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Genetic Modification of Respiratory Capacity in Potato[1]

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Mitochondrial respiration was altered in transgenic potato (*Solanum tuberosum*) lines by overexpression of the alternative oxidase *Aox1* gene. Overexpressing lines showed higher levels of *Aox1* mRNA, increased levels of alternative oxidase protein(s), and an unusual higher molecular weight polypeptide, which may be a normal processing/modification intermediate. Evidence suggests that the alternative oxidase protein is further processed/modified beyond removal of the transit peptide. Addition of pyruvate to mitochondria oxidizing succinate or NADH increased the alternative pathway capacity but did not eliminate the difference in the capacity between these two substrates. Induction of alternative pathway capacity by aging of tubers appeared to be more dependent on increased levels of alternative oxidase protein than changes in its oxidation state. In leaf and tuber mitochondria, overexpressing lines possessed higher alternative pathway capacity than the control line, which suggests that changing the alternative oxidase protein level by genetic engineering can effectively change alternative pathway capacity.

In addition to the cyanide-sensitive Cyt pathway of electron transport, plant mitochondria possess a cyanide-resistant, SHAM-sensitive alternative pathway. The alternative pathway consists of an alternative terminal oxidase that accepts electrons from the ubiquinone pool, reduces O_2 to H_2O, and does not conserve energy (Day et al., 1980; Lance et al., 1985; Moore and Siedow, 1991).

Potato (*Solanum tuberosum*) tubers provide a model system in which to study induction of the alternative pathway. The respiration of fresh tuber slice mitochondria is almost entirely cyanide sensitive, whereas mitochondria from aerobically aged slices exhibit elevated alternative pathway respiration (Dizengremel and Lance, 1976; Theologis and Laties, 1978; for review, see Day et al., 1980). The potato tuber alternative oxidase was reported to be an approximately 36-kD protein that was not detected in mitochondria from fresh tuber tissue but appeared in mitochondria from aged slices (Hiser and McIntosh, 1990). This supports the concept that increased alternative path capacity in aged tuber slice mitochondria is, to some degree, due to de novo synthesis of the protein during the aging process. De novo synthesis of alternative oxidase proteins was correlated with transcription of the alternative oxidase gene in tobacco suspension-cell cultures treated with antimycin A, a specific inhibitor of the Cyt pathway (Vanlerberghe and McIntosh, 1992, 1994), which suggests that alternative oxidase can be regulated at the level of transcription/mRNA stability. Whether such regulation is involved in the aging process of potato tuber slices remains unknown, since the potato alternative oxidase message is of an abundance too low to be detected on northern blots of either fresh or aged potato tuber mRNA (Hiser and McIntosh, 1994).

Although monoclonal antibodies against the *Sauromatum guttatum* alternative oxidase (Elthon et al., 1989b) and the alternative oxidase structural gene *Aox1* (Rhoads and McIntosh, 1991) are available for use as molecular probes, investigation of the function and regulation of the alternative pathway in higher plants has been slowed by a lack of naturally occurring mutants in the alternative oxidase. However, transformation with sense and antisense constructs has recently proven effective in altering the alternative pathway in tobacco (Vanlerberghe et al., 1994). Genetic manipulation of the alternative pathway in potato is advantageous, since potato provides a variety of metabolically different tissues (leaf, root, tuber) in which to compare alternative oxidase capacities and proteins, as well as a system in which alternative pathway capacity can be induced (tuber slice aging). Part of the difficulty of studying the alternative pathway in non-aroid higher plant tissues such as potato tuber is due to the lower natural level of alternative pathway capacity. The alternative pathway capacity has been correlated with the amount of alternative oxidase protein in higher plants (Elthon and McIntosh, 1987; Obenland et al., 1990; Kearns et al., 1992; Rhoads and McIntosh, 1992; Vanlerberghe and McIntosh, 1992; Vanlerberghe et al., 1994), including potato (Hiser and McIntosh, 1990). Increasing the levels of the alternative oxidase protein by overexpression could increase alternative pathway capacity and facilitate the study of alternative pathway function and regulation in non-aroid plants.

Here we report the production of transgenic potato plants that overexpress the alternative oxidase structural gene *Aox1*. These plants have higher levels of alternative oxidase mRNA and protein; possess an additional, characteristic alternative oxidase polypeptide in leaves, roots, and tubers; and show increased alternative pathway capacity in leaf and tuber mitochondria, thereby demonstrating that

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Abbreviations: βME, β-mercaptoethanol; SHAM, salicylhydroxamic acid.

overexpression of *Aox1* in transgenic potato can successfully alter the alternative pathway.

MATERIALS AND METHODS

Cloning and Sequencing of the Potato *Aox1* Gene

Russet-type potato (*Solanum tuberosum*) tuber slices were aged for 24 h (Hiser and McIntosh, 1990), and total RNA was isolated (McIntosh and Cattolico, 1978). mRNA was isolated from total RNA (Cashmore, 1982) and sent to Stratagene Cloning Systems, where a cDNA library was constructed in the λ ZapII vector. The phagemid library was screened using the *Sauromatum guttatum* alternative oxidase gene (Rhoads and McIntosh, 1991) as the probe. The insert from one positive plaque was subcloned into pUC119 for sequencing. Single-stranded DNA (Viera and Messing, 1987) was prepared from the clone, several deletions were made (Dale et al., 1985), and both strands of the clone and each deletion were sequenced using the Sequenase, version 2.0, kit (United States Biochemical). The SEQANAL secondary structure predictor program (Crofts, 1987) was used for sequence analysis.

Construction of the Binary Vector pSt11 and Plant Transformation

The binary vector pGA748 and the *Escherichia coli* host strain MC1000 (Casadaban and Cohan, 1980) were obtained from Dr. Gynheung An (Washington State University, Pullman). The entire cDNA insert carrying the potato *Aox1* gene was cloned into the *Eco*RI site of the polylinker of pGA748 behind the cauliflower mosaic virus 35S promoter. MC1000 transformants were screened to obtain a clone carrying the alternative oxidase gene in the sense orientation. The resultant vector pSt11 was transferred directly (An, 1987) to *Agrobacterium tumefaciens* strain LBA4404 (obtained from Dr. William Belknap, U.S. Department of Agriculture-Agricultural Research Service, Albany, CA). *Agrobacterium* transformants were screened according to the method of An et al. (1988).

The FL1607 line of potato plants was obtained from Dr. William Park (Texas A & M University, College Station, TX) and maintained in tissue culture (Wenzler et al., 1989). *Agrobacterium*-mediated leaf disc transformations (Wenzler et al., 1989) produced kanamycin-resistant plants that were shown by Southern blotting to contain the desired inserted T-DNA (data not shown). Plants were transferred to soil and moved to growth chambers under 16-h days at 20°C and 80% RH. Although some of the primary transformants had unusual growth traits, phenotypes were not further characterized in progeny plants because the overexpressing lines did not flower under conditions in which the FL1607 control line flowered.

