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Differential Expression of the Multigene Family Encoding the Soybean Mitochondrial Alternative Oxidase[1]

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The alternative oxidase (AOX) of the soybean (*Glycine max* L.) inner mitochondrial membrane is encoded by a multigene family (*Aox*) with three known members. Here, the *Aox2* and *Aox3* primary translation products, deduced from cDNA analysis, were found to be 38.1 and 36.4 kD, respectively. Direct N-terminal sequencing of partially purified AOX from cotyledons demonstrates that the mature proteins are 31.8 and 31.6 kD, respectively, implying that processing occurs upon import of these proteins into the mitochondrion. Sequence comparisons show that the processing of plant AOX proteins occurs at a characteristic site and that the AOX2 and AOX3 proteins are more similar to one another than to other AOX proteins, including soybean AOX1. Transcript analysis using a polymerase chain reaction-based assay in conjunction with immunoblot experiments indicates that soybean *Aox* genes are differentially expressed in a tissue-dependent manner. Moreover, the relative abundance of both *Aox2* transcripts and protein in cotyledons increase upon greening of dark-grown seedlings. These results comprehensively explain the multiple AOX-banding patterns observed on immunoblots of mitochondrial proteins isolated from various soybean tissues by matching protein bands with gene products.

The AOX is a cyanide-resistant, hydroxamic-acid-sensitive terminal oxidase found in the inner mitochondrial membranes of plants, some fungi, and trypanosomes (Moore and Siedow, 1991; Day and Wiskich 1995; Day et al., 1995). The activity mediates the non-proton-translocating transfer of electrons from the UQ pool to molecular O_2 to form water. Electrons flowing through AOX bypass proton-translocating complexes III and IV of the Cyt-mediated electron transport chain, causing the oxidative potential energy to be lost as heat.

The function of AOX in plants during normal vegetative growth and development remains problematic, except in the tightly regulated process of thermogenesis in aroid species. During floral maturation in species such as *Arum maculatum* and *Sauromatum guttatum*, a massive increase in AOX activity occurs in the appendix tissue of the floral spadix (Meusse, 1975). The high levels of AOX activity in thermogenic tissues has encouraged the development of protocols for the partial purification of the enzyme from several species (Huq and Palmer, 1978; Rich, 1978; Elthon and McIntosh, 1986; Berthold and Seidow, 1993; Zhang et al., 1996a). Polyclonal antisera (Elthon and McIntosh, 1987) and monoclonal antibodies (Elthon et al., 1989) have been produced against the AOX protein from *S. guttatum*. The monoclonal antibody AOA has been particularly useful because it immunoreacts with the same proteins as the polyclonal antisera. Immunological studies in which these antibodies are used indicate that de novo protein synthesis is largely responsible for the induction of AOX activity during thermogenesis (Elthon and McIntosh, 1987; Rhoads and McIntosh, 1992). In thermogenic species, the anti-AOX antibodies recognize only a single 35- to 37-kD polypeptide in mitochondria from prethermogenic tissues (Elthon and McIntosh, 1987). In organelles from thermogenic tissues this protein is detected in dramatically higher abundance and is joined by two or three other proteins of similar size and greater amount (Elthon and McIntosh, 1987; Elthon et al., 1989).

In nonthermogenic plants AOX activity is present in most tissues but may be quite low. The magnitude of the activity is tissue-dependent and developmental stage-dependent and can vary with growth conditions (Obenland et al., 1990; Kearns et al., 1992; Johns et al., 1993; Conley and Hanson, 1994; Hiser and McIntosh, 1994; Cruz-Hernandez and Gomez-Lim, 1995). Typically, mitochondria from nonthermogenic plants contain one or more AOX proteins of 32 to 39 kD that cross-react with the AOA monoclonal antibody. For example, in cotyledons from young, light-grown soybean (*Glycine max* L.) seedlings, the AOA antibody detects a single protein that is joined in cotyledons from older seedlings by a second protein with lower mobility (Obenland et al., 1990). In contrast, in etiolated cotyledons the lower-mobility species is found at all ages, whereas the higher-mobility protein appears only in older tissues. In soybean roots, a single protein co-

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Abbreviations: AOX, alternative oxidase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase PCR; SMP, submitochondrial particle; UQ, ubiquinone.

migrating with the lower-mobility band found in cotyledons is detected, whereas in leaves the immunoreactive proteins are identical in mobility and similar in abundance to those in cotyledons (Kearns et al., 1992). Determinations of the apparent molecular sizes of the immunoreactive species in soybean vary but are in the range of 33 to 39 kD. It has been presumed that the multiple proteins recognized by AOA in any single species represent different forms of AOX, although the basis of the multiplicity has not been investigated in detail.

In nonthermogenic plants AOX activity can be induced by numerous stress treatments, including inhibition of electron flow through the Cyt pathway (Vanlerberghe and McIntosh, 1992; Wagner et al., 1992), cycloheximide (Morohashi et al., 1991) or chloramphenicol (Zhang et al., 1996b) treatment, oxidative stress (Wagner, 1995), chilling (McCaig and Hill, 1977; Vanlerberghe and McIntosh, 1992), and wounding (Hiser and McIntosh, 1990). Possible elicitors of induction include salicylic acid (Kapulnik et al., 1992; Rhoads and McIntosh, 1992) and citrate (Vanlerberghe and McIntosh, 1996). Induction of AOX capacity in nonthermogenic plants typically involves de novo protein synthesis, because the total abundance of the immunoreactive proteins in both induced and noninduced systems generally parallels activity levels. Recently, the enzyme has been found to be stimulated by α-keto acids (Millar et al., 1993) and regulated by the oxidation state of intermolecular disulfide bonds, with reduced dimers being the active form (Umbach and Siedow, 1993).

A cDNA representing a plant *Aox* gene was first isolated from a *S. guttatum* library by antibody screening (Rhoads and McIntosh, 1991). Since then, *Aox* cDNA sequences from soybean (Whelan et al., 1993), tobacco (*Nicotiana tabacum*; Vanlerberghe and McIntosh, 1994; Whelan et al., 1995b), *Arabidopsis thaliana* (Kumar and Soll, 1992), mango (*Mangifera indica*; Cruz-Hernandez and Gomez-Lim, 1995), and potato (*Solanum tuberosum*; Hiser et al., 1996) have been reported. AOX sequences have also been determined for the fungi *Hansenula anomola* (Sakajo et al., 1991) and *Neurospora crassa* (Li et al., 1996) and for the protist *Trypanosoma brucei* (Chaudhuri and Hill, 1996). The availability of nucleic acid probes has allowed the analysis of *Aox* transcripts in induced and noninduced plant cell cultures (Vanlerberghe and McIntosh, 1996) and in whole plant organs in which AOX is overexpressed (Hiser et al., 1996) or highly induced, such as in thermogenic spadices (Rhoads and McIntosh, 1992) and ripening fruit (Cruz-Hernandez and Gomez-Lim, 1995). In those cases in which de novo protein synthesis is required for the induction of AOX activity, transcript levels increase, suggesting that gene transcription may be involved. These probes, however, have not allowed the general analysis of *Aox* transcripts in noninduced, vegetative tissues because of the low abundance of the messages (Hiser and McIntosh, 1994; Hiser et al., 1996). In fact, PCR-based techniques appear to be the only recourse for detecting *Aox* transcripts in these tissues (Hiser and McIntosh, 1994; Whelan et al., 1996a).

