AOX1c, a novel rice gene for alternative oxidase; comparison with rice AOX1a and AOX1b

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A novel gene for alternative oxidase (AOX) was isolated from rice (Oryza sativa L.) and characterized. The deduced amino acid sequence of the novel AOX gene contains features that are conserved among other AOXs. This AOX gene was designated AOX1c based on a phylogenetic analysis of the AOX genes. Northern hybridization analyses revealed that AOX1c and AOX1a/AOX1b transcripts accumulated differently in various rice organs and rice seedlings under low temperature conditions. AOX1c mRNA was mainly present in young leaves under constant light, mature leaves and panicles after heading, but it was not detected in young etiolated leaves and young roots of seedlings or young panicles. On the other hand, the mRNAs of the rice AOX1a and AOX1b genes were mainly present in young roots and mature leaves. Under low temperature conditions, the steady-state mRNA levels of the rice AOX1a and AOX1b genes clearly increased with time but the rice AOX1c gene was apparently not responsive to low temperature. The rice AOX gene family and differences in their regulation are discussed.

INTRODUCTION

Higher plant mitochondria have two respiratory electron transport pathways; one is a cyanide-sensitive cytochrome pathway and the other is a cyanide-insensitive alternative pathway. The cytochrome pathway is coupled with ATP synthesis, whereas the alternative pathway is uncoupled (reviewed by Day et al., 1995; Moore et al., 1995; Vanlerberghe and McIntosh, 1997; Siedow and Umbach, 2000).

The physiological function of the alternative pathway is well characterized in thermogenic plants. In the flowers or inflorescences of these plants, heat produced by way of electron flow through the alternative pathway is used to volatilize insect attractants, facilitating pollination (Meeuse and Raskin, 1988). As for non-thermogenic plants, some hypotheses have been proposed for the function(s) of the alternative pathway (Bowsher and Tobin, 2001). In cultured tobacco cells overexpressing alternative oxidase (AOX), the terminal oxidase in the alternative pathway, the production of reactive oxygen species was significantly lowered (Maxwell et al., 1999). This suggests that the alternative pathway plays a role in protecting against oxidative stress in plants. In some plant species, it has been proposed that the alternative pathway allows the TCA cycle to continue to operate under conditions where the cytochrome pathway has become limiting, thus allowing replenishment of TCA cycle intermediates that have been directed into biosynthetic pathways (reviewed by Mackenzie and McIntosh, 1999). Expression of the AOX genes is upregulated under environmental stresses such as lower temperature (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992; Ito et al., 1997) and wounding (Hiser and McIntosh, 1990). On the other hand, increases in AOX mRNA or protein have been detected during pollen development in maize (Wen and Chase, 1999), rice (Abe et al., 1997) and common bean (Johns et al., 1993). The detailed functions of the alternative pathway or AOX remain unclear.

The AOX proteins are encoded by a multigene family in several plants such as tobacco (Vanlerberghe and McIntosh, 1994; Whelan et al., 1995), soybean (Finnegan et al., 1997; McCabe et al., 1998), Arabidopsis thaliana (Saisho et al., 1997) and mango (Considine et al., 2001). These AOX genes have been reported to be differentially expressed. The AOX genes in soybean seedlings are differentially expressed depending on light (Finnegan et al., 1997) and the developmental stage (McCabe et al., 1998). Treatment of Arabidopsis with antimycin A, an inhibitor of the cytochrome pathway, greatly increases the AOX1a mRNA level, whereas it does not affect the transcript levels of other AOX genes (Saisho et al., 1997). In mango, each AOX gene is differentially...
expressed during fruit ripening (Considine et al., 2001). These findings raise the possibility that the AOXs have different roles.

Previously, we identified two rice AOX genes, AOX1a and AOX1b, whose transcript levels increased under low temperature conditions (Ito et al., 1997). Genomic Southern hybridization analysis suggested that there are additional AOX genes in the rice genome (Abe et al., 1997; Ito et al., 1997). In this report, we describe a novel rice AOX gene, AOX1c, and analyzed its mRNA level in various rice organs and rice seedlings under low temperature conditions.