RNA Analysis of Transgenic Plants

Total RNA was isolated from tissue-culture-grown leaves (Verwoerd et al., 1989); 50 μg of total RNA per lane were separated on agarose/formaldehyde gels and transferred to nitrocellulose (Seldon, 1987). Northern blots were probed with a ^{32}P-end-labeled (Sambrook et al., 1989) oligonucleotide designed to detect only sense mRNA: 5′-CGATTGACATGTCCGACTCGTACGCCTCCCAT-3′.

Isolation of Mitochondria

Mitochondria were isolated and Suc gradient purified from fresh and 24-h-aged potato tuber tissue as previously described (Hiser and McIntosh, 1990).

Leaf mitochondria were isolated from tissue-culture-grown plants essentially as described by Boutry et al. (1984). The discontinuous gradient contained 0.25 mL of 50% (v/v) Percoll, 0.5 mL of 26% Percoll, and 0.25 mL of 13.5% Percoll in a Beckman TLS-55 rotor tube, which was centrifuged in a Beckman TL-100 ultracentrifuge at 20,000 rpm for 15 min. The mitochondrial layer at the 26/50% Percoll interface was collected and pelleted as described (Boutry et al., 1984) and then resuspended in 25 μL of assay buffer (Hiser and McIntosh, 1990).

Leaf mitochondria were isolated from growth-chamber-grown plants by a variation of the method of Day et al. (1985). Approximately 150 g of leaf tissue was homogenized in 1.5 L of isolation buffer (0.3 M Suc, 30 mM Mops, 25 mM Na PPi, 10 mM KH_2PO_4, 2 mM Na_2EDTA, 2 mM Gly, 20 mM ascorbic acid, 1% (w/v) fatty-acid-free BSA, 1% (w/v) PVP-40, pH 7.5) and then filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem). The homogenate was centrifuged at 4,000*g* for 5 min, and the supernatant was recentrifuged at 20,000*g* for 20 min. The pellets were resuspended in wash buffer (0.3 M Suc, 10 mM Tes, 1 mM Gly, 0.1% (w/v) fatty-acid-free BSA, pH 7.2) and centrifuged at 4,300*g* for 4 min, and the supernatant was recentrifuged at 12,000*g* for 20 min. These washed mitochondrial pellets were resuspended in 6 to 8 mL of wash buffer, layered over 35 mL of gradient solution (wash buffer plus 30% [v/v] Percoll and a linear gradient, top to bottom, of 0–10% [w/v] PVP-25) in four Sorvall SS-34 rotor tubes, and centrifuged at 40,000*g* for 20 min. Purified mitochondria were collected from a zone near the bottom of the tube, diluted 5-fold with wash buffer, pelleted at 15,000*g* for 16 min, and resuspended in 200 μL of assay buffer (Hiser and McIntosh, 1990).

Respiration Assays

Respiration assays on isolated leaf or tuber mitochondria were performed at 25°C with a Rank Brothers (Cambridge, UK) O_2 electrode as previously described (Hiser and McIntosh, 1990) except that the chamber was covered with a black cloth during experiments with leaf mitochondria. Substrates were 10 mM succinate (in the presence of 0.1 mM ATP to activate succinate dehydrogenase) or 10 mM NADH, and the mitochondria were uncoupled with 0.5 μM *p*-trifluoromethoxycarbonylcyanide. In some assays, 0.5 mM pyruvate was included as an activator of the alternative oxidase (Millar et al., 1993). Cyt pathway capacity was taken to be that portion of uncoupled respiration inhibited by 1 mM KCN. Alternative pathway capacity was measured as that portion of uncoupled respiration inhibited by 1 to 2 mM SHAM in the presence of KCN. Residual respi-

ration was the O_2 uptake that remained in the presence of both KCN and SHAM. Results are the average (± SD) of at least three replicate isolations with at least three measurements for each treatment per isolation.

Western Analysis of Mitochondrial Proteins

Mitochondrial protein concentrations were determined by a modified Lowry method (Larson et al., 1986). Samples of leaf or tuber mitochondria (50 or 500 μg of mitochondrial protein per lane, respectively) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with the AOA monoclonal antibody against the *S. guttatum* alternative oxidase (Elthon et al., 1989b) as previously described (Hiser and McIntosh, 1990). To visualize the oxidized form of the alternative oxidase (Umbach and Siedow, 1993), however, the βME normally used as the reductant in the SDS-PAGE sample buffer was omitted from certain samples.

Protein Isolation and Sequencing

Samples of leaf mitochondria from the overexpressing line 11–6 were separated by SDS-PAGE as described above but were electroblotted at 0.2 A overnight onto polyvinylidene difluoride membranes (Problot, Millipore) in a transfer buffer containing 25 mM Tris, pH 8.3, 192 mM Gly, 20% (v/v) methanol, and 0.05% (w/v) SDS. The membranes were washed extensively with distilled water, stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol/10% (v/v) acetic acid, and destained with 50% (v/v) methanol. The higher molecular weight alternative oxidase band characteristic of overexpressing lines was excised from the membranes. N-terminal protein sequencing was performed directly on the excised bands by Dr. Joe Leykam at the Michigan State University Biochemistry Department Macromolecular Structure Facility using an Applied Biosystems 477A protein sequencer with model 120A analyzer.

RESULTS

The Potato Alternative Oxidase Gene *Aox1*

The deduced amino acid sequence of the potato alternative oxidase gene *Aox1* is shown in Figure 1. The potato cDNA was 1254 bp long with a 344-codon open reading frame that could potentially encode a 41-kD polypeptide. The *S. guttatum* alternative oxidase was shown to be made initially as a 42-kD precursor with a predicted 63-amino acid transit sequence (Rhoads and McIntosh, 1991). By homology to the *S. guttatum* deduced amino acid sequence, the potato sequence appears to encode a transit peptide of 59 amino acids. Comparison of the potato alternative oxidase deduced amino acid sequence to those from *S. guttatum* (Rhoads and McIntosh, 1991), tobacco (Vanlerberghe and McIntosh, 1994), soybean (Whelan et al., 1993), *Arabidopsis thaliana* (Kumar and Soll, 1992), and the yeast *Hansenula anomala* (Sakajo et al., 1991) shows regions of high sequence identity, including two potential transmembrane helices (amino acids 168–186 and 236–250 in the potato

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potato     MMSSRFAGTAL-RQLGPVLFAS-APGARAAAEPAYALLAGAPAAAPT-R  46
Saurom.     .....LV....C...SH.PVPQYL.AL.PT.DT.SS..H.CS....AQ.
tobacco    MMTRG-ATRMT-RTVLGHMGPRYFSTAIFRNDAGTGVMSGAAVFMHGVP
soybean  MMM...RS--.AN--.VANTAM.VA-----KGLSGEVGG.-----------.