Genomic-blot hybridization analysis in which stringent washing conditions are used reveals only a single *Aox* gene in Arabidopsis (Kumar and Soll, 1992), *S. guttatum* (Rhoads and McIntosh, 1993), and soybean (Whelan et al., 1996a). However, PCR analysis of genomic DNA has definitively shown that soybean and tobacco AOX are encoded by multigene families comprising at least three and two members, respectively (Whelan et al., 1996a). This finding necessitates more careful evaluation of the *Aox* copy number in the genomes of other plant species.

In this report we present the complete amino acid sequences for the soybean *Aox2* and *Aox3* gene products as deduced from the cognate cDNAs, and we examine the sequence conservation among AOX subunits from different species. The relative abundance of transcripts from the three known soybean *Aox* genes in roots and cotyledons from seedlings grown under normal conditions and in dark-grown cotyledons exposed to light has been estimated using RT-PCR. Amino acid sequencing of proteins in partially purified AOX preparations has allowed us to match genes and protein bands on western blots. These analyses have allowed us to attribute the presence of individual polypeptides to the differential expression of specific *Aox* genes, thus providing the first comprehensive rationalization of the multiple AOX protein patterns observed in soybean.

MATERIALS AND METHODS

Plant Growth

Soybean (*Glycine max* L. Merrill cv Stevens) seeds were planted on water-saturated vermiculite in a controlled-environment cabinet. Cotyledons and roots were harvested from the same plants after 7 d of growth at 28°C. A photoperiod of 14 h of light (100 μmol m^{-2} s^{-1})/10 h of dark was used for light-grown tissues. In greening experiments, cotyledons from seeds germinated for 7 d without light in aluminum foil-covered trays were harvested after exposure to continuous light (100 μmol m^{-2} s^{-1}) for the times indicated in the figure legends.

Primers

The primers used in this study were synthesized by the Biomolecular Resource Facility at the Australian National University (Canberra, Australia) or by Bresatec (Adelaide, Australia). The primers used for RACE were the vector-specific λ-forward (GGTGGCGACGACTCCTGGAGCCCG), the adaptor specific AP1 (CCATCCTAATACGACTCACTATAGGGC), and the *Aox*-specific Aox2/3-R (CGGCATGAAGCAGTTCCA) and degenerate HAO2-R (GCYTCYTCYTCNARR TANCCNAC) (Whelan et al., 1996a) primers. The primers used for competitive RT-PCR were the *Aox1*-specific primers Aox1-F (GAAGCACCATGCTCCAAC) and Aox1-R (CCCTTGATAGTGAATGTCC), the *Aox2*-specific primers Aox2-F (GGGAGAGTTATCGTTCAAAC) and Aox2-R (GTGATAACCAATAGGAGCC), and the *Aox3* specific primers Aox3-F (GACAAGGTTGCTTTTAGGG) and Aox3-R (CAGTGACAACATCCTTGAG). These primer pairs were designed so that the two annealing sites were in different exons, assuming that *Aox2*

and *Aox3* contain introns in positions homologous to the introns in *Aox1* (Day et al., 1995). The positions corresponding to the *Aox*-specific primer sequences are shown in Figure 1. Primers with sense-strand sequences are designated "-F" (Forward), and antisense primers are designated "-R" (Reverse).

PCR Conditions

Each 10 μL of PCR contained 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [w/v] gelatin), 0.2 mM each dATP, dCTP, dGTP, dTTP, *Taq* DNA polymerase, primers, and template DNA. For RACE, each reaction contained 1 unit of *Taq* polymerase (AmpliTaq Gold, Perkin-Elmer), and for RT-PCR amplifications, 0.35 unit of sequencing-grade *Taq* polymerase (Promega) was used. The degenerate primer HAO2-R was used at 20 pmol per reaction, whereas all other primers were used at 2 pmol per reaction. The program used on the capillary thermal cycler (Corbett Research, Mortlake, NSW, Australia) was 1 cycle of 95°C, 9 min; 53°C, 15 s; 72°C, 1 min; 40 cycles of 94°C, 15 s; 53°C, 15 s; 72°C, 1 min; and 1 cycle of 72°C, 5 min; 25°C, 1 min, except for the RT-PCR assays, in which the initial denaturation period was shortened to 3 min.

Manipulation of DNA

Cloned DNA fragments were maintained, digested with restriction enzymes (Boehringer Mannheim; AMRAD Pharmacia), analyzed by electrophoresis, purified, subcloned, and radiolabeled using standard methods (Sambrook et al., 1989). Templates for DNA sequencing were obtained either by subcloning overlapping restriction fragments or by treating linearized plasmids with exonuclease III (Henikoff, 1984) and mung bean nuclease before religation. The nucleotide sequences of double-stranded DNA templates were determined by the dideoxynucleotide termination method using dye-labeled, vector-specific primers, a thermal cycle-