MATERIALS AND METHODS

Plant materials, growth conditions and treatments For extraction of total RNA, rice (Oryza sativa L. cv. Nipponbare) was grown under light or dark conditions at 28°C for eleven days, and the leaves and the roots of seedlings were collected separately. Mature leaf blades, mature leaf sheaths and young panicles were prepared from 3-month-old plants, and panicles after heading were prepared from 3.5-month-old plants.

To determine the effect of low temperature on gene expression, 5-day-old rice seedlings grown in constant light at 28°C were transferred to 4°C in darkness. The control plants were transferred to darkness at 28°C. Rice leaves were collected just before the treatment started (0 hour), and at 24 and 48 hours after the treatment started.

Oligonucleotides used in PCR or RT-PCR Twelve oligonucleotides were synthesized:

- **P1**: 5’-CTGAGCAGCTCGCTGATGTT-3’
- **P2**: 5’-GTTTCGACCTCCTGATGTT-3’
- **P3**: 5’-GATGGTCTGCTCAGGCAGT-3’
- **P4**: 5’-ATGTGACTATATAATCTGACTGCTGC-3’
- **P5**: 5’-TCATCATTCTCAACGGCAGTCG-3’
- **P6**: 5’-TGTCAGCTCAGGATCCAGGCAGCAGC-3’
- **P7**: 5’-CTGAGAAAATCTTACGGCAGG-3’
- **P8**: 5’-CCAACAGATAACAGGACGCAGC-3’
- **P9**: 5’-ATCAGACGCAGCAGCAGTC-3’
- **P10**: 5’-AAGACGACGACGCGTCTCTGAGC-3’
- **P11**: 5’-GCGCGCTAGATTTCTTCAGC-3’
- **P12**: 5’-AACAAGCGCGACTGCTGAC-3’

where V is equal to A, C or G, R is equal to A or G, N is equal to A, C, G or T, K is equal to G or T, and Y is equal to C or T.

Probe labeling Probes were made by PCR following the method of Ito et al. (1997) using a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) following the supplier’s instructions. Primers P1 and P2 were used to obtain a probe for the conserved region of rice AOX genes, P3 and P4 were used to obtain a probe specific for AOX1a, P5 and P6 were used to obtain a probe specific for AOX1b, and P7 and P8 were used to obtain a probe specific for AOX1c. A genomic clone that contains the rice AOX1c gene was used as the DNA template (see below).

Screening of the genomic library of rice A genomic DNA library was constructed with the Lambda FIX II vector (Stratagene, La Jolla, CA, USA) following the supplier’s instructions. The library was screened by plaque hybridization using the conserved AOX probe and the rice AOX1a- and AOX1b-specific probes (see above). In order to obtain novel rice AOX-related sequences, we selected plaques that hybridized to the conserved probe but not to the AOX1a probe or AOX1b probe. Signals were detected with the DIG DNA Labeling and Detection Kit according to the manufacturer’s instructions.

Cloning and sequence analysis Isolated genomic clones were subcloned into a pBlueScript SK+ vector. Nucleotide sequences of the inserts were determined by the shotgun sequencing method described in Kubo et al. (2000) using an automatic DNA sequencer (model 373S, Perkin Elmer ABD, Foster City, CA, USA). DNA sequencing data were analyzed with GENETYX Software (Software Development, Tokyo, Japan).

Extraction of total RNA and Northern hybridization Total RNA was extracted by the method of Kawakami and Watanabe (1988). Northern hybridization was performed as described previously (Ohtsu et al., 1999).

RT-PCR cDNA was synthesized from total RNA extracted from panicles after heading with Superscript II RNase H’ Reverse Transcriptase (GIBCO BRL, Rockville, MD, USA). To obtain cDNA fragments containing the whole rice AOX1c coding region, the first PCR following the reverse transcription was done with primers P9 and P12. The second PCR was done with P10 and P11.