potato     --------AAVWLVR-FPLSRAASTMSAPAAPEGETAAKG----DVDVTKKA  85
Saurom.    --------.GL.PPSW.SPP.HA..L....QDG.KEK.A.----TAGKVPPG
tobacco    ANPSEKAVVTWVRHFPVMG..SAMS.AL----NDKQH---DKKAENGSAAA-
soybean    --------.-LY----GGGV.SE..LAL----SEKEK--IEKKVGLSS----
Arabid.           MDTR-APAIGGM.FA..ITL-GEKTMKEEDANQKKTENESTGGD
Hansen.    MIKTYQYRSILNSRNVGTI.FLK.L.--PSPHSKDPNSKSIFDIGTK--LI

potato     EGDTEQK---AVVSYWGVPPSRVTKEDGSPWRWACFRPWEAYESDMSIDLKK 134
Saurom.    .DGGAE.E--.....A....K.S.....E...T......T.QA.L....H.
tobacco    TGGGDGGDEKS.....Q..K.....TE.K.N......T.KA.L....T.
soybean    -AGG-NAEEKVI.....IQ..KI..K..TE.K.N..S..GT.KA.L....E.
Arabid.    AAGGNN.GDKGIA.....E.NKI......E.K.N......T.KA.IT.....
Hansen.    VNPPPQMADNQY.THPLE.HPKYSD..-------.EAVHFV----------

potato     HHAPTTFLDKMAFWTVKSLRWPTDIFFQRRYGCRA----------------- 169
Saurom.    ..V...I...L.LR...A.............A...-----------------
tobacco    ..........F.Y.......Y.............-----------------
soybean    .MP.............V..Y...V..........-----------------
Arabid.    ..V......RI.Y...........L.........-----------------
Hansen.    .RE.K.IG..I.DRG..F--------------...SFDVNGMLKSWEGTRYE

potato     ----------MMLETVAAVPGMVGGLLLHLKSLRRFEHSGGWIKALLEEAEN 211
Saurom.    ----------.............V.................R........
tobacco    ----------.............M...C.......Q......T..D....
soybean    ----------.............A.M...C............F........
Arabid.    ----------.............M...C.......Q..............
Hansen.    MTEEKWLTRCIF..S..G.....AAFIR..H...LLKRDKA..ET..D..Y.

potato     ERMHLMTFMEVSQPRWYERALVLAVQGVFFNAYFLGYLLSPKFAHRVVGYLE 263
Saurom.    ..........A.....................................
tobacco    ..........AK.N.......F...........VT......L...I.....
soybean    ..........AW.K.......IT.....................MF....
Arabid.    ..........AK.K.......IT.............I.......M.....
Hansen.    .....L..IKIGN.S.FT.FIIYMG....A.LF..V..IK.RYC..F.....

potato     EEAIHSYTEFLKEIDK---GTIDNVPAPAIALDYWR-LPPGSTLRDVVMVVR 312
Saurom.    ............D..S---.A.QDC...........-..Q........T...
tobacco    ............L..---.N.E........I..C.-..KD...L...L...
soybean    ............L..---.N.E........I...Q-..............
Arabid.    ............L..---.N.E........I....-..ADA.........
Hansen.    ...VST..HLI.D..SKRLPKF.D.NL.E.SWL..TD.NEK..F..LIQRI.

potato     ADEAHHRDVNHFASDVHYQGHQLKEAPAPLGYH                   344
Saurom.    ............D......ALE..TT.......
tobacco    ..............I....Q...DS...I...
soybean    ..............I....RE.R..A..I...
Arabid.    ..............I...DLE.......I...
Hansen.    ...SK..E...TLANLEQKKDRNPFALKVEDVPKEQQPDEYSLKTPHPEGWN

Hansen.    REQMRL
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Figure 1. Comparison of alternative oxidase deduced amino acid sequences from the higher plants potato, *S. guttatum* (*Saurom.*) (Rhoads and McIntosh, 1991), tobacco (Vanlerberghe and McIntosh, 1994), soybean (Whelan et al., 1993), *A. thaliana* (*Arabid.*) (Kumar and Soll, 1992), and the yeast *H. anomala* (*Hansen.*) (Sakajo et al., 1991). Numbering refers to the potato sequence. Dots denote amino acids identical with those in the potato sequence and dashes indicate breaks in the sequences inserted to maximize alignment. The starts of the mature proteins, as proposed by the authors, are in boldface type. The conserved Cys's are underlined.

sequence; for a model of alternative oxidase topology, see Siedow et al., 1992). Recent reports have proposed, as a potential means of regulation of alternative oxidase activity, interconversion between a reduced form of the alternative oxidase and an oxidized form linked by a disulfide bridge(s) (Umbach and Siedow, 1993; Umbach et al., 1994). Two conserved Cys's (amino acids 117 and 167 in the potato sequence) could potentially contribute to disulfide bond formation.

Although potato is an advantageous system in which to study the alternative pathway because of its variety of tissues and the inducibility of alternative pathway capacity in tubers by aging, it has a relatively low natural level of alternative path capacity even in mitochondria from aged tuber tissue (24 nmol O_2 min^{-1} mg^{-1} protein; see Fig. 6, FL1607 control line), as compared to aroid spadices (up to 265 nmol O_2 min^{-1} mg^{-1} protein on the day of flowering; Elthon et al., 1989a). It would therefore be useful to in-

crease the levels of the alternative oxidase protein and capacity to facilitate the study of its function and regulation. To overexpress the alternative oxidase, the entire potato *Aox1* cDNA was cloned into a binary vector in the sense orientation behind a strong promoter, and this construct was used to transform the FL1607 line of potato plants.

Alternative Oxidase Transcripts in Potato Leaves

Overexpression of *Aox1* allowed alternative oxidase mRNA to be detected in potato leaves. In both tubers and leaves of untransformed potato plants, alternative oxidase mRNA is of low abundance. No signal was detected on northern blots of total RNA or mRNA isolated from tubers (data not shown), although a message was detected previously in both fresh and aged tuber tissue using reverse transcription of RNA samples followed by PCR amplification (Hiser and McIntosh, 1994). When total RNA from leaves of tissue-culture-grown plants was examined on a northern blot (Fig. 2A), no signals appeared for the FL1607 control plants (lane FL1607). However, a transcript of approximately 1650 nucleotides appeared in most of the overexpressing lines and was particularly abundant in line 11–6 (lane 11–6). The increase in potato alternative oxidase message levels could be due to a higher level of transcription of the inserted gene, which was downstream of a strong promoter. The transcript produced by the inserted gene, which is predicted to contain some additional upstream and downstream sequences and no introns, may be more stable than the native message. Increased transcription of the native *Aox1* gene or increased stability of its mRNA cannot be excluded, particularly since the size of the native transcript in potato could not be determined. (Although the isolated potato cDNA was 1254 bp long, in *S. guttatum* [Rhoads and McIntosh, 1991], *H. anomala* [Sakajo et al., 1991], and tobacco [Vanlerberghe and McIntosh, 1994], the isolated cDNA clones were shorter than the mRNA detected by northern blotting.) Although the mechanism could not be elucidated here, overexpression of *Aox1* clearly increased the steady-state level of alternative oxidase mRNA in potato.

