A single nucleotide polymorphism in the alternative oxidase gene among rice varieties differing in low temperature tolerance

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Abstract **Alternative oxidase (AOX) is encoded in a multigene family, and multiple isoforms have been observed in various plant species. We found for the first time an allelic variation in the same *AOX* locus. On SDS–gel blots of callus protein of rice (*Oryza sativa* L.), varieties without the QTL for low temperature tolerance showed a 32-kDa AOX band, whereas those with the QTL showed a 34-kDa band. The variation was attributed to the substitution of Lys^{71} for Asn^{71} caused by a single nucleotide polymorphism between alleles of *OsAOX1a*, and was tightly linked to the presence of the QTL.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Alternative oxidase; Isoforms; Single nucleotide polymorphism; Low temperature tolerance; *Oryza sativa* L

1. Introduction

Higher plants have two mitochondrial electron transport pathways from ubiquinone to O_2 [1]. One is an electron transfer through the cytochrome pathway coupled to ATP synthesis where the terminal oxidase is inhibited by cyanide. The other is an electron flow from ubiquinone through the cyanide-insensitive respiratory pathway (alternative pathway) which is not coupled to ATP production. A terminal oxidase of the alternative pathway is alternative oxidase (AOX), which is encoded by a nuclear gene. The genes encoding AOX consist of a multigene family. Three members have been reported in soybean [2] and rice [3,4], and four members in *Arabidopsis thaliana* [5] and mango [6].

The monoclonal antibody raised against the AOX protein from *Sauromatum guttatum* [7] has been reported to cross-react with AOX protein in many plant species. In general, the antibody identifies some protein bands on an SDS–gel blot. For example, three kinds of AOX proteins were recognized with the antibody in thermogenic tissues [7–9]. The 37-kDa band of AOX was present before thermogenesis, and the appearance of the 36-kDa and 35.5-kDa bands of AOX was correlated with thermogenesis [9]. Two kinds of AOX proteins have been detected in soybean cotyledons [10,11]. The 34-kDa band of AOX was observed at all developmental stages, whereas the 36-kDa band of AOX did not appear until several days after germination [10]. Direct N-terminal sequencing of AOX proteins of soybean showed that the 34-kDa and 36-kDa bands of AOX protein found in soybean cotyledon mitochondria were the products of the *GmAOX2* and *GmAOX3* genes, respectively [12]. The molecular basis for the presence of multiple isoforms of AOX proteins, however, has not been elucidated for other species. No allelic variation of each *AOX* gene has been reported.

In rice, three distinct *AOX* genes, *OsAOX1a*, *OsAOX1b*, and *OsAOX1c*, were identified [3,4]. Although expression of these gene transcripts has been studied, the molecular size of each gene product was unknown. Our purpose is to determine if there are any isoforms which differ in apparent molecular mass and to ascertain the molecular basis of multiple isoforms in rice.

In this study, we found an allelic variation of the apparent molecular mass of AOX protein on SDS–PAGE among rice varieties and showed that this variation was tightly linked to the presence of the QTL for low temperature tolerance. The variation was shown to be attributed to a single nucleotide polymorphism in *OsAOX1a*, which caused a single amino acid substitution. The possible relationship between AOX and low temperature tolerance is discussed.

2. Materials and methods

2.1. Plant materials

Genotype of rice plant materials used in this study is listed in Table 1. A *japonica* breeding line Hokkai 241 and commercial variety Kirara 397, and a *javanica* variety Silewah were used as plant materials. Silewah is known to be highly tolerant to low temperature at booting stage [13]. We also used two parental lines. Norin-PL8 contains genes for low temperature tolerance of Silewah, which was introgressed into the Hokkai 241 by the backcross method [13]. Hokkai-PL5 is also a low temperature tolerant variety whose low temperature tolerance was introduced from a *javanica* variety, Lambayque 1, into Hokkai 241. One of the loci for low temperature tolerance of both Norin-PL8 and Hokkai-PL5 was mapped on chromosome 4 by QTL analysis [14] (Fig. 1B). BT4-76-2 and BT4-9-7 are near-isogenic lines that were selected from backcrossed progenies of Kirara 397/Norin-PL8//Kirara 397 by marker-assisted selection. These lines were characterized in terms of low temperature tolerance at the booting stage and anther length: BT4-76-2 has the QTL for low temperature tolerance on chromosome 4, and BT4-9-7 does not have the QTL [14] (Fig. 1C).

