The gene for alternative oxidase-2 (AOX2) from Arabidopsis thaliana consists of five exons unlike other AOX genes and is transcribed at an early stage during germination

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We investigated the expressions of genes for alternative oxidase (AOX1a, AOX1b, AOX1c and AOX2) and genes for cytochrome c oxidase (COX5b and COX6b) during germination of Arabidopsis thaliana, and examined oxygen uptakes of the alternative respiration and the cytochrome respiration in imbibed Arabidopsis seeds. A Northern blot analysis showed that AOX2 mRNA has already accumulated in dry seeds and subsequently decreased, whereas accumulation of AOX1a mRNA was less abundant from 0 hours to 48 hours after imbibition and then increased. The increase of the capacity of the alternative pathway appeared to be dependent on the expressions of both AOX2 and AOX1a. On the other hand, steady-state mRNA levels of COX5b and COX6b were gradually increased during germination, and the capacity of the cytochrome pathway was correlated with the increase of expressions of the COX genes. Antimycin A, the respiratory inhibitor, strongly increased the expression of AOX1a but had no effect on the expression of AOX2. A 5'RACE analysis showed that AOX2 consists of five exons, which is different from the case of most AOX genes identified so far. Analysis of subcellular localization of AOX2 using green fluorescent protein indicated that the AOX2 protein is imported into the mitochondria.

INTRODUCTION

In plant mitochondria, there are two ubiquinol-oxidizing respiratory pathways: the cyanide-sensitive, cytochrome pathway and the cyanide-insensitive, alternative pathway. The former pathway is coupled with ATP production and the terminal oxidase is cytochrome c oxidase (COX). The latter pathway is not coupled to ATP synthesis and O2 is converted to H2O in a one-step reaction by alternative oxidase (AOX), which is thought to function as a homodimeric protein (Umbach and Siedow, 1993, 1996; Vanlerberghe et al., 1998; Rhoads et al., 1998). AOX is encoded by a multigene family in the nuclear genome in several plants, including soybean (Finnegan et al., 1997), tobacco (Vanlerbergh and McIntosh, 1994; Whelan et al., 1995), rice (Ito et al., 1997) and Arabidopsis (Saisho et al., 1997). Expressions of AOX genes are induced by various biotic and abiotic stresses (Vanlerbergh and McIntosh, 1996, 1997), and the expressions are also influenced by the stage of development (reviewed by McIntosh, 1994; Day et al., 1995; Siedow and Umbach, 1995). For example, the regulation of soybean AOX genes has been shown to depend on the postgerminative development of soybean cotyledons (Finnegan et al., 1997; McCabe et al., 1998). The amount of AOX2 mRNA decreased during germination of soybean, whereas the amount of AOX3 mRNA, which was low in young cotyledons, gradually increased, and this subsequently increased the capacity for cyanide-insensitive respiration.

When plant seeds are imbibed, numerous metabolic activities are rapidly initiated. It is known that oxygen uptake and carbon dioxide release increase in seeds during imbibition (reviewed by Botha et al., 1992; Bewley, 1997). Ehrenshaft and Brambl (1990) showed that the cytochrome pathway was activated in germinating em-
bryos of maize, showing that the energy production during germination of seeds was dependent on the cytochrome electron transport and coupling oxidative phosphorylation in mitochondria. On the other hand, in some plants, cyanide-insensitive respiration is required for germination (reviewed by Botha et al., 1992). For example, in cocklebur, the alternative pathway was activated after imbibition of seeds (Esashi et al., 1981). So far, however, physiological mechanisms of coordinative regulation between the cytochrome respiration and the alternative respiration are not fully understood.

We previously reported that the Arabidopsis AOX is encoded by a multigene family, consisting of at least four copies (Saisho et al., 1997). In this study, to understand the regulation of expressions of AOX genes and COX genes during germination of Arabidopsis thaliana, we examined the expression patterns of AOX genes and the nuclear-encoded COX genes (COX5c and COX6b), and oxygen uptake by the alternative and the cytochrome respiratory pathways after imbibition of Arabidopsis seeds. We also analyzed the effect of treatment with a respiratory inhibitor, antimycin A on the expression of AOX genes during germination. Furthermore, we used a 5'RACE analysis of mRNA isolated from imbibed seeds to examine the exon/intron structure of the AOX2 gene.