Tissue-Specific Differences in Alternative Oxidase Proteins

The alternative oxidase proteins in potato leaves and roots differed from those of tubers. When western blots of mitochondrial proteins were probed with the monoclonal antibody against the *S. guttatum* alternative oxidase (Elthon et al., 1989b), a closely spaced doublet of proteins was present in leaf mitochondria from both FL1607 and the overexpressing lines (Fig. 2B). The doublet was also present in root mitochondria from FL1607 (Hiser and McIntosh, 1994) and the overexpressing lines (data not shown), although at lower levels than in leaves. The potato alternative oxidase was previously reported to be a single protein of about 36 kD in Russet Burbank tubers (Hiser and McIntosh, 1990). Likewise, only one protein was detected in FL1607 tuber mitochondria (Hiser and McIntosh, 1994; see also Fig. 2C). Therefore, the presence of a doublet of

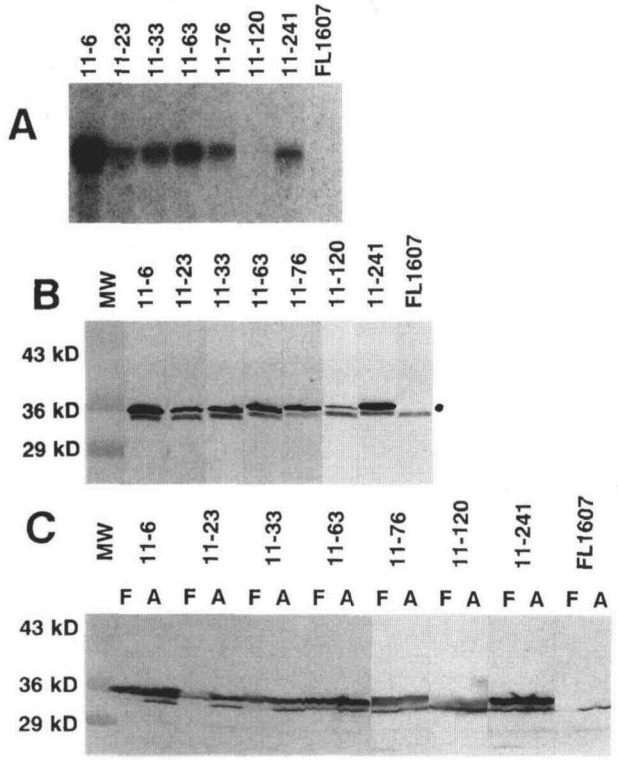

Figure 2. Northern blot of potato leaf RNA and western blots of leaf and tuber mitochondrial proteins from individual overexpressing transformant lines (numbers above each figure) and the FL1607 control line (FL1607). MW, Molecular mass standards. A, Northern blot of potato leaf total RNA (50 μg per lane) probed with an oligonucleotide designed to detect alternative oxidase mRNA (see "Materials and Methods"). B, Western blot of leaf alternative oxidase proteins (50 μg of mitochondrial protein per lane) probed with monoclonal antibodies against the *S. guttatum* alternative oxidase (Elthon et al., 1989b). The dot indicates the higher molecular weight overexpressor protein. C, Western blot of tuber alternative oxidase proteins (500 μg of mitochondrial protein per lane) from fresh (F) and aged (A) tuber tissue probed with the same antibody.

alternative oxidase proteins in potato leaves and roots and a single protein in tubers is a tissue-specific difference and not the result of using different potato lines in the present and former experiments. Tissue-specific differences in the number of alternative oxidase proteins have been observed previously in soybean and siratro (Obenland et al., 1990; Kearns et al., 1992; Day et al., 1994). Such differences could result from differential processing/modification of an alternative oxidase precursor polypeptide (see "Discussion").

Alternative Oxidase Proteins in Overexpressing Potato Lines

Overexpression of *Aox1* altered the number of alternative oxidase proteins in potato plants. In addition to the doublet of proteins present in all lines, the overexpressing lines possessed a higher molecular weight polypeptide in their leaves (Fig. 2B, dot). The relative amount of this polypeptide varied among the overexpressing lines; all lines except

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potato                50 WLVR-FPLSRAASTMSAPAAPEGETAAKGDVD 80
Sauromatum guttatum   54 WPPSWFSPPRHASTLSAPAQDGGKEKAAGTAG 85
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Figure 3. Comparison of the N-terminal protein sequence of the potato higher molecular weight alternative oxidase overexpressor protein from overexpressing line 11–6 and the N-terminal protein sequence of the 36-kD inducible alternative oxidase protein from *S. guttatum* (Rhoads and McIntosh, 1991). Amino acids 50 to 80 from potato and 54 to 85 from *S. guttatum* are shown, with the directly sequenced areas underlined and proposed N termini shown in boldface type.

11–120 possessed it at higher levels than the doublet. Line 11–6 produced this polypeptide at sufficiently high levels to be seen on a Coomassie blue-stained gel, although the doublet of proteins was not discernible (data not shown). All overexpressing lines possessed the same three proteins in the same relative ratios in their roots as well as in their leaves but at overall lower levels (data not shown). In all overexpressing lines, the higher molecular weight polypeptide was also present in fresh and aged tuber tissue in addition to the single tuber alternative oxidase protein present in the control FL1607 line (Fig. 2C). The appearance of this extra, higher molecular weight polypeptide (for clarity, hereafter referred to as the "overexpressor protein") in leaves, roots, and tubers is therefore characteristic of overexpression of *Aox1*.

The common system to induce alternative pathway capacity in potato is aging of tuber slices (reviewed by Day et al., 1980). This process was shown to induce de novo synthesis of the 36-kD tuber alternative oxidase protein, which was not apparent in fresh tubers, in the Russet Burbank line (Hiser and McIntosh, 1990). To determine the effect of alternative oxidase overexpression on this inducible system, aging of tuber tissue was investigated in the FL1607 control line and the overexpressing lines (Fig. 2C). Unlike the Russet Burbank line, the FL1607 line possessed discernible alternative oxidase protein in fresh tuber mitochondria (Hiser and McIntosh, 1994; and Fig. 2C, lane FL1607-F); its level, however, increased upon aging (Hiser and McIntosh, 1994; and Fig. 2C, lane FL1607-A). In four of the seven overexpressing lines, both the 36-kD protein and the overexpressor protein were present at higher levels in mitochondria from aged tubers than from the corresponding fresh tubers. Aging apparently induced synthesis of the overexpressor protein as well as the 36-kD protein. In the other three overexpressing lines, the levels of both proteins appeared to be approximately equal in fresh and aged tuber tissue.

