Characterization of two non-homoeologous nuclear genes encoding mitochondrial alternative oxidase in common wheat

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(Received 7 February 2002, accepted 15 March 2002)

Mitochondrial alternative oxidase (AOX) is the terminal oxidase responsible for cyanide-insensitive and salicylhydroxamic acid-sensitive respiration. We have isolated two non-homoeologous genes (*Waox1a* and *Waox1c*) encoding AOX proteins from common wheat (*Triticum aestivum* L.). These two genes were orthologous to rice *AOX1a* and *AOX1c*, and their exon/intron structure was conserved, as it is in most other plant AOX genes. Southern blot analysis indicated that both *Waox1a* and *Waox1c* were located in at least three homoeologous loci and that additional AOX genes with lower homology were present in the genome of common wheat. The *Waox1a* and *Waox1c* loci were respectively assigned to the homoeologous group 2 and 6 chromosomes. The steady-state level of *Waox1a* and *Waox1c* transcripts increased under cold stress, while only that of *Waox1a* was increased by cyanide treatment.

INTRODUCTION

In plant mitochondria, there exists a unique respiratory pathway called the cyanide-insensitive and salicylhydroxamic acid (SHAM)-sensitive alternative pathway, besides the main cyanide-sensitive cytochrome pathway (Henry and Nyns, 1975). The alternative pathway is a non-phosphorylating electron transport pathway branching from the cytochrome pathway at the ubiquinone pool, and electron flow through the pathway reduces oxygen to water without conservation of energy in the form of ATP (Lambers, 1982; Siedow, 1982). Because no proton motive force develops in this pathway, all the free energy is released as heat (Moore et al., 1978). It has been reported that the activity of the alternative pathway was enhanced or changed under various conditions including environmental and developmental changes (McIntosh, 1994). When plants are placed under low temperature conditions, the capacity of the alternative pathway increases (Vanlerberghe and McIntosh, 1992a; Purvis and Shewfelt, 1993). There have been reports describing transition or shift of the respiratory pathways during seed germination (Wilson and Bonner, 1971; Yentur and Leopold, 1976; Burguillo and Nicolas, 1977). Other

reports suggested that the earliest stage of germination might entirely depend on the activity of the alternative pathway (Poljakoff-Mayber and Evenari, 1958; Wilson and Bonner, 1971). In wheat, the initial growth of germinating seedlings depends essentially on the alternative pathway (Kasai et al., 1998a). The alternative pathway was also suggested to work as an antioxidative defense system in durum wheat when the photorespiratory cycle is highly active, as in the case of exposure to environmental stress (Pastore et al., 2001).

Alternative oxidase (AOX) or ubiquinol oxidase is the terminal oxidase of the cyanide-insensitive alternative pathway, and the AOX proteins are encoded by nuclear genes. Through the alternative pathway electrons from reduced ubiquinone are transferred to oxygen directly by AOX. This reaction consists of a single step that is solely mediated by AOX (Kumar and Söll, 1992; Cruz-Hernández and Gómez-Lim, 1995). cDNA clones of the AOX genes have been isolated from plant species such as *Arabidopsis thaliana* (Kumar and Söll, 1992; Saisho et al., 1997; Saisho et al., 2001), tobacco (Vanlerberghe and McIntosh, 1994; Whelan et al., 1995), mango (Cruz-Hernández and Gómez-Lim, 1995), soybean (Whelan et al., 1993; Wagner and Krab, 1995; Finnegan et al., 1997), potato (Hiser et al., 1996) and rice (Ito et al., 1997; Saika et al., 2002). In soybean, *Arabidopsis* and rice, the AOX proteins are encoded by multigene families. Ito et al.

Edited by Yoshio Sano

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(1997) reported the presence of tandemly arranged AOX genes and demonstrated that steady-state levels of mRNA from both AOX genes increased under low temperature conditions. They concluded that the enhancement of the capacity of the alternative pathway in higher plants by low temperature can be partly explained by the enhanced expression of the AOX genes at the transcriptional level.

