 3). The size of this transcript is the same as that seen in the thermogenic plant *S. guttatum*, the only other plant species for which such data are published (Rhoads and McIntosh, 1992).

We examined changes in the level of the *Aox1* transcript in tobacco suspension cells in response to the inhibition of CP respiration. The CP was specifically inhibited by the addition of 2 μM AA, a concentration shown to decrease CP respiration by >95% in these suspension cells (Vanlerberghe and McIntosh, 1992b) as well as in suspension cells of *Petunia hybrida* (Wagner et al., 1992b). This reduction in CP respiration resulted in a rapid increase in *Aox1* mRNA abundance, which increased 6-fold within 2 h and 22-fold after 4 h (Fig. 3). We also examined the mRNA level of two other genes encoding respiratory chain proteins (the nuclear gene *Cc1*, encoding the apoprotein of Cyt *c*, and the mitochondrial gene *cox1*, encoding Cyt oxidase subunit I). We saw single transcripts for each of these genes on northern blots. There were minor changes in their levels in response to AA, but these changes were small compared with the changes in *Aox1* mRNA (Fig. 3).

Previous work has shown that inhibition of the CP by AA in suspension cells is transient (probably due to degradation or dilution of the AA over time; Vanlerberghe and McIntosh, 1992b; Wagner et al., 1992b). Therefore, we examined changes in *Aox1* gene expression both in response to inhibition of the CP and during its recovery. We simultaneously measured changes in key respiratory characteristics of the cells in an attempt to correlate them with changes in gene expression.

Before AA addition, AP capacity was approximately 7% of the total capacity (AP capacity + CP capacity) of electron transport measured in whole cells (Fig. 4A). Addition of AA immediately decreased CP capacity by >95%, and this "apparent" capacity remained low for the next 16 h. The term apparent CP capacity is used here because AA addition presumably does not affect the in vivo capacity of the pathway (as measured before AA addition) but rather chemically blocks electron flow through the pathway (Vanlerberghe and

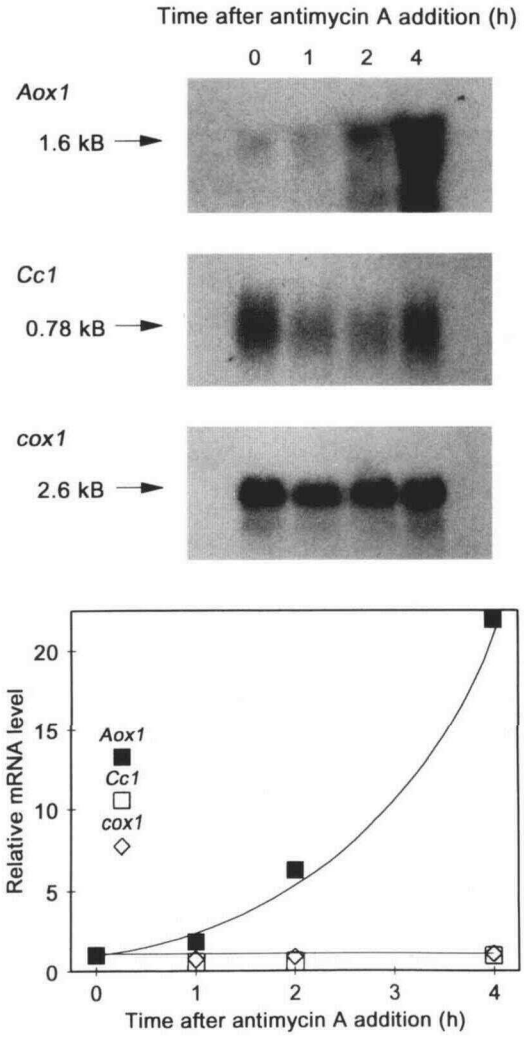

Figure 3. Changes in specific mRNA levels of NT1 tobacco suspension cells in response to the addition of 2 μM AA at 0 h. Northern blots of total tobacco RNA (30 μg) were hybridized with cDNAs representing the AOX gene of tobacco (*Aox1*), the apoprotein of the Cyt *c* gene of tobacco (*Cc1*), and the Cyt oxidase subunit I gene of maize (*cox1*). The blots were analyzed by autoradiography (upper panels) and with a phosphorimager (lower graph).