The unexpected appearance of the extra alternative oxidase polypeptide in the overexpressing lines suggested that the product of the inserted gene may not have been processed correctly. Incomplete removal of the transit peptide was one possible explanation; to test this possibility, N-terminal sequencing was performed on the overexpressor protein isolated from leaves of line 11–6. This N-terminal sequence could not be compared to that from the doublet of leaf alternative oxidase proteins or to the 36-kD tuber protein, since they were of an abundance too low to be isolated for sequencing. When compared to the only other available direct N-terminal alternative oxidase protein sequence, that from the 36-kD inducible *S. guttatum* alternative oxidase polypeptide (Rhoads and McIntosh, 1991), the two proteins showed the same N terminus (Fig. 3). Thus, the putative transit peptide of the overexpressor protein was correctly removed upon import into the mitochondria; any altered processing or modification that created the overexpressor protein occurred elsewhere in the polypeptide. When mitochondria from growth-chamber-grown FL1607 leaves were used for western blotting, a faint band appeared that co-migrated with the overexpressor protein (Fig. 4A, lane 1). Therefore, the overexpressor protein may represent a normal processing/modification intermediate; overexpression of *Aox1* may have overwhelmed a processing or modification step, allowing the normal intermediate to build up to readily detectable levels in overexpressing lines.

Oxidation/Reduction of Alternative Oxidase Proteins in Potato

In several different plant species, the alternative oxidase has been reported to exist both in a reduced form and in an

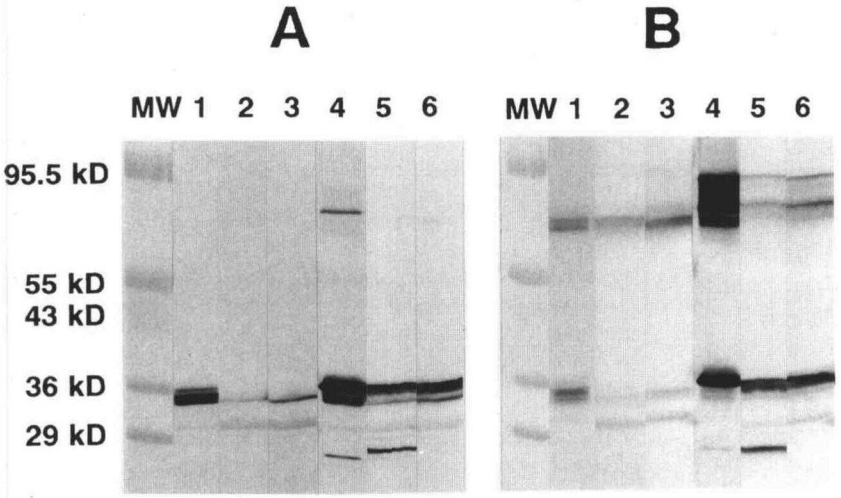

Figure 4. Oxidation/reduction of potato alternative oxidase proteins. Western blots of leaf (lanes 1 and 4), fresh tuber (lanes 2 and 5), and aged tuber (lanes 3 and 6) mitochondria (50, 500, or 500 μg of total mitochondrial protein per lane, respectively) from the FL1607 control line (lanes 1–3) and the 11–6 overexpressing line (lanes 4–6) were probed with monoclonal antibodies against the *S. guttatum* alternative oxidase (Elthon et al., 1989b). MW, Molecular mass standards. A, The reductant βME was included in the SDS-PAGE sample buffer such that only the reduced forms of the alternative oxidase are apparent. B, The reductant βME was omitted from the SDS-PAGE sample buffer to permit visualization of the oxidized forms of the alternative oxidase.

oxidized form linked by disulfide bridge(s) (Umbach and Siedow, 1993; Day et al., 1994; Lennon et al., 1995), and interconversion between forms could be involved in alternative oxidase regulation (Umbach and Siedow, 1993; Umbach et al., 1994). Variations between different tissues in the reduced/oxidized ratios have been noted (Umbach and Siedow, 1993; Day et al., 1994), as well as developmental changes in the ratio (Day et al., 1994; Lennon et al., 1995). Since potato possesses several different tissues and a system in which the alternative oxidase can be induced, the oxidized form(s) of potato leaf and tuber alternative oxidase proteins was investigated by omission of the reducing agent βME from the buffer of some SDS-PAGE samples (Fig. 4).

In FL1607 leaf mitochondria, the level of the doublet of alternative oxidase proteins normally seen when βME was used in the sample buffer (Fig. 4A, lane 1) was lower when βME was omitted and high molecular weight oxidized forms were present (Fig. 4B, lane 1). Likewise in FL1607 fresh and aged tuber mitochondria (Fig. 4A, lanes 2 and 3), the levels of the normal 36-kD protein were lower in the minus-βME samples and oxidized forms occurred (Fig. 4B, lanes 2 and 3). When βME was omitted from 11–6 leaf (Fig. 4B, lane 4) and fresh (Fig. 4B, lane 5) and aged (Fig. 4B, lane 6) tuber samples, a complex pattern of oxidized forms was present, and the levels of the normal doublet of leaf alternative oxidase proteins or the normal 36-kD tuber protein were considerably lower. The levels of the overexpressor protein, however, did not vary much between the plus-βME samples (Fig. 4A, lanes 4–6) and minus-βME samples (Fig. 4B, lanes 4–6), which suggests that the overexpressor protein was less involved in oxidation/reduction. Although alternative pathway capacity is induced in potato by aging of tuber slices, no major differences in the relative ratio of reduced to oxidized forms of the protein between fresh (Fig. 4B, lanes 2 and 5) and aged (Fig. 4B, lanes 3 and 6) tuber mitochondria were observed in the minus-βME samples from either the FL1607 control line or the 11–6 overexpressing line; rather, aging appeared to increase the levels of all forms of the oxidase. In minus-βME samples, additional reduced form bands sometimes appeared beneath the usual bands; such bands were reported previously in soybean cotyledon (Umbach and Siedow, 1993) and root (Day et al., 1994) mitochondria and attributed to an electrophoresis artifact induced by omission of the reductant from the sample buffer.

Variation between leaves and tubers in the ratio of reduced to oxidized forms of the alternative oxidase was noted in the minus-βME samples (Fig. 4B). However, the pattern of variation differed between the FL1607 control line and the 11–6 overexpressing line. In FL1607 leaf mitochondria (Fig. 4B, lane 1), the relative levels of the oxidized and reduced forms appeared approximately equal. In 11–6 leaf mitochondria (Fig. 4B, lane 4), however, the oxidized forms of the normal leaf doublet of alternative oxidase proteins appeared to be at higher levels than reduced forms, although the levels of the two forms of the overexpressor protein appeared similar. In FL1607 and 11–6 tuber mitochondria, the amount of the 36-kD alternative oxidase protein in the oxidized form relative to the reduced form varied between preparations (data not shown). In the FL1607 line, the oxidized form appeared to be at equal or higher levels than the reduced form; in the 11–6 line, the oxidized form appeared at equal or lower levels.