A number of alloplasmic lines have been produced and used as valuable experimental materials for studying cytoplasmic diversity and nucleus-cytoplasm interactions in *Triticum* and *Aegilops* species (reviewed by Tsunewaki, 1993). Based on studies using these alloplasmic lines, it was suggested that the AOX gene might be one of the nuclear-encoded genes playing important roles in nucleus-cytoplasm interactions in wheat and related species (Nakamura et al. 1991a, b; Kasai et al. 1998b). To examine such interactive diversity modulating respiratory characteristics at the molecular level, the isolation of AOX genes is essential. Here, we report the cloning of two non-homoeologous genes encoding the AOX proteins from common wheat. The response of these genes to cold stress and respiratory inhibitors was studied.

MATERIALS AND METHODS

Plant materials. Two cultivars, 'Mironovskaya 808' (M808) and 'Chinese Spring' (CS), of hexaploid or common wheat (*Triticum aestivum* L., genome constitution AABBDD) were grown in a controlled-climate cabinet at 25°C. A tetraploid cultivar (*T. durum* cv. 'Langdon', AABB) and a diploid wheat accession (*T. monococcum*, AA) were also used. A nullitetrasonic series of CS produced by Sears (1966) was used for chromosome assignment of the isolated AOX genes.

Isolation and sequencing of wheat AOX cDNA and genomic clones. A cDNA library was constructed from cold-acclimated seedlings of the winter-hardy common wheat cultivar M808 (Tsvetanov et al., 2000). This library was screened by plaque hybridization using the rice AOX1a cDNA (Ito et al. 1997) as a probe to obtain wheat homologues.

To determine the 5' and 3' ends of the *Waox1a* and *Waox1c* transcripts (for their designations, see RESULTS AND DISCUSSION), 5' and 3' RACE-PCRs were conducted using a Marathon cDNA Amplification Kit (CLONTECH). Single-stranded cDNA was produced from total RNA extracted from young spikelets of CS using a first-strand cDNA synthesis kit (TOYOBO, Japan). The cDNA was used for the RACE-PCR. The following two gene-specific primers were designed for the 5' RACE-PCR based on the isolated cDNA sequence and the partial sequence information of a wheat AOX gene

from the database (accession number AF174004); 5'-AGGAACTCGGTGTAGGAGTGGATG-3' and 5'-CCACCGACATCTTCTTCCAGAGGA-3'. For the 3' RACE-PCR, two gene-specific primers, 5'-CGACATCTTCTTCCAGAGGAGGTAC-3' and 5'-TCCACTCCTACACCGAGTTCCTCC-3', were designed in a similar way. The RACE-PCR products were amplified using the Advantage cDNA Polymerase Mix (CLONTECH) as follows: denaturation at 94°C for 30 sec, annealing and DNA synthesis at 72°C (first 5 cycles), 70°C (second 5 cycles) and 68°C (final 25 cycles) for 4 min per cycle.

To isolate the complete open reading frames (ORFs) of *Waox1a* and *Waox1c*, RT-PCR was conducted using the total RNA extracted from young spikelets of CS as a template. The following two gene-specific primer sets were designed: 5'-GATTGTGATTTCGCGGAGGCGTTC-3' and 5'-TAGCTCTCATTTCTCTGCCTTCCAAAC-3' for *Waox1a* and 5'-GCGAGCCTACGACCACGAGTTT-3' and 5'-CTAGGCGGTCTGGGCATTCATC-3' for *Waox1c*. The RT-PCR was performed using a first-strand cDNA synthesis kit (TOYOBO, Japan).

To obtain the genomic sequences covering the complete ORFs of *Waox1a* and *Waox1c* and to examine their exon/intron structure, the gene-specific primer sets described above were used for the amplification using total DNA of CS. The PCR was performed using Advantage cDNA Polymerase Mix (CLONTECH).