RESULTS AND DISCUSSION

A novel rice AOX gene Screening the rice genomic library yielded three clones that contained novel AOX-related sequences. The nucleotide sequences of the inserts, when compared with the sequences of previously identified AOX genes, suggested that only one clone contained a complete AOX coding region. The other two clones appeared to contain partial AOX sequences (data not shown). The sequence of the novel AOX gene suggested that it was divided into four exons (Fig. 1A). To test this, we used RT-PCR using primers P9 and P12 and primers P10 and P11 (Fig. 1B) to amplify the cDNA frag-
A novel rice AOX gene containing the whole coding region of the novel AOX gene. Total RNA extracted from rice panicles after heading was used as a template. A cDNA fragment of about 1.1 kb was amplified and sequenced (data not shown). Three introns were identified in the novel AOX gene by comparing its sequence with the genomic

Fig. 1. (A) Physical map of the region in the rice genome that includes the novel AOX coding region. The filled boxes indicate the coding regions. Arrows indicate the transcriptional directions. Restriction sites are indicated for SalI (S), DraI (D), PstI (P) and EcoRI (E). Underline with an asterisk indicates the position of the probe used in the Northern hybridization analysis. (B) Nucleotide and deduced amino acid sequences of the novel AOX gene. Nucleotide sequences in lower case letters indicate introns. Arrows indicate the positions and directions of primers used in producing a gene specific probe (P7 and P8) and in RT-PCR (P9 to P12).
sequence (Fig. 1). In this respect, it resembled most of the other AOX genes.

A phylogenetic tree based on the amino acid sequences of the various AOX proteins revealed that the novel AOX is highly homologous to rice AOX1a in the AOX1-group (Fig. 2). Therefore, the novel AOX was named rice AOX1c.

The cysteine residue at position 118 was conserved in the deduced amino acid sequence of AOX1c (Fig. 3). This cysteine residue is involved in dimerization of the AOX protein via an S-S bond and in activation of the AOX protein by α-keto acids (Rhoads et al., 1998; Vanlerberghe et al., 1998). Four helical regions including two iron-binding motifs that have been assumed to be involved in formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund, 1999; Siedow and Umbach, 2000; Berthold et al., 2000) were observed (Fig. 3). Two helices that are proposed to form a membrane-binding domain (Andersson and Nordlund, 1999; Siedow and Umbach, 2000; Berthold et al., 2000) were also observed (Fig. 3). In other AOX genes, these domains allow the enzyme to act as a ubiquinol oxidase in mitochondria, and so AOX1c may have a similar enzymatic ability.

The result of genomic Southern hybridization analysis using the conserved AOX probe suggests that there are AOX-related sequences other than the AOX1a and AOX1b genes in the rice genome (Ito et al., 1997). The three clones obtained in this study are likely to correspond to these AOX-related sequences. As described above, two of the clones contained only partial AOX sequences (data not shown). Furthermore, one of these AOX-related sequences contained an internal stop codon in the predicted coding region as a result of a point mutation (data not shown), suggesting that the sequence represents a

![Fig. 2. Phylogenetic tree of 17 deduced AOX proteins from plants, fungi and Trypanosoma constructed by the neighbor-joining method (Saitou and Nei, 1987). Branches are drawn proportional to genetic distances. References to published sequences are as follows: rice AOX1a and AOX1b (Ito et al., 1997), A. thaliana AOX1a, AOX1b, AOX1c and AOX2 (Saisho et al., 1997; Saisho et al., 2001), soybean AOX1, AOX2 and AOX3 (Whelan et al., 1993; Finnegan et al., 1997), S. guttatum AOX (Rhoads and McIntosh, 1991), tobacco AOX1a (Vanlerberghe and McIntosh, 1994) and AOX1b (Whelan et al., 1995), mango AOX (Cruz-Hernández and Gómez-Lim, 1995; Considine et al., 2001), P. anomala AOX (Sakajo et al., 1991), N. crassa AOX (Li et al., 1996) and T. brucei AOX (Chaudhuri and Hill, 1996).]
A novel rice AOX gene pseudogene of AOX. Other AOX genes (AOX2 or AOX3) have been identified in soybean (Finnegan et al., 1997), mango (Cruz-Hernández and Gómez-Lim, 1995; Considine et al., 2001) and Arabidopsis AOX1a (AtAOX1a; Saisho et al., 1997) and S. guttatum AOX (SgAOX; Rhoads and McIntosh, 1991). The alignments were generated by the CLUSTAL W algorithm (Tompson et al., 1994). Black boxes represent identical amino acids. Open circle above position 118 of OsAOX1c indicates the cysteine residue that is involved in dimerization of the AOX protein via an S-S bond. This residue is involved in activation by α-keto acids (Rhoads et al., 1998; Vanlerberghe et al., 1998). Lines above the amino acid sequences indicate helical regions that are assumed to be involved in the formation of a hydroxo-bridged binuclear iron center (Andersson and Nordlund, 1999; Siedow and Umbach, 2000; Berthold et al., 2000). Arrowheads indicate an iron-binding motif. Two-headed arrows above the amino acid sequences indicate possible membrane-binding domains (Andersson and Nordlund, 1999; Siedow and Umbach, 2000; Berthold et al., 2000).