Effects of *Aox1* Overexpression on Mitochondrial Respiration

Based on preliminary respiration assays using leaf and tuber mitochondria from all of the overexpressing lines (data not shown), line 11–6 was chosen for more intensive study because it possessed consistently high levels of alternative pathway capacity.

Overexpression of *Aox1* altered the respiratory capacity in isolated leaf mitochondria (Fig. 5). With either succinate or NADH as the exogenous substrate, line 11–6 possessed lower total (uncoupled, uninhibited) respiration, a lower Cyt pathway capacity (light gray bars), and a higher alternative pathway capacity (dark gray bars) than the comparable treatment of the FL1607 control line. The other overexpressing lines showed the same trends in the preliminary assays (data not shown). In both the FL1607 control line and the 11–6 overexpressing line, a lower alternative pathway capacity was measured with NADH than with succinate. This substrate effect has often been noted in other

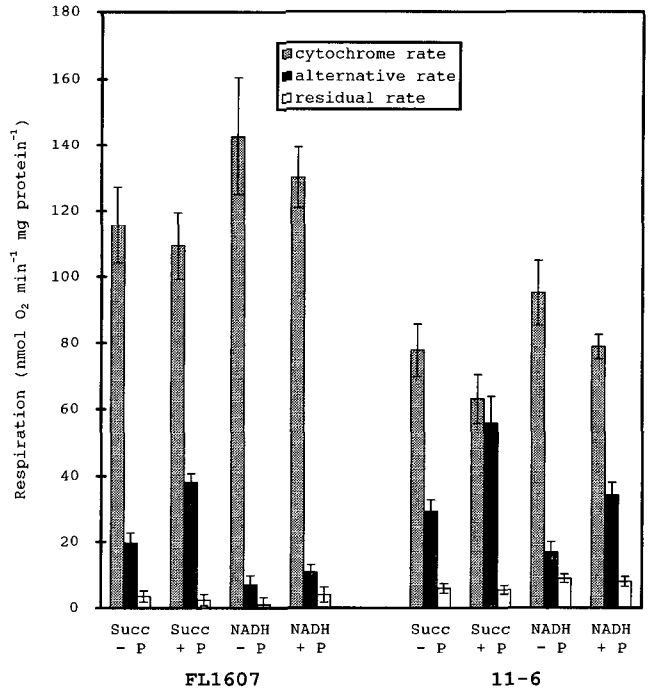

Figure 5. Respiration of potato leaf mitochondria isolated from the FL1607 control line or the 11–6 overexpressing line. Respiration attributable to the Cyt path (light gray bars), alternative path (dark gray bars), or residual O_2 uptake (open bars) is expressed as nmol O_2 min^{-1} mg^{-1} protein. Substrates were either 10 mM succinate (Succ) or 10 mM NADH, with (+P) or without (−P) pyruvate as an activator of the alternative oxidase. Results are the means (± SD) of four (FL1607) or seven (11–6) replicate isolations with at least three measurements per treatment per isolation.

plants (for reviews, see Lance et al., 1985; Moore and Siedow, 1991). Inclusion of pyruvate, an activator of the alternative oxidase (Millar et al., 1993; Umbach et al., 1994), in the assay medium increased the alternative pathway capacity in both FL1607 and 11–6 lines using either substrate. Pyruvate did not significantly increase the total respiration but appeared to shift the proportion of respiration away from the Cyt pathway to the alternative pathway. This observation may be related to a report that pyruvate shifted the threshold value of quinone pool reduction at which the alternative oxidase became active in soybean cotyledon mitochondria (Umbach et al., 1994). Although the substrate differences and pyruvate effects were similar in both the overexpressing and control lines, overexpression of *Aox1* in line 11–6 led to an increased alternative pathway capacity in leaf mitochondria regardless of the substrate used or presence/absence of pyruvate.

Overexpression of *Aox1* had a similar effect in isolated tuber mitochondria (Fig. 6). In either fresh or aged tuber mitochondria with succinate as the substrate, line 11–6 possessed a lower total respiration rate, lower Cyt pathway capacity (light gray bars), and higher alternative pathway capacity (dark gray bars) than the comparable treatment of the control FL1607 line. Line 11–6 even possessed significant alternative pathway capacity in fresh tissue. When NADH was provided as the substrate, similar trends were observed (data not shown). As in leaf mitochondria, addition of pyruvate to tuber mitochondria did not significantly increase the total respiration rate but caused a shift in the respiratory capacity from the Cyt pathway to the alternative pathway. Aging of either FL1607 or 11–6 tuber tissue caused an increase in total respiration due to increases in the capacities of both the Cyt and alternative pathways, although these increases were not as dramatic as reported for Russet Burbank tuber mitochondria (Hiser and McIntosh, 1990). Under every treatment, however, the overexpressing line 11–6 had a significantly higher alternative pathway capacity than the control FL1607 line. This suggests that alteration of level of alternative oxidase protein by genetic engineering can effectively change alternative pathway capacity in different tissue types.

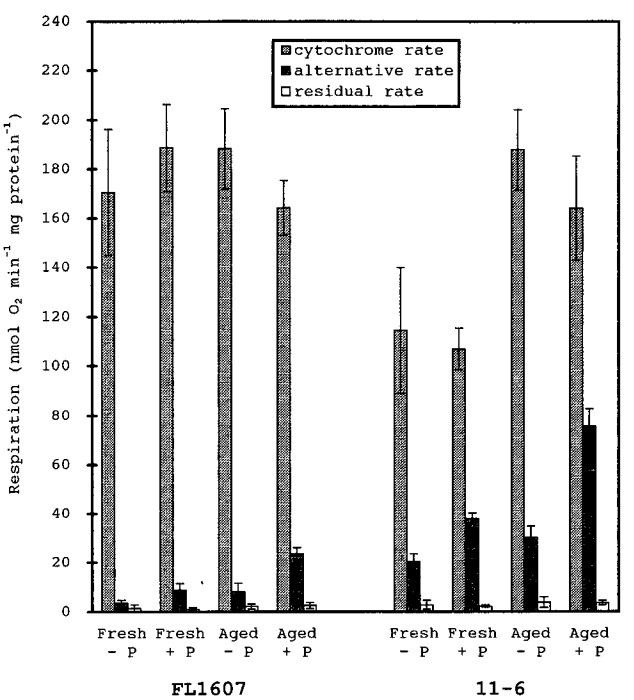

Figure 6. Respiration of mitochondria isolated from fresh and aged potato tuber tissue from the FL1607 control line or the 11–6 overexpressing line. Respiration attributable to the Cyt path (light gray bars), alternative path (dark gray bars), or residual O_2 uptake (open bars) is expressed as nmol O_2 min^{-1} mg^{-1} protein. The supplied substrate was 10 mM succinate, with (+P) or without (−P) pyruvate as an activator of the alternative oxidase. Results are the means (± SD) of three to four replicate isolations with at least three measurements per treatment per isolation.