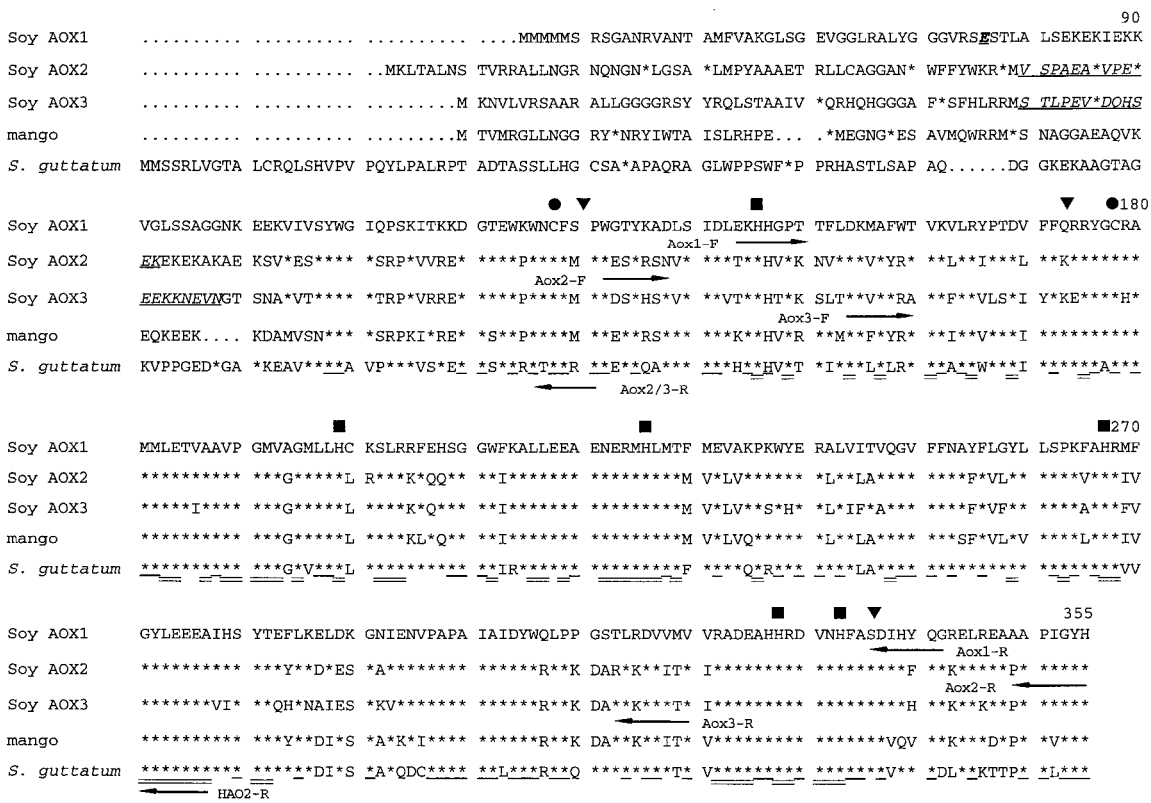

Figure 1. A comparison of AOX amino acid sequences. The sequences from soybean (Soy), mango, and *S. guttatum* are shown. The determined N-terminal sequences of mature soybean AOX2 and AOX3 (underlined italics) and the putative N terminus of mature soybean AOX1 (underlined bold italics) are indicated. Residues identical to the soybean AOX1 sequence are marked with asterisks (*), residues in the *S. guttatum* sequence that are absolutely conserved in all species examined are double-underlined, and those absolutely conserved in plants are single-underlined. The Cys residues that are present in all plant sequences (●) and the His residues that are present in all species (■) are shown. The positions of introns in *Aox1* of soybean (Day et al., 1995) and *S. guttatum* (Rhoads and McIntosh, 1993) are marked (▼). Sequences corresponding to the locations of PCR primers are underlined in bold with arrowheads showing the direction of priming. Numbers correspond to positions within the alignment. The sequences compared were from soybean AOX1 (Whelan et al., 1993), AOX2 (accession no. U87906), and AOX3 (accession no. U87907), mango (Cruz-Hernandez and Gomez-Lim, 1995), *S. guttatum* (Rhoads and McIntosh, 1991), tobacco AOX1-a (Vanlerberghe and McIntosh, 1994) and AOX1-b (Whelan et al., 1995b), Arabidopsis (Kumar and Soll, 1992), potato (Hiser et al., 1996), *H. anomola* (Sakajo et al., 1991), *T. brucei* (Chaudhuri and Hill, 1996), and *N. crassa* (Li et al., 1996).

sequencing kit, and an automated sequencer (Applied Biosystems). Contiguous sequences were managed and analyzed using the SeqEd (Applied Biosystems), MacVector (Kodak), and AssembyLIGN (Kodak) software packages. Sequence alignments were constructed using the Clustal V program in the Lasergene software package (DNASTAR, Madison, WI).

Purification of RNA

Total RNA was purified from plant tissues ground to a fine powder under liquid N_2 using the guanidine isothiocyanate/organic extraction method of Chomczynski and Sacchi (1987). Approximately 1.5 g of tissue was used during library construction, and approximately 100 mg of tissue was used for transcript analysis by RT-PCR. Poly(A)$^+$ RNA was isolated from total RNA by batch treatment with oligo(dT)$_{25}$-conjugated paramagnetic particles (Dynal, Oslo, Norway) according to the manufacturer's instructions, except that NaCl was substituted for LiCl in all buffers.

Library Screening and Analysis

An amplified cDNA library of poly(A)$^+$ RNA from 10-d-postemergent seedlings cloned into the *Eco*RI site of λgt11 (Clontech Laboratories, Palo Alto, CA) was screened by nucleic acid hybridization, and the inserts of plaque-purified positive clones were subcloned into plasmid vectors (Sambrook et al., 1989). An adaptor-ligated cDNA library was also constructed from 1 μg of poly(A)$^+$ RNA isolated from an entire 7-d-old light-grown soybean seedling. Oligo(dT)$_{18}$-primed cDNA synthesis and adaptor ligation were performed using a kit (Marathon, Clontech) following the manufacturer's instructions. Aliquots (1 μL) of the λgt11 library or dilutions of the adaptor ligated cDNA library were used as templates for RACE. The resulting PCR products were separated on low-melting-temperature agarose gels containing 1× Tris-acetate-EDTA buffer (Sambrook et al., 1989). The desired fragments were purified from these gels using a DNA purification system (WIZARD, Promega) and cloned into a T-tailed vector (pGEM-T, Promega), as recommended by the manufacturer.

Isolation, Blotting, and Immunodetection of Mitochondrial Proteins

Mitochondria were isolated using the Percoll (AMRAD, Pharmacia) gradient method of Day et al. (1985). Submitochondrial particles were prepared essentially by the method of Rasmusson and Møller (1991). AOX was partially purified by the method of Zhang et al. (1996a). Aliquots of mitochondrial, SMP, or partially purified AOX protein were separated by SDS-PAGE according to the method of Laemmli (1970) after dissociation by heating at 90°C for 5 min in sample buffer containing SDS and DTT. Proteins for immunoblotting were transferred to nitrocellulose membranes (Hybond-C, Amersham) by the method of Towbin et al. (1979) using a semidry blotting system (Millipore). AOX subunits were detected using a mouse monoclonal antibody raised against the thermogenesis-induced subunits of the *S. guttatum* enzyme (Elthon et al., 1989) and visualized by chemiluminescence using a commercially available kit (Boehringer Mannheim). Polypeptides for N-terminal sequence determination were electroblotted to PVDF membranes (Bio-Rad) using 10 mM Tris-Gly buffer, pH 8.3, 0.04% (w/v) SDS, and 20% (v/v) methanol. Protein bands were excised from blots stained with 0.1% (w/v) Coomassie blue R-250 in 50% (v/v) methanol, destained with 50% (v/v) methanol, and air-dried.

Construction of a Standard Template for Competitive RT-PCR

A PCR mutagenesis strategy using *Aox*-specific primer pairs was used to construct a standard template. The final product is an 851-bp chimera of DNA sequences from the three soybean *Aox* cDNAs with an additional 180-bp fragment of non-*Aox* DNA inserted. The starting materials were the purified PCR products resulting from the amplification of *Aox1* and *Aox2* cDNAs by their respective gene-specific primer pairs. The amplicons were combined, denatured by heating at 100°C for 5 min, and allowed to re-form duplexes by slowly cooling to ambient temperature. The heteroduplexes were end-filled with the Klenow fragment of *Escherichia coli* DNA polymerase I (Sambrook et al., 1989), and the resulting sense and antisense *Aox1*/*Aox2* hybrid strands were selectively amplified in separate reactions using Aox1-F/Aox2-R and Aox1-R/Aox2-F primer pairs, respectively. The products of these reactions were purified, combined, denatured, annealed, and end-filled as above, before amplifying the final product with the Aox2-F/Aox2-R primer pair. The product was cloned and individual inserts were sequenced to verify that mutagenesis had progressed as expected. A second round of mutagenesis was performed to introduce the *Aox3*-specific primer sequences and an artificial intron into the *Aox1*/*Aox2* chimeric fragment. In this case the product of the first mutagenesis and an *Aox3* cDNA with a 180-bp fragment of unrelated DNA blunt-end cloned into the single *Bsm*I site served as the starting templates. After the final amplification with the Aox2-F/Aox2-R primer pair, the product was cloned and the sequence of the final mutant was verified.