Steady-state mRNA levels of AOX genes AOX1c transcript levels were found to be high in leaves of light-grown 11-day-old seedlings and leaf blades of 3-month-old plants, but somewhat lower in panicles after heading (Fig. 4A). On the other hand, relatively-high mRNA levels of AOX1a were observed in the roots of light-grown and dark-grown seedlings and in leaf blades and leaf sheaths of mature plants (Fig. 4A). The locations and levels of mRNA of the rice AOX1b gene were almost the same as those of the rice AOX1a gene (data not shown). These differences, especially in the seedlings and the flowers, suggest that the expression of the rice AOX1c and AOX1a/AOX1b genes is differentially regulated among the different organs of rice.

In 11-day-old seedlings, the AOX1c transcript was detected only in leaves that had been under constant light (i.e., not in etiolated leaves) (Fig. 4A). There is some evidence that the expression of AOX1c and the activity of the
Fig. 4. Northern hybridization analyses showing steady-state mRNA levels of rice AOX1c and AOX1a genes in various organs (A) and after exposure to 4°C (cold) for up to 48 h (B). The 3’UTR of the rice AOX1a gene was used as the probe for AOX1a as described in Ito et al. (1997). The position of the probe for the rice AOX1c gene is described in Figure 1A. 17 and 5 µg of total RNA per lane were used in the assays for the rice AOX1c and AOX1a gene, respectively. Equal loading of total RNA was checked by ethidium bromide (EtBr) staining. The larger AOX1a band (2.9 kb) in (B) is thought to be the immature transcript of the gene (Ito et al., 1997).
alternative pathway are regulated by light. In soybean, the amounts of AOX2 mRNA and AOX2 protein increased when dark-grown cotyledons were exposed to continuous light (Finnegan et al., 1997). In potato leaves, AOX protein clearly accumulated in the panicles after heading; however, no AOX protein was detected in leaves (Fig. 4B). On the other hand, the AOX1c protein may play a similar role in green leaves. A similar increase in AOX1c expression with time was observed with the soybean AOX1c protein (McCabe et al., 1998).

It is interesting that the mRNA of rice AOX1c was clearly present in the panicles after heading; however, no signals were detected in the total RNA of the young panicles (Fig. 4A). This suggests that rice AOX1c is mainly expressed at the late stage in rice flower development.

**Effect of low temperature on steady-state mRNA level of AOX1c** After 48 hours of exposure to low temperature, the AOX1c mRNA signal was almost undetectable in the leaves (Fig. 4B). On the other hand, the steady-state mRNA level of rice AOX1a increased with time, as was found previously (Ito et al., 1997). Therefore, the expressions of the AOX1c and AOX1a/AXO1b genes appear to be differentially regulated in rice under low temperature conditions (in darkness). As mentioned above, other AOX genes from soybean, Arabidopsis and mango are also differentially expressed under different conditions or developmental stages (see Introduction).

In conclusion, the novel AOX gene, AOX1c in rice has common features with the other AOX genes, but the regulation of its expression appears to be different from that of the rice AOX1a and AOX1b genes.

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