**MATERIALS AND METHODS**

**Plant materials, growth conditions and chemical treatment.** Arabidopsis thaliana (L.) Heynh. ecotype Columbia gl1 was grown at 24°C under continuous light, whose intensity was 45 μE/m²/sec. For investigation of expression of AOX genes in early stage of development, seeds were imbibed with Murashige-Skoog (MS) medium with or without 5mg/l antimycin A (SIGMA, St. Louis, MO, USA).

**Oligonucleotides.** The following 12 oligonucleotides were synthesized:
P1: 5'-CGGTAGAGCCTATAAAGACGACA-3',
P2: 5'-TCCCTCTTCTACGGAGTTTTTCT-3',
P3: 5'-AAGTCCGATAGGATACCGGAC-3',
P4: 5'-CATCCTTTGCTGTTCTATTCCC-3',
P5: 5'-CAGGAAACAGATATGACCAT-3',
P6: 5'-CTTCCAGCTCAAGTACTGAGT-3',
P7: 5'-AGGAGCAGATTACGGAGA-3',
P8: 5'-TAGATGACATCTCC-3',
P9: 5'-GTGCCGGTTATCTCTACGGAG-3',
P10: 5'-TCAAGGCTTCTCCGTATTTCCC-3',
P11: 5'-GTGTCGACTATGAGTCAACTCA-3',
P12: 5'-GTCCATGGCCATCCACTCAAGTTAAATTTCC-3'.

**Probe labeling.** The fragments that corresponded to the 5' untranslated regions (5'UTRs) of AOX1a and AOX2 were amplified from genomic clones by PCR using primers P1–P2 and primers P3–4, respectively. Amplification of the fragments corresponded to the 5'UTRs of COX5b and COX6b by PCR was performed using cDNA clones and primers P5–6 and P5–7, respectively. The amplified fragments were labeled to be used as probes with the DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany).

**Extraction of total RNA and Northern hybridization.** Total RNA was extracted from imbibed seeds as described by Naito et al. (1988). Poly-A+ RNA was prepared using Oligotex-dT30 super (Takara Shuzo, Kyoto, Japan).

Total RNA was denatured by treatment with formaldehyde and fractionated in a 1% denaturing agarose gel. Subsequently, the gel was stained with ethidium bromide and blotted onto a nylon membrane (Magna Pure Nylon; Micron Separation, Westborough, MA, USA). Northern hybridization was performed with the DIG DNA Labeling and Detection Kit (Roche Diagnostics).

**Measurement of oxygen uptake of imbibed seeds.** Oxygen uptake by dry and imbibed seeds was measured using an oxygen electrode (Hansatech, Norfolk, UK) in 1 ml reaction buffer (50 mM HEPES, pH 7.2, 0.5 mM CaSO4; Ishii et al., 1977). The capacity of the alternative pathway was expressed as the rate of oxygen uptake in the presence of 2 mM KCN or 10 mM NaN3, which are inhibitors of the cytochrome respiration. The capacity of the cytochrome pathway was expressed as the rate of oxygen uptake in the presence of 5 mM salicylhydroxamic acid (SHAM), which is an inhibitor of the alternative respiration. The residual oxygen uptake (after addition of both SHAM and KCN/NaN3) was subtracted from all rates.

**5'RACE, cloning and sequence analysis.** 5' rapid amplification of cDNA end (5'RACE) was carried out with the 5'-Full RACE Core Set (Takara Shuzo) according to the manufacturer's instructions. The cDNA was synthesized from DNase-treated mRNA (0.5 μg) extracted from imbibed seeds of Arabidopsis using AMV reverse transcriptase XL (Takara Shuzo) and a 5'-end phosphorylated P8 primer at 50°C for 60 min. The cDNA was ligated and was concatenated using T4 RNA ligase (Takara Shuzo). PCR was performed with LA Taq DNA polymerase (Takara Shuzo) using primers P9 and P10 and the concatenated cDNA.

The 5'RACE-generated fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA), and were completely sequenced in the directions of sense and antisense strands with an automatic DNA sequencer (model 373S; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's inst-
the accumulation of AOX2 (nucleotide numbers 2868–3204 of accession number D89875), AOX1b (nucleotide numbers 120–394 of accession number D89875), AOX1c (nucleotide numbers 1999–2324 of accession number AB003175) and AOX2 (nucleotide numbers 886–1029 of accession number AB003176), we found that previous study, we did not detect 48 hours after imbibition, with maximum level at 12 hours, and subsequently decreased (Fig. 1A, -AA). In our previous study, we did not detect AOX2-specific transcripts in rosette leaves, stems, roots, and flowers and buds by Northern hybridization (Saisho et al., 1997). Therefore, it is suggested that AOX2 is mainly transcribed at an early stage during germination of Arabidopsis. On the other hand, accumulation of AOX1a mRNA was less abundant from 0 hours to 48 hours after imbibition, and then increased (at 72 hours and 96 hours after imbibition; Fig. 1B, -AA). By contrast, no signals specific to AOX1b and AOX1c were detected (data not shown).