The isolated cDNA clone and the cloned PCR products were sequenced using the automated fluorescent dye deoxy terminator cycle sequencing system and an ABI PRISM™ 310 Genetic Analyzer (PE Applied Biosystems, USA). The DNA sequences and the deduced amino acid sequences were analyzed using GENETYX software (Software Development, Japan), and sequence homology was searched for using BLAST algorithm (Karlin and Altschul, 1993). Multiple alignment was performed using GENETYX software and a phylogenetic tree was constructed by the unweighted pair group method with the arithmetic mean (UPGMA method) (Sneath and Sokal, 1973).

Southern and Northern blot analyses. Total DNA from diplotid, tetraploid and hexaploid wheat accessions was digested by *EcoRI* and *DraI* for genomic Southern blot analysis. The blots were probed with the 5' RACE-PCR product and the entire cDNA sequence for *Waox1a* and an internal 424-bp sequence (nucleotide positions 680 to 1104) for *Waox1c*. For RNA extraction, seedlings of M808 and CS were grown for 2 weeks at 25°C under a 16-hour photoperiod at a light intensity of 55 to 65 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps. The seedlings were cold acclimated for 5 days at $4 \pm 0.5^\circ\text{C}$ under the same photoperiod conditions. The 2-week-old seedlings were also treated by spraying with 3mM potassium cyanide (KCN) or 3mM SHAM and kept for

24 hours under the normal temperature and light conditions. Poly(A)⁺ mRNAs were extracted using a QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). The methods of electrophoresis, Southern and Northern blotting, hybridization with ³²P-labeled probes, and autoradiography were as described by Ohno et al. (2001).

RESULTS AND DISCUSSION

Cloning and sequencing of two wheat AOX cDNAs. To isolate cDNA clones encoding the mitochondrial AOX proteins of wheat, a cDNA library constructed from poly(A)⁺ mRNA of cold-acclimated M808 seedlings was screened with a rice *AOX1a* cDNA probe. One positive clone was isolated from 11,240 plaques. The insert of this cDNA clone was 1,297-bp in length. The insert lacked both of a translation start codon and a poly(A) sig-

nal, but it showed high homology to the rice *AOX1a* cDNA. To determine the 5' and 3' ends of the wheat AOX cDNAs, RACE-PCR was conducted using a cDNA template synthesized from the mRNA of young spikelets of CS. One of the 5'- and 3'-RACE-PCR products was identical to our cDNA clone. Another 5' and 3' RACE-PCR product showed high homology to the wheat AOX cDNA sequence published in the database (AF174004). Based on the nucleotide sequences of the RACE-PCR products, two primer sets were designed to isolate cDNAs containing complete ORFs of the wheat AOX genes. Two types of cDNAs encoding wheat AOX proteins were amplified; they were respectively 1,119-bp and 1,136-bp in length (Fig. 1).

The 1,119-bp cDNA contained an ORF encoding a polypeptide of 328 amino acid residues (Fig. 1A). This nucleotide sequence showed highest homology to the rice *AOX1a* gene. The 1,136-bp cDNA encoded a polypeptide

Fig. 1. Nucleotide and deduced amino acid sequences of two cDNAs encoding wheat alternative oxidase, *Waox1a* (A) and *Waox1c* (B). Underlines indicate primers used for amplification of the wheat AOX cDNAs and genomic sequences. The sites of introns are indicated by filled triangles, and * indicates stop codons.