DISCUSSION

When the potato *Aox1* was overexpressed in transgenic potato plants, plant lines showed increased levels of alternative oxidase mRNA and protein and increased alternative pathway capacity in their leaf and tuber mitochondria. This demonstrates that the alternative pathway can be successfully altered in potato by genetic manipulation.

The major unexpected result of *Aox1* overexpression in potato was the appearance of the characteristic overexpressor protein in leaves, roots, and tubers, in addition to the protein(s) present in those tissues in the FL1607 control line. This phenomenon was difficult to explain as merely a cloning anomaly. Translation was expected to begin at the same start site as for the normal gene product; however, correct processing of the transit peptide should have removed additional N-terminal amino acids contributed by an upstream start site in the vector sequence. Translation was also expected to end at the normal stop codon present in the inserted gene. One possible explanation is that the overexpressor protein, although imported into mitochondria, was not processed correctly. However, the transit peptide was apparently correctly removed from the precursor. The N-terminal sequence of the overexpressor protein from line 11–6 matched that obtained from one of the *S. guttatum* proteins (Fig. 3). This region was also demonstrated to be the N-terminal processing site in soybean (Whelan et al., 1995a). Therefore, it is unlikely that any incorrect processing occurred at the N terminus; potential incorrect processing at other locations in the polypeptide remains to be investigated. (The overexpressor protein from line 11–6 was not sequenced in its entirety [data not shown].)

One interesting observation is that, in both leaves and tubers of the 11–6 overexpressing line, the levels of the overexpressor protein did not shift much from reduced forms to oxidized forms under conditions in which oxidized forms could be detected (Fig. 4). However, the levels of the usual protein(s) present in leaves and tubers of both 11–6 and the control line decreased and oxidized forms not present in the control line were visible in the overexpressing line. Such results would be consistent with the overexpressor protein being a partially processed/modified form

that could undergo oxidation/reduction but not as well as the mature, fully processed/modified form.

Two lines of evidence suggest that the overexpressor protein represents a normal processing intermediate not previously observed. First, a band that co-migrated with the overexpressor protein on western blots was seen in samples of FL1607 leaf mitochondria (Fig. 4). Second, overexpression in tobacco of the tobacco *Aox1* gene was accompanied by the appearance of a similar (but more difficult to discern) higher molecular weight band in some leaf and root mitochondrial samples (Vanlerberghe et al., 1994). Overexpression of *Aox1* may have produced enough protein to overwhelm a normal processing or modification step, allowing a normal intermediate to accumulate to high levels in overexpressing potato lines. Evidence from soybean cotyledons and roots (Whelan et al., 1995a) strongly suggests that some form of modification of the alternative oxidase occurs beyond removal of the transit peptide. Such modification(s) could explain the normal presence of multiple alternative oxidase proteins in potato leaves (Figs. 2B and 4) and many other plant tissues (Obenland et al., 1990; Kearns et al., 1992). The *S. guttatum* spadix has three alternative oxidase proteins (Elthon et al., 1989a, 1989b); these are likely to be produced by different posttranslational modifications, since a single precursor polypeptide was reported (Rhoads and McIntosh, 1991) that appeared to be encoded by a single nuclear gene (Rhoads and McIntosh, 1993). However, the discovery of a second alternative oxidase gene (allele?) in tobacco (Whelan et al., 1995b) opens the possibility that different proteins could be different gene products in other plants. However, we have found no evidence thus far for additional alternative oxidase gene(s) in potato.

It has been suggested that interconversion between the oxidized and reduced forms of the alternative oxidase via formation or reduction of disulfide bond(s) may regulate alternative oxidase activity in situ, with the reduced form being more active (Umbach and Siedow, 1993; Umbach et al., 1994). Exogenous manipulation of the disulfide bond redox status in soybean seedling mitochondria showed that maximum alternative oxidase activity was achieved (in the presence of pyruvate) in mitochondria treated with reductant (Umbach et al., 1994). Two reports have correlated an increase in the alternative pathway respiration with a shift toward the reduced form during a normal developmental process. Increasing alternative oxidase activity in the developing appendix tissue of *S. guttatum* was associated with an increasing proportion of the alternative oxidase proteins in the reduced form (Umbach and Siedow, 1993). In leaves of pea seedlings, alternative oxidase activity increased with age, and this correlated with a shift toward the reduced forms of the protein (Lennon et al., 1995). In contrast, a decline in the alternative pathway with age in soybean roots did not correlate with increased levels of the oxidized form of the protein (Day et al., 1994). In the comparable inducible system of potato, aging of tuber slices led to increased alternative pathway capacity (with and without pyruvate) in mitochondria from both FL1607 control and 11–6 overexpressing tubers (Fig. 6). However, no significant differences in the proportion of reduced/oxidized forms of the alternative oxidase were observed; rather, a general increase in the levels of all forms of the oxidase was seen in aged tuber mitochondria from either line (Fig. 4B). Therefore, during aging of potato tuber tissue, the increase in alternative pathway capacity was apparently not dependent on major changes in the disulfide bond redox status but was more dependent on the amount of alternative oxidase protein.

As in many plant tissues (for reviews, see Lance et al., 1985; Moore and Siedow, 1991), a difference between NADH and succinate oxidation was observed in potato leaf and tuber mitochondria. Some authors had noted that NADH was not utilized as well as succinate (e.g. in potato tuber mitochondria: Dizengremel and Lance, 1976), although others had found similar rates of respiration with both substrates (e.g. in potato tuber callus mitochondria: Wagner et al., 1989). Here, potato leaf and tuber mitochondria from both the FL1607 control line and the 11–6 overexpressing line oxidized NADH as well as they oxidized succinate (Fig. 5 and data not shown). As reported for many plants, including potato (Dizengremel and Lance, 1976; Wagner et al., 1989; Liden and Akerlund, 1993), a lower alternative pathway capacity was measured with NADH than with succinate (Fig. 5 and data not shown). This observed difference may have been due to the ability of succinate (but not NADH) to produce pyruvate endogenously and thereby activate the alternative oxidase (Millar et al., 1993; Day et al., 1994). However, addition of pyruvate to potato leaf (Fig. 5) or tuber (data not shown) mitochondria did not eliminate the difference in measured alternative pathway capacity between NADH and succinate, as was reported for soybean cotyledon mitochondria (Millar et al., 1993; Day et al., 1994). The substrate effect in potato, therefore, may have been partially mediated by other factors. Stimulation of NADH oxidation in potato tuber callus mitochondria by succinate has been reported (Wagner et al., 1989), and succinate itself might have activated the alternative oxidase directly (Liden and Akerlund, 1993). However, the difference may also have occurred at the level of the dehydrogenases or substrate uptake and may not have been directly related to the alternative oxidase.