To examine expression patterns of nuclear-encoded COX genes during germination, Northern blot analyses were performed using probes specific to COX5b and COX6b. Transcripts of COX5b and COX6b accumulated at a low level in dry seeds and gradually increased from 6 hours to 96 hours after imbibition (Fig. 2). These rapid increases of expression of COX genes may be responses to demands for energy production by oxidative phosphorylation for efficient germination of Arabidopsis.

Construction and visualization of a GFP fusion protein. To study the subcellular localization of AOX2, a recombinant plasmid, named AOX2pre-GFP, was constructed as follows: The sequence corresponding to the predicted precursor protein (residues 1–72) was amplified by PCR from the cDNA clones using primers P11 and P12. Because the primers P11 and P12 contained an Sall site and an Ncol site near their 5′-ends, respectively, the PCR-amplified fragment was digested with both Sall and Ncol and doned in-frame into the Sall and Ncol sites of the CaMV35S-sGFP(S65T)-NOS vector (Chiu et al., 1996), kindly provided by Dr. Y. Niwa, University of Shizuoka. The AOX2pre-GFP plasmid is shown in Fig. 5. Its sequence was checked before proceeding.

Tobacco (Nicotiana tabacum) BY-2 cells were cultured by the method of Nagata et al. (1981). Cells cultured for 4 or 5 days were transformed by particle bombardment. The bombardment was carried out as described previously (Nakazono et al., 2000). Tobacco BY-2 cells were cultured for 36 hours after transformation in the dark. The transformed cells were then observed at × 40 magnification with a confocal laser scanning microscopy as described by Arimura et al. (1999). To visualize localization of mitochondria in tobacco BY-2 cells, the cells were treated with 500 nM MitoTracker Orange CM-H$_2$TMRos (Molecular Probes, Eugene, OR, USA), a mitochondrial-specific dye, for 30 min.

RESULTS AND DISCUSSION

Expressions of nuclear-encoded AOX genes and COX genes during germination of Arabidopsis thaliana. To understand regulation of expressions of AOX genes and COX genes during germination of Arabidopsis thaliana, Northern hybridization was performed using total RNAs extracted from dry seeds and germinating seeds imbibed for 6, 12, 18, 24, 36, 48, 72 and 96 hours. Under our conditions, the radicles emerged between 72 and 96 hours after imbibition. Using probes specific to individual AOX genes [AOX1a (nucleotide numbers 2868–3204 of accession number D89875), AOX1b (nucleotide numbers 120–394 of accession number D89875), AOX1c (nucleotide numbers 1999–2324 of accession number AB003175) and AOX2 (nucleotide numbers 886–1029 of accession number AB003176)], we found that AOX2 mRNA was already accumulated in dry seeds, and the accumulation of AOX2 transcript was maintained to 48 hours after imbibition, with maximum level at 12 hours, and subsequently decreased (Fig. 1A, -AA). In our previous study, we did not detect AOX2-specific transcripts in rosette leaves, stems, roots, and flowers and buds by Northern hybridization (Saisho et al., 1997). Therefore, it is suggested that AOX2 is mainly transcribed at an early stage during germination of Arabidopsis. On the other hand, accumulation of AOX1a mRNA was less abundant from 0 hours to 48 hours after imbibition, and then increased (at 72 hours and 96 hours after imbibition; Fig. 1B, -AA). By contrast, no signals specific to AOX1b and AOX1c were detected (data not shown).

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Capacities of the alternative respiration and the cytochrome respiration in germinating seeds. To clarify whether the expression patterns of AOX genes and COX genes correspond to the respective capacities of the alternative respiration and the cytochrome respiration during germination, we measured oxygen uptake in dry seeds and germinating seeds at 12, 36 and 72 hours after imbibition (Fig. 3). It was revealed that the total respiration rate of dry seeds was extremely low, whereas during germination, both of the alternative respiration and the cytochrome respiration gradually increased (Fig. 3). Oxygen uptakes via the cytochrome respiration at 12, 36 and 72 hours after imbibition were three times, nine times and seven times higher than oxygen uptakes via the alternative respiration at each time point, respectively (Fig. 3).