2.2. Callus induction and culture

Rice callus was induced in solid MS [15] medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid, 300 mg/l casein acid hydrolysate and

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10 mM proline from mature rice seeds and cultured in a growth chamber under a light condition at 28°C. The secondary callus was transferred to a fresh medium every 3 weeks, and 10-day-cultured callus was subjected to the following experiments.

2.3. Western blot analysis

Extraction of crude mitochondrial protein was performed as described by [16]. Thirty micrograms of crude mitochondrial protein extracted from rice callus were subjected to electrophoresis in an SDS–13.5% polyacrylamide gel, and then electroblotted onto a polyvinylidene fluoride membrane (Immobilon; Millipore). The membrane was reacted with the monoclonal antibody against the AOX of *S. guttatum* [7]. Immunoreactive proteins were visualized using the High Sensitive Immunoblotting (M) Kit (Wako, Tokyo, Japan).

2.4. Northern blot and reverse transcription (RT)-PCR analysis

Probes for Northern blot analysis were labeled with digoxigenin using the PCR DIG Probe Synthesis kit (Roche Dyagnostics). Primers P1 and P2 were used to obtain a probe specific for *OsAOX1a*, P3 and P4 for *OsAOX1b*, and P5 and P6 for *OsAOX1c* (accession numbers of *OsAOX1a*, *OsAOX1b* and *OsAOX1c* are AB007452, AB004865 and AB074005, respectively). Primer sequences are listed as follows: P1, 5′-GATGTTTGTCTACTGCCGAG-3′; P2, 5′-AACAGGCCCAATTCAGATAG-3′; P3, 5′-TCATCATTCATCAACGGGCGAT-3′; P4, 5′-CACGTGGATACTTCTACTCC-3′; P5, 5′-TCAGCTCTGCAGTTGTTACC-3′; P6, 5′-TTAACCCACAAACAGTGGCT-3′. PCR was performed as described by [17]. Southern blot analysis using these probes was used to confirm the specific detection of each gene (data not shown).

Poly(A)+RNA was isolated from rice callus using the Micro-Fast-Track mRNA Isolation kit (Invitrogen), and 3 μg of poly(A)+RNA was subjected to Northern blot analysis. Hybridization, washing and detection were performed according to the manufacturer's instructions of the DIG DNA Labeling and Detection kit (Roche Dyagnostics). The same blots were reprobed by β-tubulin cDNA to check the integrity of mRNA. The tubulin probe was obtained using primers as described by [17].

For RT-PCR, the first-strand cDNA was synthesized from 150 ng of poly(A)+RNA isolated from rice callus using the First-Strand cDNA Synthesis kit (Amersham Bioscience). First-strand cDNA was used as a template for PCR amplification with specific primers for *OsAOX1a*, *OsAOX1b* and *OsAOX1c* as described above. The tubulin-specific primers were used to amplify the β-tubulin gene as a constitutive control.

2.5. Sequence analysis

Oligonucleotide primers to amplify the *OsAOX1a* coding region are listed as follows: P7, 5′-CGCCTCTAAGAATTCCTCCC-3′; P8, 5′-ATCACCCGGAGAATCAAACG-3′; P9, 5′-GCTTCCCCACTGATATCTTC-3′; P10, 5′-TGATGCAAATCCTCGGCAGTAG-3′. Primers P7 and P8 were used to amplify the exon 1 to 2 of *OsAOX1a* using rice genomic DNA as a template. Primers P9 and P10 were used to amplify the exon 3 to 4 of *OsAOX1a* using rice callus first-strand cDNA as a template. Amplified fragments were purified and directly sequenced.