McIntosh, 1992b). During this same 16-h period, AP capacity gradually increased to approximately 10-fold its initial level (from 7.6 natoms O mg^{-1} fresh weight h^{-1} to greater than 75 natoms O mg^{-1} fresh weight h^{-1}) (Fig. 4A). Before AA addition, the AP was not engaged in O_2 consumption ($\rho \cong 0$) (Fig. 4B) and respiration rate was under adenylate control, being stimulated 2.2-fold by addition of the uncoupler FCCP (Fig. 4B). When the CP was suppressed (during the first 16 h after AA addition), the AP was completely engaged ($\rho \cong 1$) in respiration (Fig. 4B). Respiration during this period proceeds almost exclusively through the AP and is thus unaffected by FCCP (Fig. 4B). Suppression of the CP by AA resulted in a 28-fold increase in *Aox1* mRNA within 4 h (Figs. 4C and 5). After 16 h, with the CP still largely suppressed, *Aox1* mRNA remained high (6-fold higher than before AA addition) (Figs. 4C and 5).

Between 16 and 25 h after AA addition there was a large recovery of the CP (to 70% of its initial level by 25 h) and there was a decreased engagement of the AP ($\rho = 0.75$ by 25 h) (Fig. 4, A and B). This was accompanied by a drop in *Aox1* mRNA to a level similar to that observed before AA addition (Figs. 4C and 5). Between 25 and 48 h after AA addition the CP completely recovered. This recovery was accompanied by a gradual loss of AP capacity (Fig. 4A) and engagement (Fig. 4B). These changes resulted in a progressive increase in the

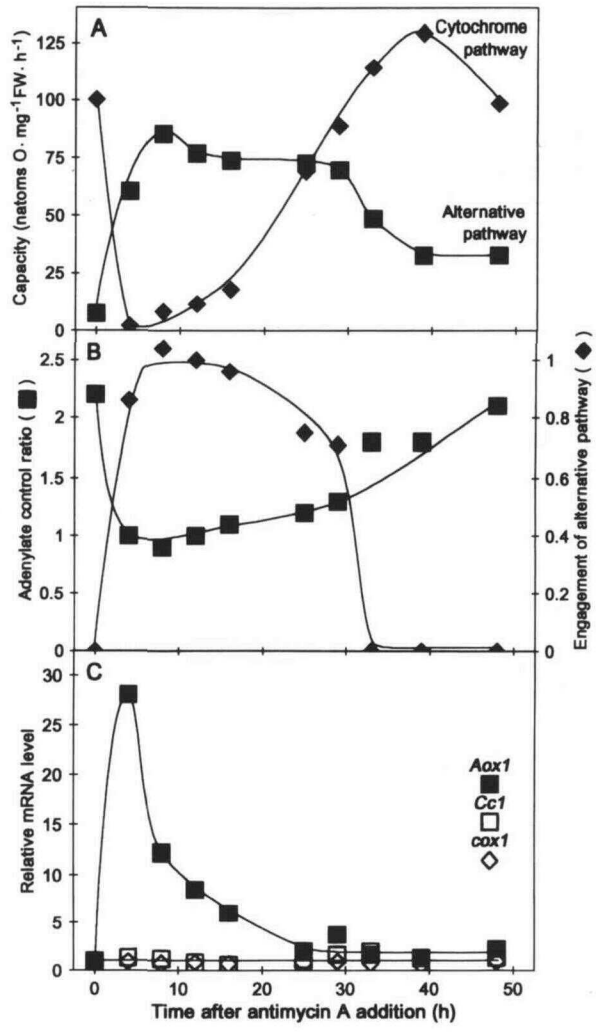

Figure 4. Changes in respiratory characteristics and levels of specific mRNAs of NT1 tobacco suspension cells in response to the addition of 2 μM AA at 0 h. A, The capacities of the AP and the CP to support O_2 consumption. B, The left axis shows adenylate control of respiration (defined as the respiration rate in the presence of FCCP divided by the respiration rate in the absence of FCCP); the right axis shows the degree of engagement of the AP, which is the fraction (0–1) of the AP capacity that is engaged in respiratory O_2 consumption. C, mRNA level of genes encoding AOX (*Aox1*), the apoprotein of Cyt *c* (*Cc1*), and Cyt oxidase subunit I (*cox1*) analyzed with a phosphorimager. Autoradiograms of the *Aox1* and *Cc1* data are presented in Figure 5.