Fig. 2. Phylogenetic comparison of the wheat AOX proteins with AOX proteins of other organisms. (A) A phylogenetic tree constructed for WAOX1a, WAOX1c and 18 AOX proteins identified from *Sauromatum guttatum* (Rhoads and McIntosh, 1993), *Arabidopsis* (Saisho et al., 1997), tobacco (accession numbers X79768 and S71335), soybean (Finnegan et al., 1997), rice (Ito et al., 1997; Saika et al., 2002), *Aspergillus niger* (AB016540), *Neurospora crassa* (L46869), *Magnaporthe grisea* (AB005144), *Chlamydomonas reinhardtii* (AF047832) and *Trypanosoma brucei* (Q26710). The tree was constructed by the UPGMA method based on Nei's genetic distance. (B) Multiple alignment of the deduced amino acid sequences of Waox1a and Waox1c and 17 previously reported AOX proteins. Amino acid residues identical to those of WAOX1a are presented as dots. Deletions are shown by minus signs. Open triangles indicate the sites of two cysteines that are involved in dimerization of the AOX protein by S-S bond formation (Umbach and Siedow 1993). Filled triangles indicate the iron-binding motifs and underlines indicate α -helical regions.

of 347 amino acid residues and was highly homologous to the rice AOX1c (Fig. 1B). These results indicate that the two wheat cDNAs were derived from two different AOX gene loci and that they represent wheat orthologues of the rice AOX1a and AOX1c genes, respectively. We therefore named these two cDNAs Waox1a and Waox1c based on their orthologous features compared with the rice AOX1 genes. Our findings suggest that the wheat genome, like the rice genome, contains at least two non-homoeologous loci encoding two functionally active AOX1 proteins.

The amino acid sequences of WAOX1a and WAOX1c showed much higher homology with plant AOX proteins than with AOX proteins in other organisms. A phylogenetic tree of the AOX proteins was constructed by the UPGMA method (Fig. 2A). Comparison among the AOX proteins of plants and fungi revealed that the predicted amino acid sequences of the two wheat AOX proteins showed the highest levels of identity with the AOX proteins of rice (69% to 75%). These rice and wheat AOX proteins constituted one cluster and were clearly distinguished from the other plant AOX proteins. Multiple

alignment of the deduced amino acid sequences of the wheat and rice AOX proteins is shown in Fig. 2B. The N-terminal regions, which were assumed to be mitochondrial-targeting signals, had limited homology among the wheat and rice AOX1 proteins. All these proteins contained two cysteine residues that are well conserved among all higher plant AOX proteins and involved in dimerization by S-S bond formation (Umbach and Siedow, 1993). Four predicted α -helix regions that are involved in formation of a hydro-bridged binuclear iron center (Siedow et al., 1995) and the iron binding motifs (Andersson and Nordlund, 1999) were also conserved in the wheat AOX proteins. The structural conservation strongly suggested that Waox1a and Waox1c encode functional AOX proteins.

Exon/intron structure of the Waox1a and Waox1c genes. Most of the *Arabidopsis* and rice AOX genes contain four exons and three introns (Ito et al., 1997; Saisho et al., 1997; Saika et al., 2002). Exceptions include the *Arabidopsis* AOX2 gene, which harbors four introns (Saisho et al., 2001), and the rice AOX1b gene, which

lacks the second intron (Ito et al., 1997). The rice *AOX1b* gene is a tandemly duplicated version of the *AOX1a* gene and the AOX1b protein lacks several features conserved among the plant AOX proteins (Ito et al., 1997). To study the exon/intron structure of the *Waox1a* and *Waox1c* genes, PCR products were amplified from total DNA of CS using the same two primer sets used for amplification of the cDNAs containing complete ORFs and for their sequencing (Fig. 1). The PCR products from the *Waox1a* and *Waox1c* loci were respectively 2,045-bp and 1,419-bp in length and their sequences corresponded with those of the two cDNAs. Both *Waox1a* and *Waox1c* contained four exons and three introns in their ORFs, like rice *AOX1a* and *AOX1c*. The nucleotide lengths of the first, second and third introns were 178, 665 and 144-bp, respectively, in *Waox1a*. In *Waox1c*, the first, second and third introns were respectively 73, 80 and 130-bp in length.