Competitive RT-PCR Assay

Total RNA from target tissues was reverse-transcribed and the relative abundance of cDNAs corresponding to *Aox* transcripts was estimated by competitive PCR using the *Aox*-specific primer pairs. Each estimation involved the titration of a constant amount of cDNA template against varying amounts of a linearized plasmid containing the competing standard template. The standard template was constructed (see above) so that the amplicons of the standard and experimental templates could be discriminated by size.

Reverse transcription of 1 μg of total RNA was performed by incubating 20-μL reactions containing 1× PCR buffer, 50 pmol oligo(dT)$_{18}$, 1 mM each dATP, dCTP, dGTP, and dTTP, 5 mM DTT, 20 units of RNA Guard (Pharmacia), and 20 units

of Molony murine leukemia virus reverse transcriptase (Epicentre Technologies, Madison, WI) at 42°C for 90 min. A two-step approach was then taken to estimate the relative abundance in the cDNA of reverse transcripts from the three *Aox* genes. First, the abundance of each cDNA was estimated using the *Aox*-specific primer pairs separately in three PCR assays containing 1000-fold serial dilutions of the standard template. This estimate was further refined in a set of reactions that titrated the cDNA against 2-fold serial dilutions of the standard template. The range of dilutions was chosen to encompass the estimate obtained during the previous step. After PCR the products were separated on 2% (w/v) agarose gels in 1× Tris-borate-EDTA buffer (Sambrook et al., 1989) and visualized by ethidium bromide staining. Ethidium bromide fluorescence was measured (FluorImager SI, Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software (Molecular Dynamics). The signal from each PCR product fell within the linear response range of the imager for ethidium bromide fluorescence. The signals obtained were corrected for differences in length between the standard and experimental templates and plotted as the log of the ratio of the signals obtained from the experimental and standard templates against the log of the amount of input standard template. From this, the amount of standard template necessary to give a signal ratio of unity was calculated.

O_2 Consumption Assays and UQ Determinations

AOX activity, as myxothiazol-resistant O_2 consumption, was measured using an O_2 electrode (Rank Bros., Cambridge, UK) at 25°C in 2 mL of reaction medium containing 0.3 M Suc, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% (w/v) BSA, 10 mM Tes, pH 7.2, 2.5 mM DTT, 1 mM NADH, 5 μM myxothiazol, and 1 mM pyruvate. Approximately 200 μg of SMPs from cotyledon and root mitochondria was used in each assay. Pyruvate concentration was varied as indicated in determining the $K_{½}$ for pyruvate stimulation. Orientation of SMPs was determined by the oxidation of reduced Cyt *c* in the presence and absence of 0.01% (v/v) Triton X-100 (Rasmusson and Møller, 1991). The protein content of the SMP samples was estimated by the technique of Lowry et al. (1951); the extraction of UQ from SMP samples and analysis by reverse-phase HPLC was conducted according to the method of Ribas-Carbo et al. (1995). A C_{18} μBondapak column (250 mm × 10 mm; Millipore) was used on a system (LKB-Pharmacia) with an isocratic mobile phase of ethanol:methanol (7:3) at a flow rate of 0.5 mL min^{-1}, and the effluent from the column was monitored at 275 and 290 nm. Commercial preparations (Sigma) of UQ_9 and UQ_{10} were reduced to the respective quinols by the method of Rich (1978). Retention times of the reduced and oxidized standards were determined for comparison with SMP extracts.

RESULTS

Soybean AOX Is Encoded by a Multigene Family

The use of degenerate primers derived from highly conserved AOX peptide sequences in PCR amplification of soybean genomic DNA led to the isolation of 170-bp fragments representing three *Aox* genes (Whelan et al., 1996a). The soybean *Aox1* gene product had been characterized earlier (Whelan et al., 1993), so the putative new genes were designated *Aox2* and *Aox3*. In the present study, the *Aox2* and *Aox3* PCR fragments have been used to isolate respective cDNA sequences encoding full-length ORFs (Fig. 1). Initially, a seedling cDNA library was screened by hybridization, but the presence in *Aox2* cDNAs of two internal *Eco*RI sites that were cleaved during library construction necessitated the use of PCR-based RACE to isolate 5′ proximal regions of this cDNA. In the first instance, the *Aox*-specific degenerate primer HAO2-R (Fig. 1) and the vector-specific λ-forward primer were used. When this approach failed to complete the ORF, an adaptor-ligated cDNA library was amplified with the gene-specific primer Aox2/3-R (Fig. 1) and the adaptor-specific primer AP1. The resulting composite cDNA sequence covered 1295 bp and encoded the entire 999-bp *Aox2* ORF. The first in-frame Met residue 16 codons downstream of an in-frame stop codon is presumed to be the initiation codon. To ensure the fidelity of the cDNA sequences isolated by PCR, a number of individual cloned PCR products were sequenced. Screening the library with the *Aox3*-specific probe identified a clone with a 1228-bp insert that encoded a complete 978-bp ORF and possessed an in-frame stop codon 13 codons upstream of the first in-frame Met.

The calculated molecular masses of the deduced 333-residue *Aox2* and the 326-residue *Aox3* primary translation products (Fig. 1) are 38.1 and 36.4 kD, respectively. The N-terminal regions of both primary translation products resemble mitochondrial targeting signals in that they possess a high proportion of basic and hydroxylated amino acids and only one acidic residue (Hartl et al., 1989). In both proteins this region is followed by a short segment containing a high proportion of Glu and Lys residues. These characteristics are common to the N-terminal region of the soybean *Aox1* primary translation product (Fig. 1), which has been demonstrated to possess a cleavable presequence capable of targeting the protein into isolated mitochondria (Whelan et al., 1995a). Using a 12-amino-acid residue-sliding window, we examined the hydrophobic moment along the N-terminal presequence of each of the three *Aox* gene products. This analysis indicates that each presequence has the potential to fold into an amphipathic helix, indicative of many mitochondrial targeting signals.