2.6. Site-directed mutagenesis and plasmid construction

The Lys^{71} codon (AAG) of the *OsAOX1a* cDNA [16] was converted to asparagine (AAT) by using a QuikChange Site-Directed Mutagenesis kit (Stratagene). The mutagenic oligonucleotides were 5′-GGCGGAGGCCAATAAGGCGGACGCGG-3′ and 5′-CCGCGTCCGCCTTATTGGCCTCCGCC-3′ in which the bases exchanged are underlined. A *Kpn*I restriction site was added upstream of Met^{54} which is deduced as an N-terminus residue of mature protein of OsAOX1a. A *Hin*dIII restriction site was also added downstream of the termination codon of the wild-type (Lys^{71}) and mutagenized (Asn^{71}) *OsAOX1a* by PCR amplification using the primers 5′-TTGGGTACCATGTCCACGTCGTCCGTGGC-3′ and 5′-CAAAAGCTTTCAGTGATATCCGATCGGCG-3′. The products were cloned into pBluescript II SK(-) (Stratagene), sequenced to confirm their identities and then subcloned as *Kpn*I–*Hin*dIII fragments into the expression plasmid, pQE-30 (Qiagen), placing each Lys^{71} and Asn^{71} *OsAOX1a* downstream of the histidine-tag.

2.7. Expression of recombinant OsAOX1a in Escherichia coli

Lys^{71} and Asn^{71} *OsAOX1a* cloned into pQE-30 were introduced into *E. coli* M15 cells. Expression of recombinant proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside, and cells were harvested after 5 h of induction. Cells boiled in SDS sample buffer were separated by SDS–PAGE, and recombinant proteins were detected with the antibody as described above.

3. Results

3.1. Mapping position of OsAOX1a

A rice *AOX* gene, *OsAOX1a*, was mapped by RFLP analysis and integrated in the high-resolution RFLP map [18] and was shown to be tightly linked to the RFLP marker S2486 on chromosome 4 (Fig. 1A). The QTL for low temperature tolerance of anthers at the booting stage of rice derived from *javanica* variety, Silewah, has been well characterized in Norin-PL8. Norin-PL8 has been shown to contain at least two QTLs for low temperature tolerance on chromosome 4, and one of them has been mapped between RFLP marker R2737 and XNpb102 which has a 5.8 cM interval between them [14] (Fig. 1B). The mapping position of *OsAOX1a* was included in the position of the QTL for low temperature tolerance.

3.2. The difference of AOX protein among rice varieties

To investigate if there are any differences of AOX among varieties with or without the QTL for low temperature tolerance, we carried out Western blot analysis of callus protein of seven rice varieties. A single band of 32 kDa was detected in *japonica* varieties without the QTL, Hokkai 241 and Kirara 397 (Fig. 2, lanes 1 and 5). In contrast, a single band of 34-kDa was detected in *javanica* variety with the QTL, Silewah (Fig. 2, lane 2). The same 34-kDa band was also detected in Norin-PL8 and Hokkai-PL5 (Fig. 2, lanes 3 and 4), which contain the QTL (Table 1). The AOX protein was then compared between near-isogenic lines with or without the QTL (see Fig. 1C). BT4-76-2 containing the QTL exhibited a 34-kDa band (Fig. 2, lane 6), whereas, BT4-9-7 not containing the QTL showed a 32-kDa band (Fig. 2, lane 7). Thus the 34-kDa band of AOX protein was completely correlated with the presence of the QTL for low temperature tolerance.