The nuclear exon/intron junctions of the first and second introns of the *Waox1* genes showed typical characteristics, i.e. 5'-AG/GTAAGT and TGCAG/G-3' at the splice sites (Lorkovic et al., 2000). The third introns, however, had CG/GTACGC at the 5' splice site. The intron insertion sites of the *Sauromatum guttatum Aox1* gene and all four AOX genes of *Arabidopsis* are conserved with respect to one another (Rhoads and McIntosh, 1993; Saisho et al., 1997). The intron positions of rice *AOX1a*, *AOX1c* and *AOX1b* are also conserved except for the missing second intron of the last (Ito et al., 1997). These insertion sites were also conserved in the wheat AOX genes.

Southern blot analysis and chromosome assignment. To study the copy number of the *Waox1a* and *Waox1c* genes in the wheat genome, Southern blot analysis was conducted using total DNA isolated from a single accession each of hexaploid, tetraploid and diploid wheat

Fig. 3. Genomic distribution of *Waox1a* and *Waox1c*. (A), (B) Southern blot analysis of the two wheat alternative oxidase genes. DNAs from hexaploid (M808), tetraploid (Ldn) and diploid (Mcc) wheat accessions were digested with *Eco*RI and *Dra*I and Southern blotted. The blots were probed with the 32 P-labeled entire cDNA sequence of *Waox1a* (A) or 424-bp sequence of *Waox1c* (B). (C), (D) Chromosome assignment of the *Waox1a* and *Waox1c* genes. DNAs from the nulli-tetrasomic series of CS were digested with *Dra*I and Southern blotted. The blots were probed with the 32 P-labeled 5' RACE-PCR product for *Waox1a* (C) and an internal 424-bp sequence of *Waox1c* (D). Nulli-tetrasomic lines are abbreviated, e.g. N2BT2A for the nulli-2B and tetra-2A. Only the blots for three (C) and four (D) nulli-tetrasomic lines for the homoeologous group 2 and 6 chromosomes are shown.

species. The two cDNA clones showed different Southern blot patterns (Figs. 3A and 3B), consistent with that they are non-homoeologous clones. Southern blotting of *Waox1a* generated six bands when the entire cDNA sequence was used as a probe (Fig. 3A). This suggested that *Waox1a* constituted a small multigene family in the wheat genome. However, using the 5' RACE-PCR product of *Waox1a* as a probe, three major bands were generated together with several minor bands in the common wheat genome (Fig. 3C). In the case of *Waox1c* probed with an internal sequence, Southern blotting generated three, two and one major bands and some additional minor bands in the hexaploid, tetraploid and diploid wheat accessions, respectively (Fig. 3B).

To assign the *Waox1a* and *Waox1c* loci to wheat chromosomes, Southern blot analysis was conducted using the nulli-tetrasomic series of CS. For *Waox1a*, the gene-specific 5' RACE-PCR product was used as a probe. In the nulli2B-tetra2A line, the largest fragment was missing and a smaller fragment was more prominent than the other fragments (Fig. 3C). The second-largest fragment of *Waox1a* was missing in the two nulli2D lines. These results strongly suggest that the *Waox1a* loci were located on the three homoeologous chromosomes 2A, 2B and 2D. In addition, a minor fragment was missing in nulli3B (data not shown), suggesting that one of the paralogous copies of the *Waox1a* gene is on the chromosome 3B. As for *Waox1c*, the largest fragment was missing and a dose of the smaller fragment was doubled in the nulli6D-tetra6A line (Fig. 3D). Similarly, a middle fragment was more prominent than the other two fragments in the tetra6A lines. These results suggest that the *Waox1c* genes were located on the homoeologous group 6 chromosomes.

The mitochondrial targeting signals of the *Waox1* genes might be shared with unrelated genes. To obtain a more precise estimate of the copy number of the *Waox1a* gene, a probe that lacks this sequence should be used. In conclusion, the common wheat genome possesses at least three homoeologous loci for each of the *Waox1a* and *Waox1c* genes on the three component A, B and D genomes.