The AOX2 and AOX3 Proteins Possess Cleavable Presequences

When mitochondrial proteins isolated from light-grown soybean cotyledons are separated by SDS-PAGE and probed with the AOA antibody, two prominent bands with apparent molecular masses of 34 and 36 kD are typically seen upon visualization of the immunoreaction with an alkaline phosphatase-coupled colorimetric assay (Obenland et al., 1990; Kearns et al., 1992). The relative amounts of these proteins are variable and appear to depend on the age of the cotyledon, as well as on the presence or absence of light during growth (Obenland et al., 1990). In an at-

tempt to match the AOX proteins previously detected on immunoblots with their cognate genes, the N-terminal sequences of the immunoreactive proteins present in soybean cotyledons were determined directly. Partially purified AOX from cotyledons of 7-d-old soybean seedlings (Zhang et al., 1996a) was separated by SDS-PAGE. Although numerous bands were observed upon Coomassie blue staining (results not shown), only abundant proteins of 34 and 36 kD cross-reacted with the AOA antibody. The proteins corresponding to the immunoreactive bands were excised from an identical blot and subjected to direct N-terminal sequencing. Comparison of the ascertained sequences with those deduced from soybean *Aox* cDNAs revealed that the 34- and 36-kD species correspond to the *Aox2* and *Aox3* gene products, respectively (Fig. 1). These results establish that *Aox2* and *Aox3* are expressed in soybean and that the primary translation products have cleavable presequences of 57 and 50 residues, respectively, which presumably act as mitochondrial targeting signals and are removed upon import. The mature AOX2 and AOX3 subunits have deduced masses of 31.8 and 31.6 kD, respectively. The basis of the discrepancy between the molecular masses calculated from the deduced amino acid sequences and those determined by SDS-PAGE is not known.

The Mature AOX2 and AOX3 Sequences Are Distinct from Other AOX Subunits

Alignment of the determined or predicted mature AOX sequences from plants *H. anomola*, *N. crassa*, and *T. brucei* shows a high level of sequence identity across genera, especially in the C-terminal two-thirds of the protein (Fig. 1). The sequence identity is most pronounced over four regions spanning residues 183 to 204, 216 to 229, 271 to 282, and 322 to 333 of the comparison. The region covered by residues 297 to 309 is especially well conserved in plants. Within soybean, AOX2 and AOX3 are 75% similar to one another and 68 and 63% similar to AOX1, respectively. Although the three soybean sequences are highly conserved, soybean AOX1 is more similar to AOX subunits from all other plants except mango, which is the most dissimilar plant sequence, than it is to either AOX2 or AOX3 of soybean (Fig. 2). Conversely, AOX2 and AOX3 are more similar to one another than to any other plant sequences.

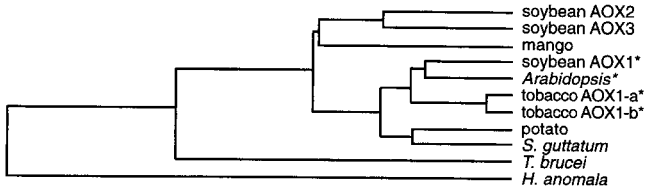

Figure 2. A dendrogram demonstrating the sequence relatedness of mature (processed) AOX proteins. The sequences for the mature proteins are those deduced previously for soybean AOX1 (Whelan et al., 1995a) or postulated from the published AOX sequences of tobacco (AOX1-a and AOX1-b), Arabidopsis, mango, and potato (Hiser et al., 1996), *H. anomola* (Sakajo et al., 1991), and *T. brucei* (accession no. U52964). Alignment was performed using the Clustal method. The mature AOX proteins with a −10 Arg are indicated with an asterisk (*).

Soybean *Aox* Genes Are Expressed in a Tissue-Dependent Manner

We have demonstrated that the 34- and 36-kD proteins in soybean cotyledon mitochondria, which readily immunoreact with the AOA antibody (Obenland et al., 1990; Kearns et al., 1992), correspond to mature AOX2 and AOX3, respectively. In contrast to cotyledons, a single 36-kD protein is detected when mitochondria from soybean roots are examined (Kearns et al., 1992). To investigate whether these differences in protein pattern between tissues are due to differences in expression of the three *Aox* genes, a semiquantitative, competitive RT-PCR assay was developed. Reverse transcripts from total cellular RNA were used as PCR templates in reactions containing *Aox* transcript-specific primer pairs. The amplicons for the *Aox1*-, *Aox2*-, and *Aox3*-specific primer pairs are 603, 671, and 499 bp in length, respectively. If *Aox2* and *Aox3* have genomic organizations similar to that of *Aox1* (Day et al., 1995), then each amplicon spans an exon-exon boundary. This would eliminate possible confusion arising from the amplification of genomic DNA contaminants in the RNA preparations. In every case, the omission of reverse transcriptase resulted in no products being formed, demonstrating that genomic contamination of the RNA preparations was negligible.

The relative amount of each *Aox* cDNA in the total population was determined by amplifying a constant amount of cDNA in several reactions containing a single gene-specific primer pair and a range of known standard template amounts. The standard template is a fragment of soybean *Aox2* cDNA mutagenized to contain the sequences of the *Aox1*- and *Aox3*-specific primers in their homologous locations (Fig. 3A). To distinguish the PCR products derived from the cDNA and standard templates, a 180-bp sequence was introduced between the three pairs of convergent primer sequences on the standard template. For example, when cDNA generated from total RNA of cotyledons of light-grown seedlings was amplified with the *Aox2*-specific primer pair, two products of varying relative intensity were obtained (Fig. 3B, inset). The 671-bp product resulted from the RT-PCR amplification of *Aox2* transcripts, and the 851-bp product corresponds to the amplification of the standard template.

A logarithmic relationship exists between the amount of standard template added to each reaction and the ratio of the fluorescent signals obtained from the target and standard template amplicons (Fig. 3B). From this relationship the amount of standard template that yields a signal ratio of unity was calculated. The calculated value represents an estimate of target template levels in the cDNA population, because at this point there would be equal competition of the two templates for reagents in the PCR. Although not strictly quantitative due to possible differential efficiencies in cDNA synthesis between samples, this assay allows the estimation of the relative amounts of transcripts from the three *Aox* genes in a given RNA population, because the

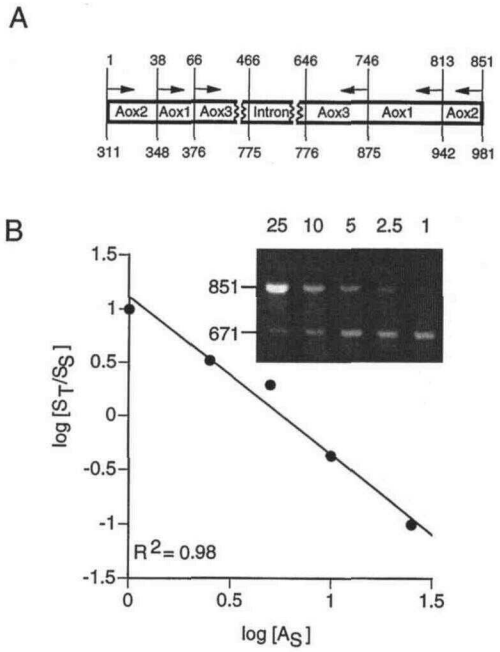

Figure 3. Logarithmic competition between the standard template and soybean AOX cDNAs during PCR. A, The positions of *Aox* cDNA sequences in the standard template are shown. Coordinates of sequence boundaries in the standard template are shown across the top, with the corresponding *Aox2* cDNA coordinates shown across the bottom. Arrows mark the position and direction of the *Aox* gene-specific primers described in Figure 1, which were used to construct the standard template and for the RT-PCR assay. B, A representative plot of $\log(A_S)$ versus $\log(S_T/S_S)$, where A_S is the initial amount of standard template added to the PCR assay and S_T and S_S are the fluorescent signals generated by the target and standard template product bands, respectively. The inset shows the gel used to generate the plot. The amount in nanograms of standard template added to each PCR is indicated above each track. The sizes of the PCR products in base pairs are shown on the left.