Table 1
Genotype of rice plant materials used in this study

Variety name		Presence (+) or absence (–) of the QTL for low temperature tolerance
Hokkai 241	*japonica*	–
Kirara 397	*japonica*	–
Silewah	*javanica*	+
Norin-PL8	Silewah/Hokkai 241//3*Hokkai 241	+
Hokkai-PL5	Hokkai 241/Lambayque 1//3*Hokkai 241	+
BT4-76-2	BC_1F_5(Kirara 397/Norin-PL8//Kirara 397)/3*Kirara 397	+
BT4-9-7	BC_1F_5(Kirara 397/Norin-PL8//Kirara 397)/3*Kirara 397	–

3.3. Transcripts of AOX genes in rice callus

Three *AOX* genes, *OsAOX1a*, *OsAOX1b* and *OsAOX1c*, have been identified in rice [3,4]. In order to clarify which genes are responsible for the AOX protein detected in callus, transcripts of each gene were investigated by Northern blot and RT-PCR analysis. In Northern blot of callus RNA probed with *OsAOX1a*, 1.4-kb bands were detected in all varieties tested (Fig. 3A). The amounts of transcripts of *OsAOX1a* were identical among varieties. No bands were detected when the blot was probed with *OsAOX1b* or *OsAOX1c* (Fig. 3A).

RT-PCR confirmed the presence of the transcripts of *OsAOX1a* (Fig. 3B, lanes 1–6) in all the varieties. No bands were detected for *OsAOX1b* and a faint band was observed for *OsAOX1c* (Fig. 3B). The intensity of bands of *OsAOX1c* was much weaker compared with the band amplified from genomic DNA (lane 7). These results indicate that the AOX protein detected in rice callus is a product of *OsAOX1a*, and the difference of AOX protein among varieties is not caused by different gene products.

3.4. The single nucleotide polymorphism of OsAOX1a among rice varieties

Since the AOX protein detected in callus was shown to be a product of *OsAOX1a*, we compared the nucleotide sequence throughout the coding region of *OsAOX1a* among varieties. The sequence of each variety was identical except for one nucleotide. The 297th nucleotide was 'G' resulting in encoding lysine (AAG) at the 71st amino acid residue in Hokkai 241, Kirara 397 and BT4-9-7, all of which showed a 32-kDa band on the SDS–gel blot. On the contrary it was 'T' resulting in asparagine (AAT) in Silewah, Norin-PL8, Hokkai-PL5 and BT4-76-2, which produced a 34-kDa protein. This single nucleotide polymorphism was completely linked to the difference of apparent molecular mass of AOX protein.

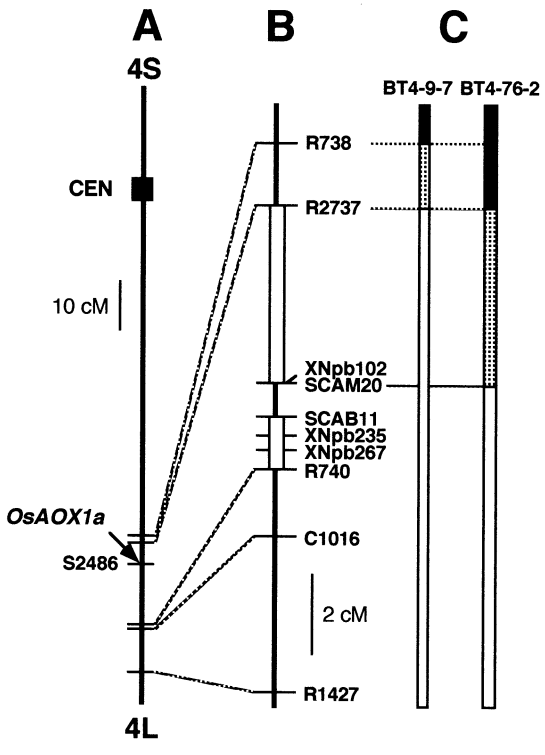

Fig. 1. Mapping positions of *OsAOX1a* and the QTL for low temperature tolerance. A: The map of chromosome 4 based on the genetic linkage map of the Rice Genome Research Program, Japan [18]. Mapping position of *OsAOX1a* is indicated by an arrow on the left. B: The map based on the recombination of near-isogenic lines in QTL analysis [14]. Two open boxes indicate the distinct regions of QTL. C: The genotype of near-isogenic lines used in this study. Solid bars and open bars represent the Norin-PL8 type and Kirara 397 type, respectively. Dotted bars represent the interval in which recombination has occurred.