Although steady-state level of AOX2 mRNA was higher at 12 hours after imbibition than at 36 hours (Fig. 1A, -AA), the capacity of the alternative pathway remained about the same between 12 hours and 36 hours after imbibition. Because AOX1a is expressed at a low level at 12 hours and 36 hours after imbibition, AOX2 protein may mainly contribute to the cyanide-insensitive respiration at 12 hours and 36 hours. The capacity of the alternative respiration at 72 hours was approximately twice the capacities at 12 hours and 36 hours (Fig. 3), and the increase of capacity of the alternative respiration corresponded to the increase of steady-state mRNA level of AOX1a at 72 hours after imbibition (Fig. 1B, -AA).

Simultaneously, oxygen uptake by the cytochrome pathway was measured in the presence of the alternative respiration inhibitor, SHAM. The capacity of the cytochrome pathway gradually increased along with the increase of steady-state mRNA levels of COX5b and COX6b (Fig. 3). In maize embryos, it is known that synthesis and activity of the cytochrome respiratory pathway are rapidly constructed during germination (Ehrenshaft and...
Fig. 1. Analysis of the steady-state levels of AOX2 (A) and AOX1a (B) transcripts during germination. Arabidopsis seeds were imbibed on MS medium without (-AA) or with 5 mg/l antimycin A (+AA) for 0, 6, 12, 24, 36, 48, 72 and 96 hours. Total RNA extracted from seeds imbibed in each time period, and Northern blot hybridization was performed using AOX2-specific and AOX1a-specific probes.

Brambl, 1990). The expressions of the COX genes and oxygen uptake via the cytochrome respiration in Arabidopsis imbibed seeds in the present study seem to be consistent with these previous results.

**AOX1a, but not AOX2, responds to treatments with antimycin A during germination.** It is known that addition of antimycin A, which inhibits electron transfer at complex III in mitochondria, enhances the synthesis of AOX protein and/or cyanide-insensitive respiration in some species (Vanlerberghe and McIntosh, 1992; Wagner and Wagner, 1997; Finnegan et al., 1998). We previously reported that expression of AOX1a was induced by treatments with antimycin A in 2-week-old Arabidopsis seedlings, and that none of expressions of other copies (AOX1b, AOX1c and AOX2) responded to the antimycin A-treatment in seedlings (Saisho et al., 1997). However, it was possible that no responses of expressions of AOX1b,
Arabidopsis alternative oxidase-2 gene

Fig. 2. Analysis of the steady-state levels of COX5b and COX6b transcripts during germination. Arabidopsis seeds were imbibed on MS medium for 0, 6, 12, 24, 36, 48, 72 and 96 hours. Total RNA extracted from seeds imbibed in each time period, and Northern blot hybridization was performed using COX5b-specific and COX6b-specific probes.

AOX1c and AOX2 genes to treatment with antimycin A were due to extremely low levels of their transcripts, and therefore we might not be able to detect the increased levels of their mRNAs even if their expressions could respond to stimulation by antimycin A. During germination of Arabidopsis, we detected an AOX2 mRNA signal by Northern hybridization. We expected that this signal could be better understood if it was affected by treating the seeds with antimycin A. As shown in Figure 1B (+AA), the amounts of AOX1a mRNA were dramatically increased by addition of antimycin A during germination. By contrast, the steady-state levels of AOX2 mRNA were the same between treatments with antimycin A and without antimycin A (Fig. 1A, +AA). These results show that, in Arabidopsis, expression of AOX2 hardly responds to stimulation by antimycin A.
AOX2 gene consists of five exons. The Northern hybridization using total RNA extracted from imbibed Arabidopsis seeds (Fig. 1A), shows that the molecular size of the detected AOX2 mRNA did not correspond to the expected size. Therefore, we examined 5'-terminus of AOX2 mRNA using poly-A+ RNA extracted from seeds

![Diagram AOX2](image1)

NH₂-Met-Ser-Gln-Leu-Ile-Thr-Lys-Ala-Ala-Leu-Arg-Val-Leu-Leu-
Val-Cys-Gly-Arg-Gly-Asn-Cys-Asn-Met-Phe-Val-Ser-Ser-Val-Ser-
Ser-Thr-Ser-Val-Met-Lys-Ser-Pro-Tyr-Glu-Ile-Thr-Ala-Pro-Met-
Arg-Ile-His-Asp-Trp-Cys-Gly-Phe-Gly-Asp-Phe-Lys-Ile-Gly-Ser-
Lys-His-Val-Gln-Gly-Asn-Phe-Asn-Leu-Arg-Trp-Met-Gly-Met-Ser-Ser-Ala----