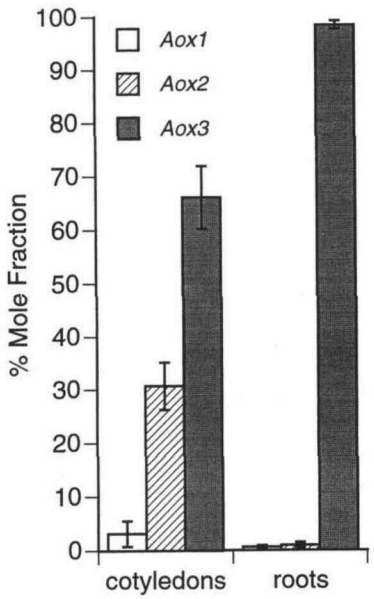

Figure 4. The relative abundance of transcripts from the three *Aox* genes in cotyledons and roots. Data are averages of three determinations with bars indicating SE.

assays for all three genes are performed using the products of a single reverse-transcription reaction.

When RNA isolated from cotyledons of 7-d-old light-grown seedlings was assayed, *Aox3* transcripts contributed 65% to the *Aox* transcript pool, whereas *Aox2* provided about 30% (Fig. 4), correlating with the detection of 36- and 34-kD proteins by immunoblotting of this tissue (Fig. 5). Transcripts from *Aox1* made up only 1 to 5% of the total pool. The *Aox* transcript pool in root RNA from the same plants, however, was almost exclusively derived from *Aox3* (Fig. 4), with *Aox1* and *Aox2* each contributing only 0.5 to 1% to the total. The preponderance of *Aox3* transcripts in root RNA confirms that the 36-kD protein found in root mitochondria is the mature AOX3 subunit, as was shown by protein sequencing to be the case in cotyledon mitochondria.

The relatively low abundance of *Aox1* transcripts in both cotyledons and roots explains the absence of an *Aox1* product on immunoblots of mitochondria from these tissues (Obenland et al., 1990; Kearns et al., 1992). However, through the course of our studies of AOX we have periodically observed a faint immunoreaction between the AOA antibody and a 32-kD protein in total mitochondrial proteins. This band is more readily detected when the signal from the AOA immunoreaction is increased by using SMPs instead of intact mitochondria as the source of target protein, in conjunction with a more sensitive chemiluminescence-based detection method (Fig. 5). When cotyledon SMPs were examined, a 32-kD species became clearly evident under these conditions (Fig. 5, lane 1), although at much lower intensities than AOX2 and AOX3. The 32-kD species had the same mobility during SDS-PAGE as the processed AOX1 observed in in vitro mitochondrial import experiments (Whelan et al., 1995a) and is therefore likely to be mature AOX1. However, we have been unable to establish this directly by N-terminal sequence analysis due to the low abundance of the protein. The single 36-kD band detected in root SMPs (Fig. 5, lane 2) is the same pattern that was reported previously (Obenland et al., 1990; Kearns et al., 1992). Thus, the protein species and amounts detected by immunoblotting with the AOA antibody generally correlate with the transcript levels of the individual *Aox* genes.

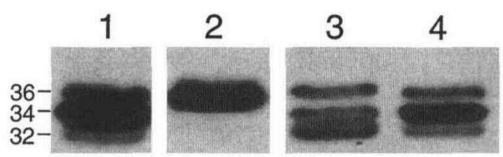

Figure 5. Cross-reactivity of AOA monoclonal antibody on immunoblots of soybean SMP proteins. Approximately 25 μg of SMP protein from light-grown cotyledons (lane 1) and roots (lane 2) or 50 μg of SMP protein from dark-grown cotyledons (lane 3) and dark-grown cotyledons subsequently exposed to 24 h of continuous light (lane 4) were separated by SDS-PAGE and immunoblotted with the AOA monoclonal antibody. The immunoreactions were detected by chemiluminescence. The apparent molecular masses of the immunodetected proteins are shown on the left.

Subunit Composition May Affect the Properties of AOX

The presence of different proportions of the three AOX subunits in root and cotyledon mitochondria raises the possibility that the properties of the enzyme may vary between these tissues. With NADH as substrate, the basal enzyme activity (i.e. in the absence of added pyruvate) in SMPs prepared from light-grown cotyledon mitochondria was about 3-fold higher than in SMPs prepared from root organelles (Table I). Pyruvate, an effector of AOX (Millar et al., 1993), stimulated the enzyme in both cotyledon and root SMPs but much more so in the latter (note that the preparation of SMPs removes any endogenous pyruvate in the mitochondria and prevents its formation from succinate). Despite the greater stimulation of the enzyme in root SMPs, the activity in the presence of pyruvate was only 75% of the stimulated activity in cotyledon SMPs. Moreover, 10-fold more pyruvate was required to reach half-maximal stimulation of AOX in root compared with cotyledon SMPs (Table I). These results may indicate that the AOX enzyme in cotyledons is less dependent on activators than on its root counterpart. However, differences were also found in the size and composition of the UQ pool between root and cotyledon mitochondria (Table I) when total UQ was extracted from mitochondrial membranes and quantified by HPLC. The UQ_{10} analog was by far the predominant species in each case, with detectable levels of UQ_9 found only in cotyledon mitochondria, where it represents 17% of the total UQ. No other UQ analogs were observed, which is consistent with other studies of soybean mitochondria from various tissues (Ribas-Carbo et al., 1995). In cotyledon mitochondria, total extractable UQ was found to be 2.5-fold higher than in root mitochondria, which may have influenced the pyruvate requirement for maximal activity. A more pronounced difference between root and cotyledon AOX was their different discrimination against $^{18}O_2$ (see table I, Robinson et al., 1992).

Table I. *A comparison of AOX properties in root and cotyledon SMPs from soybean*

O_2-consumption measurements and HPLC analysis of UQ were performed as described in "Materials and Methods." Data are means ± SE (n = 3).

Character	SMPs	
	Cotyledon	Root
Orientation (% inside out)	88 ± 2	84 ± 3
AOX activity (nmol O_2 min^{-1} mg^{-1} protein)		
Control	74 ± 5	26 ± 5
+5 mM pyruvate	137 ± 12	106 ± 24
$K_{½}$ stimulation (μM)	4.5 ± 0.9	51 ± 4
UQ (nmol mg^{-1} mg^{-1} protein)		
Total	4.2 ± 0.3	1.7 ± 0.2
UQ_{10}	3.5 ± 0.3	1.7 ± 0.2
UQ_9	0.7 ± 0.02	<0.05
$^{18}O_2$ discrimination[a] (‰)	31.2	25.6

[a] From Robinson et al. (1992), as determined in whole tissues.