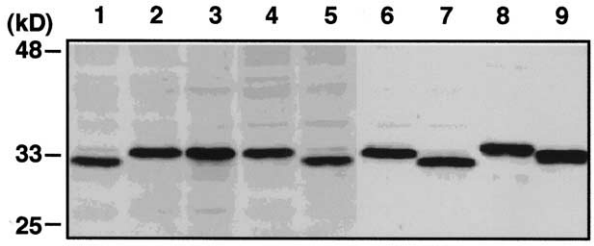

Fig. 2. The difference of AOX protein on SDS–gel blots. A crude mitochondrial protein (30 μg) extracted from rice callus of Hokkai 241 (lane 1), Silewah (lane 2), Norin-PL8 (lane 3), Hokkai-PL5 (lane 4), Kirara 397 (lane 5), BT4-76-2 (lane 6), and BT4-9-7 (lane 7) was subjected to SDS–PAGE. The recombinant proteins of Asn^{71} (lane 8) and Lys^{71} (lane 9) OsAOX1a were also subjected to SDS–PAGE. The electroblotted membrane was reacted with the monoclonal antibody to AOX. The molecular markers are indicated in kDa on the left.

3.5. Apparent molecular mass of recombinant protein with Lys^{71} and with Asn^{71}

To determine if the single amino acid substitution is responsible for the difference of the apparent molecular mass of the AOX protein, cDNA encoding mature forms of each OsAOX1a protein was constructed by removal of the mitochondrial targeting presequence and expressed in *E. coli*. Recombinant protein with Lys^{71} exhibited a 34-kDa band on the SDS–gel blot (Fig. 2, lane 9) while that with Asn^{71} showed a 35-kDa band (Fig. 2, lane 8) in the same manner as was observed in rice callus. Because the histidine-tag was added to the recombinant proteins, the mobility was lower than that in the case of rice callus. These results indicate that the difference of the apparent molecular mass of AOX protein is caused by a single amino acid substitution in OsAOX1a.

4. Discussion

There have been no reports of an allelic variation of AOX protein within the same species. We found for the first time an allelic variation of AOX protein among rice varieties differing in low temperature tolerance. The molecular basis for the multiple isoforms of AOX protein has been well characterized in soybean. 34-kDa and 36-kDa bands of AOX-detected soybean cotyledon were the products of *GmAOX2x_rm;* and *GmAOX3* genes, respectively [12]. It has been suggested that the multiple bands detected in other species result from different gene products. The molecular masses of mature GmAOX2 and GmAOX3 products calculated from the cDNA sequences were 31.8 kDa and 31.6 kDa, respectively [12]. The reason for the discrepancy between the apparent molecular masses and the calculated molecular masses has not been elucidated.

In rice, the molecular masses of mature OsAOX1a, OsAOX1b and OsAOX1c calculated from the deduced amino acid contents were 31.8 kDa, 32.3 kDa, and 33.2 kDa, respec-