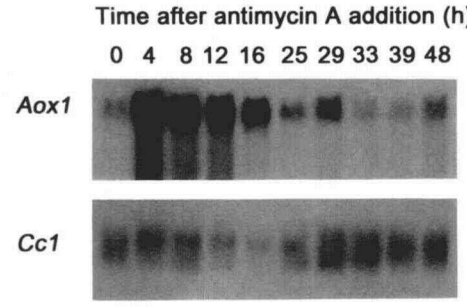

Figure 5. Changes in specific mRNA levels of NT1 tobacco suspension cells in response to the addition of 2 μM AA at 0 h. This figure is an autoradiogram of the *Aox1* and *Cc1* phosphorimager data presented in Figure 4C and shows more clearly the minor changes in *Cc1* mRNA levels.

ability of FCCP to stimulate respiration. Levels of *Aox1* mRNA remained low throughout this time (Figs. 4C and 5).

We also examined *Cc1* and *cox1* mRNA levels during the period of inhibition and recovery of the CP and saw only minor fluctuations in their levels (Figs. 4C and 5). The level of the nuclear gene transcript *Cc1* showed some decline during the first 16 h (to 43% of the 0-h level) when the CP was inhibited by AA, followed by some increase (to 183% of the 0-h level) as the CP recovered (Fig. 5). However, these changes were very slight in comparison with the changes in *Aox1* mRNA level, and, at present, we do not know the significance of these transient changes in *Cc1* mRNA level.

DISCUSSION

AOX cDNA Clone

We have isolated a cDNA representing the *Aox1* gene of tobacco (Fig. 1) and found it to have a high degree of homology to the other plant *Aox1* genes reported to date as well as significant homology to a yeast AOX gene (Fig. 2). The high degree of homology among the plant sequences indicates that the protein from thermogenic and nonthermogenic plants is structurally similar.

The pAONT1 clone encodes a deduced polypeptide of 353 amino acids. Following its translation in the cytosol, this precursor polypeptide may be further processed during import into the mitochondrion and upon insertion in the inner mitochondrial membrane. Processing (and possibly other modification) of the AOX has not been studied in any system, but the presence of multiple AOX proteins of similar size in purified mitochondria from *S. guttatum* (Elthon et al., 1989a, 1989b; Rhoads and McIntosh, 1992), soybean (Obenland et al., 1990; Kearns et al., 1992), potato (C. Hiser and L. McIntosh, unpublished data), pea (Goyal et al., 1991), and tobacco (Vanlerberghe and McIntosh, 1992b) is suggestive of multiple posttranslational processing steps. With regard to protein import, it is interesting that two common mitochondrial processing signals (a −2 Arg and a −10 Arg) are proposed to occur in the putative transit peptides of tobacco (this report), soybean (Whelan et al., 1993), and *A. thaliana* (Kumar and Soll, 1992). Protein sequence alignment indicates that these Arg's may be highly conserved among the different species (Fig. 2).

At present, the size/activity relationship of the AOX protein is not known. In NT1 tobacco suspension cells, the major immunoreactive protein on SDS-PAGE gels is estimated with molecular mass markers to be approximately 35 kD. However, there is also a second protein of a slightly higher "apparent" molecular mass (Vanlerberghe and McIntosh, 1992a, 1992b). This band is difficult to resolve and is usually less abundant than the major, 35-kD band.

AOX Gene Expression

The CP in tobacco suspension cells can be specifically inhibited by 2 μM AA, which blocks electron transfer in complex III (ubiquinol-Cyt *c* oxidoreductase; Vanlerberghe and McIntosh, 1992b). Chemical inhibition of the CP resulted in a rapid and dramatic increase in the level of the 1.6-kb *Aox1* mRNA (Figs. 3–5), suggestive of a rapid activation of transcription (and/or increased transcript stability) of the nuclear gene. We saw similar results after inhibition of the CP by azide (an inhibitor of complex IV), indicating that the response is not specific to the inhibition of complex III by AA (E. van der Knaap, G.C. Vanlerberghe, and L. McIntosh, unpublished data). Once inhibition of the CP by AA was relieved, the level of *Aox1* mRNA returned to the level seen before AA addition (Figs. 4 and 5).