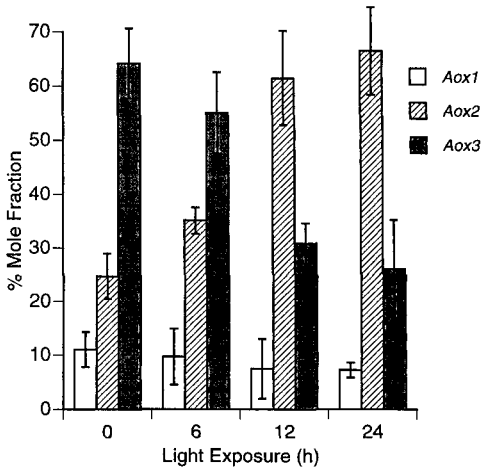

Figure 6. Response of the *Aox* transcript pool in dark-grown cotyledons to exposure to continuous light. Averages of three determinations are presented, with bars indicating SE.

Aox Gene Expression Patterns Change during the Greening of Cotyledons

The observation that the *Aox* genes are expressed in a tissue-dependent manner prompted us to investigate other situations in which *Aox* gene activity may change. Greening of etiolated shoots was investigated, because this transition represents a rapid alteration in cell physiology. Seeds germinated in the dark for 7 d were exposed to continuous light and cotyledons were harvested after 0, 6, 12, and 24 h of light exposure. Mitochondria were isolated from 0- and 24-h light-exposed tissue. The AOX activity in mitochondria from etiolated cotyledons, assayed in the presence of pyruvate with NADH as the substrate, was approximately 5-fold less on a mitochondrial protein basis than that typically found in mitochondria from 7-d-old light-grown seedlings. Upon exposure of the dark-grown seedlings to 24 h of light, cotyledon AOX activity approximately doubled. Although induced, the activity after 24 h of light was still only about 40% of that typically observed for cotyledon mitochondria from plants grown under the normal light cycle.

Immunoblotting of SMPs from etiolated cotyledons with the AOA monoclonal antibody (Fig. 5, lane 3) revealed three polypeptide bands with approximately equivalent intensities and apparent molecular sizes of 32, 34, and 36 kD, corresponding to AOX1, AOX2, and AOX3, respectively. In contrast, when SMPs from dark-grown cotyledons exposed to 24 h of light were examined (lane 4), the 34-kD AOX2 subunit was the predominant immunoreactive species.

The relative abundance of transcripts from the three *Aox* genes in dark-grown cotyledons exposed to continuous light was examined using the RT-PCR assay (Fig. 6). In dark-grown cotyledons, transcripts from *Aox3* predominated and were approximately 6- and 2.5-fold more abundant than those from *Aox1* and *Aox2*, respectively. The relative abundance of *Aox3* transcripts decreased and that

of *Aox2* increased during exposure to light. After 24 h of continuous light, the proportion of transcripts from these two genes was essentially reversed. During the period examined, the relative abundance of *Aox1* transcripts decreased slightly. The correlation between the increase in relative abundance of *Aox2* transcripts and the accumulation of the 34-kD protein during greening supports the conclusion that this polypeptide is encoded by *Aox2*.

DISCUSSION

The subunits of the AOX dimer are encoded by at least three genes in soybean (Whelan et al., 1996a). The *Aox1* gene encodes a 36.5-kD precursor protein (Whelan et al., 1993), which is cleaved to 32.2 kD upon import into isolated mitochondria (Whelan et al., 1995a). We have now characterized the two remaining known members of this multigene family and have demonstrated that both are actively transcribed and translated. The relatively steady-state transcript levels of the three *Aox* genes in cotyledon and root tissue were examined, as were the effects of greening on AOX activity, protein patterns, and relative transcript levels. These analyses, coupled with direct N-terminal protein sequencing, have allowed us to elucidate the basis of the tissue- and age-dependent polypeptide patterns that have been seen on immunoblots of cotyledon and root mitochondrial proteins probed with anti-AOX antibodies (Kearns et al., 1992).

Both *Aox2* and *Aox3* encode proteins that are processed, presumably upon import into mitochondria, to mature subunits of 31.8 and 31.6 kD, respectively. As yet, the basis for the discrepancy between the molecular masses deduced from the cDNA sequences and the apparent molecular masses determined by SDS-PAGE is unknown. The lack of overall sequence conservation at the N termini of the *Aox* primary translation products (Fig. 1) is typical of proteins requiring N-terminal signals for mitochondrial import. However, sequence comparisons have revealed conserved Arg residues at positions −2 or −10 relative to the cleavage site in a number of mitochondria-targeted plant proteins (Chaumont and Boutry, 1995). Alignment of the cleavage sites determined for AOX2, AOX3, and the *S. guttatum* subunit with that proposed for soybean AOX1 (Whelan et al., 1995a) demonstrates that an Arg residue at position −2 is highly conserved among plant AOX sequences (Fig. 7). Moreover, an Arg at position −10 is also found in the soybean AOX1 sequence. Also of note is the conservation of hydroxylated residues at positions +2 and +3 among the AOX proteins. Examination of the other plant AOX sequences revealed the presence of similar motifs in all cases (Fig. 7), allowing putative cleavage sites to be identified. Although an Arg at position −2 and hydroxylated residues at +2 and +3 are nearly universal in these sequences, the −10 Arg is found only in Arabidopsis AOX and soybean and tobacco AOX1 sequences and is not necessary for import of the soybean protein into isolated mitochondria (Whelan et al., 1996b). AOX subunits with Arg at position −10 from the presumed mitochondrial targeting signal cleavage site form a distinct sequence group (Fig. 2).

soybean AOX2	40	ANGWFFYWKRTM VSPAEA
soybean AOX3	38	GGAFGSFHLRRM STLPEV
S. guttatum	52	LWPPSWFSPPRH ASTLSA
soybean AOX1	30	GL**R**ALYGGGVRS ESTLAL
tobacco AOX1-a	58	WV**R**HFPVMGSRS AMSMAL
tobacco AOX1-b	2	WV**R**HFPVMGPRS ASTVAL
Arabidopsis	2	DT**R**APTIGGMRF ASTITL
mango	34	GLESAVMQWRRM LSNAGG
potato	48	AVWLVRFPLSRA ASTMAS

Figure 7. An alignment of the amino acid sequences around the mature N termini of AOX subunits. Double-underlined sequences represent those determined directly in this study for soybean AOX2 and AOX3 or those determined previously for *S. guttatum* AOX (Rhoads and McIntosh, 1991). Single-underlined sequences represent those deduced previously for soybean AOX1 (Whelan et al., 1995a) or postulated from published sequences (Hiser et al., 1996). Numbers refer to coordinates in the respective amino acid sequences. The Arg residues found at position −10 from the cleavage sites are in bold. The sources for the sequences are described in the legend to Figure 1.