Fig. 4. (A) Comparison of structure of AOX2 with those of AOX1a, AOX1b and AOX1c. Boxes indicate exons of AOX1a, AOX1b and AOX1c genes. The filled boxes are the coding region reported previously, and the shaded boxes are the coding region identified in this study. (B) Deduced N-terminal amino acid sequence of AOX2 with the filled boxes the coding region reported previously, and the shaded boxes the coding region identified in this study. The N-terminal region can form an amphiphilic helix. The 1st residue to the 18th residue, which gives the maximal hydrophobic moment, are plotted on a helical wheel. + indicates positively charged residues. Black box indicates hydrophobic residues. The sequence identified in this study is shown in bold.
Fig. 5. Subcellular localization of GFP fusion protein in tobacco suspension-cultured BY-2 cells. (A) Structure of the recombinant AOX2pre-GFP plasmid. The sequences corresponding to the predicted precursor protein (residues 1–72) were fused in-frame with the synthetic green fluorescent protein (sGFP; Chiu et al., 1996). CaMV 35S, cauliflower mosaic virus 35S promoter. NOS, nopaline synthase terminator. (B) Transient expression of recombinant AOX2pre-GFP plasmid in tobacco BY-2 cells. The plasmid was transformed into BY-2 cells by particle bombardment (PDS-1000: BIO-RAD). The fluorescent images were taken with excitation at 488 nm and emission at 530 nm for detection of GFP (upper panel) and at over 665 nm for detection of mitochondria (middle panel). Lower panel shows the merged GFP and MitoTracker fluorescent images.
imibed for 12 hours by the 5’RACE method. As a result, it was revealed that the 5’-terminus of AOX2 transcript is extended and the Arabidopsis AOX2 gene contains one extra exon and one extra intron in the upstream region. This finding indicates that the AOX2 gene consists of five exons and four introns (Fig. 4A). In our previous study (Saisho et al., 1997), we did not find an additional exon in the AOX2 gene. This might have been because AOX2 expression is very low in the roots of 8-week-old seedlings. Therefore, it seems likely that the fragment amplified by the previous 5’RACE method was derived from immature AOX2 mRNA.

AOX genes consist of four exons and three introns in most plants identified so far (Rhoads and McIntosh, 1993; Finnegan et al., 1997; Ito et al., 1997; Saisho et al., 1997). The position of each intron is the same among most AOX genes that have so far been identified. To our knowledge, there are only two exceptions, the AOX1b gene of rice (Ito et al., 1997) and the AOX2 gene of Arabidopsis (this report). The rice AOX1b gene has three exons because exon 2 of rice AOX1b contains regions corresponding to both exons 2 and 3 of most AOX genes (Ito et al., 1997). In contrast, exons 1 and 2 of Arabidopsis AOX2 are separated by an intron in the region that corresponds to the first exon of most AOX genes including Arabidopsis AOX1a, AOX1b and AOX1c (Fig. 4A). This is the first report of an AOX gene that contains five exons and four introns.

**Intracellular localization of the AOX2 protein.** We previously suggested that the AOX2 protein lacks a typical mitochondrial targeting signal (Saisho et al., 1997). However, it is possible that the N-terminal extended sequence, which was identified in this study, functions as a mitochondrial targeting signal. To examine this possibility, we constructed an AOX2pre-synthetic green fluorescent protein (GFP) fusion protein. GFP has been shown to be useful as a vital marker for the analysis of trafficking and subcellular localization of proteins (Chiu et al., 1996; Köhler et al., 1997; Arimura et al., 1999). The plasmid, termed AOX2pre-GFP, was constructed by fusing codons (coding for amino acid residues 1–72) of the predicted AOX2 precursor protein to the coding sequence of GFP (Fig. 5A). The plasmid was introduced into tobacco suspension-cultured BY-2 cells by particle bombardment. After 36 hours of transformation, we observed the cells using confocal laser scanning microscopy (Fig. 5B). Many fluorescent particles, indicating the presence of AOX2pre-GFP recombinant proteins, were found in the transformed cells (Fig. 5B, top panel; GFP). The locations of mitochondria in the cells were visualized by staining with the mitochondrial-specific dye, MitoTracker Orange (Fig. 5B, middle panel; MitoTracker). The GFP fluorescence corresponded to the staining pattern observed using the mitochondrial-specific dye MitoTracker.


Rhoads, D. M., and 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