We have shown that the amounts of alternative oxidase mRNA and protein and alternative pathway capacity can be successfully manipulated by transformation in potato. Overexpression of *Aox1* could prove useful in answering basic questions about the function and regulation of the alternative oxidase. Since separate isolation of the multiple forms of the "normal" alternative oxidase from non-aroid plant species is so difficult with current technology, further analysis of the overexpressor protein may be a useful method to probe normal processing and/or modification of the potato alternative oxidase. The question of disulfide bond formation as a means of alternative oxidase regulation could be investigated by creating potato lines that overexpress a version of *Aox1* in which appropriate Cys residue(s) are mutated. Further analysis of overexpressing lines, as well as construction of antisense transformants and those utilizing tissue-specific or inducible promoters,

promises to provide useful information about the regulation and function of the alternative oxidase of potato.

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LITERATURE CITED

An G (1987) Binary Ti vectors for plant transformation and promoter analysis. Methods Enzymol **153:** 292–305

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

Boutry M, Faber A-M, 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

Casadaban MJ, Cohan SN (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol **138:** 179–207

Cashmore A (1982) The isolation of poly A^+ messenger RNA from higher plants. *In* M Edelman, RB Hallick, N-H Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, New York, pp 387–392

Crofts AR (1987) SEQANAL program series. University of Illinois, Urbana

Dale RM, McClure BA, Houchins JP (1985) A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing the corn mitochondrial 18S rDNA. Plasmid **13:** 31–40

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, Vol 2. Academic Press, New York, pp 197–241

Day DA, Millar AH, Wiskich JT, Whelan J (1994) Regulation of alternative oxidase activity by pyruvate in soybean mitochondria. Plant Physiol **106:** 1421–1427

Day DA, Neuburger M, Douce R (1985) Biochemical characterization of chlorophyll-free mitochondria from pea leaves. Aust J Plant Physiol **12:** 219–228

Dizengremel P, Lance C (1976) Control of changes in mitochondrial activities during aging of potato slices. Plant Physiol **58:** 147–151

Elthon TE, McIntosh L (1987) Identification of the alternative terminal oxidase of higher plant mitochondria. Proc Natl Acad Sci USA **84:** 8399–8403

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

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

Hiser C, McIntosh L (1990) Alternative oxidase of potato is an integral membrane protein synthesized *de novo* during aging of tuber slices. Plant Physiol **93:** 312–318

Hiser C, McIntosh L (1994) Potato alternative oxidase: detection of mRNA by PCR and tissue-specific differences in the protein levels. *In* WR Belknap, ME Vayda, WD Park, eds, The Molecular and Cellular Biology of the Potato, Ed 2. CAB International, Wallingford, UK, pp 143–150

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

Lance C, Chauveau M, Dizengremel P (1985) The cyanide-resistant pathway of plant mitochondria. *In* R Douce, DA Day, eds, Higher Plant Cell Respiration. Springer-Verlag, New York, pp 202–247

Larson E, Howlett B, Jagendorf A (1986) Artificial reductant enhancement of the Lowry method for protein determination. Anal Biochem **155:** 243–248

Lennon AM, Pratt J, Leach G, Moore AL (1995) Developmental regulation of respiratory activity in pea leaves. Plant Physiol **107:** 925–932

Liden AC, Akerlund H-E (1993) Induction and activation of the alternative oxidase of potato tuber mitochondria. Physiol Plant **87:** 134–141

McIntosh L, Cattolico RA (1978) Preservation of algal and higher plant ribosomal RNA integrity during extraction and electrophoretic quantitation. Anal Biochem **91:** 600–612

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

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

Obenland D, Dietham 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

Rhoads DM, McIntosh L (1991) Isolation and characterization of a cDNA clone encoding an alternative oxidase protein of *Sauromatum guttatum* (Schott). Proc Natl Acad Sci USA **88:** 2122–2126

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

Rhoads DM, McIntosh L (1993) The salicylic acid-inducible alternative oxidase gene *aox1* and genes encoding pathogenesis-related proteins share regions of sequence similarity in their promoters. Plant Mol Biol **21:** 615–624

Sakajo S, Minagawa N, Komiyama T, Yoshimoto A (1991) Molecular cloning of cDNA for antimycin A-inducible mRNA and its role in cyanide-resistant respiration in *Hansenula anomala*. Biochim Biophys Acta **1090:** 102–108

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 11.31–11.32

Seldon RF (1987) Analysis of RNA by northern hybridization. *In* FM Ausubel, R Brent, RE Kingston, DD Moore, JD Seidman, JA Smith, K Struhl, eds, Current Protocols in Molecular Biology. Greene Publishing Associates, New York, pp 4.9.1–4.9.8

Siedow JN, Whelan J, Kearns A, Wiskich JT, Day DA (1992) Topology of the alternative oxidase in soybean mitochondria. *In* H Lambers, LHW van der Plas, eds, Molecular, Biochemical and Physiological Aspects of Plant Respiration. Academic Publishing, The Hague, pp 19–27

Theologis A, Laties GG (1978) Relative contribution of cytochrome-mediated and cyanide-resistant electron transport in fresh and aged slices. Plant Physiol **62:** 232–237

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

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. FEBS Lett **348:** 181–184

Vanlerberghe GC, McIntosh L (1992) 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

Vanlerberghe GC, Vanlerberghe AE, McIntosh L (1994) Molecular genetic alteration of plant respiration. Silencing and overexpression of alternative oxidase in transgenic tobacco. Plant Physiol **106:** 1503–1510

Verwoerd TC, Dekker BMM, Hoekema A (1989) A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res **17:** 2362

Viera J, Messing J (1987) Production of single-stranded plasmid DNA. Methods Enzymol **153:** 3–11

Wagner AM, Kraak MHS, van Emmerik WAM, van der Plas LHW (1989) Respiration of plant mitochondria with various substrates: alternative pathway with NADH and TCA cycle derived substrates. Plant Physiol Biochem **27:** 837–845

Wenzler H, Mignery G, May G, Park W (1989) A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. Plant Sci **63:** 79–85

Whelan J, Hugosson M, Glaser E, Day DA (1995a) Studies on the import and processing of the alternative oxidase by isolated soybean mitochondria. Plant Mol Biol **27:** 769–778

Whelan J, McIntosh L, Day DA (1993) Sequencing of a soybean alternative oxidase cDNA clone. Plant Physiol **103:** 1481

Whelan J, Smith MK, Meijer M, Yu J-W, Badger MR, Price GD, Day DA (1995b) Cloning of an additional cDNA for the alternative oxidase in tobacco. Plant Physiol **107:** 1469–1470