Examination of the mature protein sequences confirms that AOX subunit sequences are highly conserved among plant species and across genera. As commented on previously (McIntosh, 1994), the conservation is especially striking in the two regions proposed to be membrane-spanning helices (Fig. 1, positions 178–208 and 238–268) and the intervening amphipathic helix (Fig. 1, positions 213–234). High levels of sequence conservation are also seen in four short regions (Fig. 1, positions 274–282, 287–295, 298–308, and 317–328), resembling a binding site for a binuclear iron center that may be involved in the transfer of electrons from reduced UQ to O_2 (Siedow et al., 1995). The AOX2 and AOX3 sequences conform to this model in every respect except for a replacement in AOX3 of a His residue, which is thought to be important for iron binding by an Ile residue (Fig. 1, position 279). A His at position 285 may compensate for this change, because a similar arrangement is found in AOX from *H. anomola*, a yeast.

Separate Cys residues have been implicated in the oxidative regulation of AOX activity (Umbach and Siedow, 1993) and in the stimulation of the enzyme by pyruvate (Umbach and Siedow, 1996). Two Cys residues are conserved in all plant AOX sequences examined to date, including AOX2 and AOX3 (at residues 128 and 178 in Fig. 1). The observation that AOX2 and AOX3 possess only these two Cys residues and that at least AOX3 responds to both redox control and pyruvate activation (Day et al., 1994) supports the suggestion that any involvement of Cys in AOX regulation would be mediated through these two residues (Umbach and Siedow, 1996).

Despite the general conservation among AOX sequences, the observed variation allows them to be grouped according to relatedness (Fig. 2). With the exception of mango, all of the reported plant AOX sequences are more similar to soybean AOX1 than to either AOX2 or AOX3. This is not

surprising, since, with the exception of the Arabidopsis sequence, which was isolated because of its ability to complement a heme biosynthesis mutation in *E. coli* (Kumar and Soll, 1992), all of the sequences in the AOX1 group were isolated from libraries by hybridization with the *S. guttatum* gene sequence or with sequences isolated using that probe. The high-stringency conditions typically used for screening precluded the isolation of sequences as divergent as the three soybean *Aox* sequences (Whelan et al., 1996a). These observations, together with the association of multiple proteins with AOX activity in most species and the finding of a second *Aox* gene in tobacco (Whelan et al., 1996a), strongly suggest that *Aox* multigene families are the rule rather than the exception in plants. As yet, there is no strong evidence that any plant species possesses only a single *Aox* gene.

The AOA monoclonal antibody recognizes one to three proteins on immunoblots of mitochondrial proteins from all plants examined. In mitochondria from soybean cotyledons, 34- and 36-kD proteins are detected. Direct N-terminal sequencing of proteins in partially purified AOX preparations has now demonstrated these proteins to be AOX2 and AOX3, respectively. The finding that the most abundant *Aox* transcripts in cotyledons are those from *Aox2* and *Aox3* indicates that protein levels are to a large degree a reflection of transcript levels. This conclusion is supported by the observation that upon exposure of etiolated cotyledons to light the relative abundance of the 34-kD protein and *Aox2* transcripts increase concurrently. However, the exact relationship between transcript and protein abundance cannot be assessed with confidence, because the relative reactivity of the monoclonal antibody to each of the three AOX subunits is unknown. The single 36-kD protein detected in root mitochondria is almost certainly AOX3, because *Aox3* transcripts are overwhelmingly the most abundant in this tissue.

The 32-kD protein seen in very low abundance in cotyledon mitochondria is presumably AOX1. Although scarce in all of the tissues examined, the relative proportions of *Aox1* transcripts in the total *Aox* transcript pool in root and cotyledon RNA correlated well with the relative intensity of the 32-kD band on immunoblots of SMPs from these tissues. This protein also corresponds in size to the import product when the AOX1 precursor protein was incubated with isolated mitochondria (Whelan et al., 1995a). AOX1 has probably eluded previous detection due to its low abundance in the tissues examined and was detected here because of two main advantages of the current method: (a) the use of SMPs removes interfering soluble proteins and allows more membrane protein to be separated by SDS-PAGE, and (b) chemiluminescence is more sensitive than alkaline phosphatase-coupled colorimetric detection systems. *Aox1* may be expressed at higher levels in soybean tissues other than roots or cotyledons.

Previous RT-PCR analysis of the steady-state transcript levels of *Aox1* and *Aox3* suggested that *Aox1* transcripts were more abundant than *Aox3* transcripts in cotyledons from 7-d-old seedlings (Whelan et al., 1996a). No information was available regarding *Aox2* transcripts. Although 7-d-old cotyledons were also used in the current study, *Aox3* transcripts were found to predominate. A possible explanation for this discrepancy may lie in the use of degenerate primers in the earlier study. Differences in annealing optima for the multitude of primer pairs involved may have led to differential amplification efficiencies for the target templates, and the effects of this on the estimation of relative transcript abundance are unknown. The present work circumvents this complication by estimating transcript abundance based on an internal standard template that has exactly the same priming sites as the target templates and therefore has a similar amplification efficiency. Moreover, the use of the same standard template to assay all three genes compensates for any differences in PCR efficiency due to the presence of the artificial intron sequence and any problems associated with the use of multiple standard templates.

The differential stimulation by pyruvate of AOX from roots and cotyledons suggests that the tissue-dependent expression of the three *Aox* genes leads to enzymes with different properties. Although differences in total UQ content may explain some of the variation in pyruvate stimulation (Ribas-Carbo et al., 1995), differences in the $K_{½}$ for stimulation suggest more fundamental differences between the AOX isoforms. The presence of different AOX isoforms in roots and etiolated cotyledons compared with green cotyledons may also explain the reported difference in O_2-isotope discrimination values for AOX between photosynthetic and nonphotosynthetic tissues (Robinson et al., 1992, 1995). Furthermore, the O_2 discrimination values determined for AOX during greening of etiolated soybean cotyledons were intermediate between the values for fully green and etiolated cotyledons (Robinson et al., 1995). This correlates with the changes in AOX protein complement we have observed during greening, where AOX2 levels increase upon exposure to light, whereas AOX1 and AOX3 levels stay the same. Since AOX2 transcripts and protein have been observed only in tissues exposed to light (this study; Kearns et al., 1992), it is possible that this isoform is responsible for the different O_2-isotope discrimination between photosynthetic and nonphotosynthetic tissues in soybean.

The presence of multiple AOX subunits in at least some tissues raises the possibility of subunit heterodimerism. When soybean cotyledon mitochondrial proteins are separated by SDS-PAGE under nonreducing conditions, three higher-mass bands with similar staining intensities are found (Umbach and Siedow, 1993). Two of the latter species have masses expected for homodimers of AOX2 or AOX3 subunits. The third species, however, has an intermediate molecular mass, which potentially represents AOX2/AOX3 heterodimers. In another study, monoclonal antibodies that cross-reacted with individual *S. guttatum* AOX subunits were able to immunoadsorb, under nonreducing conditions, all three subunits found in thermogenic tissues, with the retention of the nonspecific subunits being substoichiometric (Elthon et al., 1989). These observations are consistent with the adsorption of a mixture of homo- and heterodimers. Thus, heterodimerism may be a general

phenomenon of AOX in plants. Tissue-dependent expression of AOX subunits provides a potentially important mechanism for the coarse regulation of AOX activity in response to the multitude of metabolic environments experienced by mitochondria during plant growth and development.

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