Effects of cold stress and respiratory inhibitors on the accumulation of wheat AOX1 transcripts. The rice *AOX1a* mRNA levels increase in plants grown under low temperature conditions (Ito et al., 1997). When maize and tobacco plants were placed under low temperature conditions, both the capacity of the alternative pathway and the amount of AOX proteins increased (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992a). The cold-responsiveness of *Waox1a* and *Waox1c* was studied by Northern blot analysis of mRNAs extracted from M808 plants. The specificity of the probes was confirmed by genomic Southern blot hybridization

(Fig. 3). Both *Waox1a* and *Waox1c* transcripts were detected, though at different levels, in 2-week-old plants grown under the normal temperature conditions. The steady-state level of the *Waox1a* transcript significantly increased within 5 days of cold acclimation at 4°C (Fig. 4). The amount of *Waox1c* transcript also showed a slight increase during cold acclimation.

Fig. 4. Effects of cold stress and treatments with KCN (3mM) and SHAM (3mM) on the levels of the *Waox1a* and *Waox1c* transcripts in the leaf tissues of 2-week-old seedlings of M808. Upper panel: *Waox1a*, middle panel: *Waox1c*, and lower panel: a barley actin gene as a control. C: normal temperature control, cold: cold acclimation at 4°C for 5 days.

KCN and SHAM are well-known specific inhibitors of the two respiratory pathways. KCN inhibits respiration through the cytochrome pathway by blocking a proton translocation site in complex IV. Addition of antimycin A, which inhibits complex III, enhances the synthesis of AOX protein in tobacco (Vanlerberghe and McIntosh, 1992b). Treatment of wheat seedlings with 3mM KCN for 24 hours increased the amount of *Waox1a* transcripts but not that of *Waox1c* transcripts (Fig. 4). Therefore, only *Waox1a* appeared to be responsive to the reduction of the cytochrome pathway activity. This suggests that the two *Waox1* genes are transcriptionally controlled through different regulatory pathways. SHAM affects the cyanide-insensitive pathway by inhibiting the activity of the AOX protein through competition of ubiquinone coupling (Moore and Siedow, 1991). In our study, however, treatment with 3mM SHAM for 24 hours appeared to increase the level of the *Waox1* gene transcript but not that of the *Waox1c* gene transcript (Fig. 4). The effect of SHAM on the transcription of these wheat AOX genes needs to be studied further.

Differential expression of various AOX gene family

members has been reported in *Arabidopsis*, soybean and rice (Saisho et al., 1997; Finnegan et al., 1997; Ito et al., 1997; Saika et al., 2002). In wheat, expression of the *AOX1* genes, which constitute a multigene family in the nuclear genome, are also likely to be differently regulated under various stress conditions. Using alloplasmic lines of wheat, in which different nuclear genomes are combined with different cytoplasmic genomes, cytoplasmic diversity affecting respiratory characteristics was demonstrated (Nakamura et al. 1991a; Kasai et al. 1998b). The capacity of the alternative pathway showed significant variations among the lines, irrespective of the plant tissues studied. Among wheat alloplasmic lines with distantly related *Agropyron* cytoplasm, the capacity of the alternative pathway also differed significantly (Nakamura et al. 1991b). The successful cloning of the wheat *AOX1* genes should help dissect such complex interactions that are manifested in the regulation of respiratory pathways in wheat.

We are grateful to Dr. A. Hirai for providing us with the rice *AOX1a* cDNA clone. This work was partly supported by a grant-in-aid for scientific research (to CN, No. 10044207) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The sequences of *Waox1a* and *Waox1c* reported in this paper have been deposited in the DDBJ database (accession No. AB078882 and AB078883). This is Contribution No. 144 from the Laboratory of Plant Genetics, Faculty of Agriculture, Kobe University.

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