We have previously shown that inhibition of the CP by AA in these suspension cells results in increased AOX protein and AP capacity in the mitochondria within several hours and that the protein level and capacity decline as the CP recovers (Vanlerberghe and McIntosh, 1992b). The increase in AOX protein could be prevented by inhibitors of RNA synthesis (actinomycin D) or cytosolic protein synthesis (cycloheximide). Those results and the changes in *Aox1* mRNA seen in this study suggest that changes in *Aox1* gene expression are responsible for the changes in respiratory characteristics seen after AA addition.

After CP inhibition by AA, there was no significant change in the mRNA level of two genes encoding proteins of the CP, either the nuclear gene *Cc1* (encoding the apoprotein of Cyt *c*, which transfers electrons from ubiquinol-Cyt *c* oxidoreductase to Cyt oxidase) or the mitochondrial gene *cox1* (encoding Cyt oxidase subunit I). Therefore, the increase in *Aox1* mRNA in response to the inhibition of CP respiration is not a general phenomenon of genes encoding respiratory components but is a more specific event. The regulatory signal that couples *Aox1* mRNA accumulation with CP activity is unknown. In the yeast *Saccharomyces cerevisiae*, coordinate expression of nuclear genes (such as those encoding apoproteins of Cyts of the CP) with mitochondrial status is achieved by a network of transcriptional activators/repressors responsive to regulatory signals such as carbon, oxygen, and heme (Forsburg and Guarente, 1989; de Winde and Grivell, 1993). Recently, it was shown that an AOX gene from the yeast *H. anomala* is also induced at the mRNA level by some respiratory inhibitors (Sakajo et al., 1991) and that the generation of superoxide anions in the mitochondrion may be part of the induction mechanism (Minagawa et al., 1992). In plants, the signals and mechanisms that coordinate mitochondrial

status and nuclear gene expression are unknown, although the mechanisms coordinating nuclear gene expression with chloroplast function are beginning to be elucidated (e.g. Susek et al., 1993, and refs. therein).

Physiological Significance

Studies with whole tissue and isolated mitochondria show that the AP is engaged in O_2 consumption only when the CP becomes limiting (see introduction). This suggests that the AP may act as an overflow to maintain electron transport when the CP is saturated or inhibited. At least two types of limitation of CP respiration resulting in a diversion of electrons to the AP might occur (Day and Lambers, 1983; Blacquiere and de Visser, 1984). First, it may be that the carrier capacity of one of the electron transfer components of the CP is limiting. Second, it may be that the CP becomes restricted by ADP and/or Pi supply (adenylate control of respiration) to the extent that electrons enter the AP. In this sense, the presence of two pathways may lend a degree of "flexibility" in the regulation of plant respiration. Since AP respiration is not coupled to ATP synthesis, it may allow carbon oxidation in respiration to proceed without being tightly regulated by the availability of ADP.

In addition to the above-mentioned mechanisms governing engagement of the AP, the present study indicates that a mechanism exists whereby the capacity of the AP can be adjusted in response to changes in the activity of the CP. This mechanism involves changes in *Aox1* gene expression and may be critical in situations in which the CP becomes limiting. Treatments that have been shown to suppress CP activity (resulting in an increased capacity and/or engagement of the AP) include chilling injury (Leopold and Musgrave, 1979), nutrient deprivation (Bingham and Farrar, 1989), salt stress (Jolivet et al., 1990), high CO_2 (Palet et al., 1991), and Pi limitation (Rychter et al., 1992). In some cases this suppression of CP activity occurred at the level of the carrier capacity of the pathway (e.g. Palet et al., 1991), whereas in other cases it was ascribed to increased adenylate control (e.g. Bingham and Farrar, 1989). In the present study we have limited the carrier capacity of the CP by chemical inhibition and found that a mechanism exists that influences *Aox1* gene expression. It would be interesting to know whether a similar mechanism exists in response to adenylate restriction of CP activity and what regulatory signals govern the expression of nuclear genes encoding mitochondrially localized proteins.

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