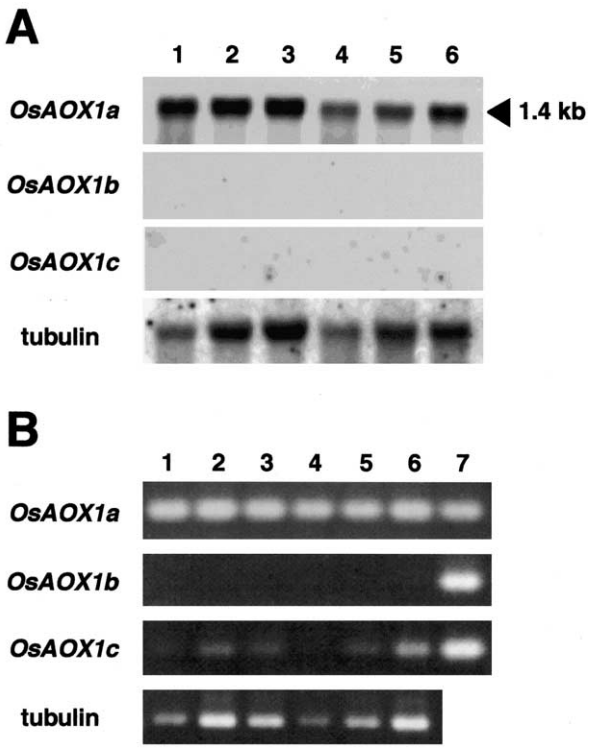

Fig. 3. Analysis of transcripts of three AOX genes among rice varieties on Northern blots (A) and RT-PCR (B). A: Poly(A)+RNA (3 μg) was isolated from rice callus of Hokkai 241 (lane 1), Silewah (lane 2), Norin-PL8 (lane 3), Hokkai-PL5 (lane 4), BT4-9-7 (lane 5), and BT4-76-2 (lane 6), and the blot was probed with *OsAOX1a, OsAOX1b,OsAOX1c* or tubulin cDNA. The size of the transcripts of *OsAOX1a* (1.4 kb) is shown by the arrowhead on the right. Transcripts of *OsAOX1b* and *OsAOX1c* were not detected in Northern blots. B: Transcripts of *OsAOX1a*, *OsAOX1b* and *OsAOX1c* were analyzed by RT-PCR using rice callus cDNA of Hokkai 241 (lane 1), Silewah (lane 2), Norin-PL8 (lane 3), Hokkai-PL5 (lane 4), BT4-9-7 (lane 5), and BT4-76-2 (lane 6) as a template. The fragment amplified from rice genomic DNA (lane 7) and tubulin gene is indicated as a positive control. No contamination of genomic DNA was checked by the absence of reverse transcriptase enzyme as a template (data not shown).

tively. The band of 32-kDa detected in this study coincided with the calculated molecular masses of OsAOX1a. The 34-kDa band detected in varieties with the QTL was larger than the calculated molecular mass. Only a single nucleotide polymorphism causing change of a single amino acid residue was found between varieties with 32-kDa protein and those with 34-kDa protein. It is possible that the single amino acid substitution may affect the modification of AOX protein or merely affect the mobility of the proteins in SDS–PAGE. A subject for future consideration is to determine whether the single amino acid substitution affects enzymatic activity or not.

Many researchers have proposed that the alternative pathway may maintain flux through the mitochondrial electron transport system in a cold condition [19–25]. But the relationship between the alternative pathway and low temperature tolerance has not been fully elucidated. Rice has evolved in tropical and subtropical areas, and hence it is vulnerable to cool weather. Anthers at booting stage are known to be most susceptible to low temperature [26–28]. Because the anther demands a high mitochondrial activity, disorder of a carbon source from the TCA cycle and adenylate control caused by environmental stress readily causes male sterility. We demonstrated previously that the *OsAOX1a* was highly expressed in anthers at the booting stage and was not expressed in mature anthers [16]. We focus on the relationship between low temperature tolerance and the *AOX* gene.

The map position of *OsAOX1a* was included in the position of the QTL for low temperature tolerance of anthers at the booting stage (Fig. 1A,B). Identity of the map position of *OsAOX1a* was also confirmed in near-isogenic lines, BT4-9-7 and BT4-76-2 (Fig. 1C). BT4-9-7 does not have the QTL and is shown to possess the OsAOX1a protein with Lys^{71}, while BT4-76-2 has the QTL and possess the OsAOX1a protein with Asn^{71}. A single nucleotide polymorphism in *OsAOX1a* was completely linked to the presence of the QTL for low temperature tolerance. These results suggest that the *OsAOX1a* is related to low temperature tolerance. An interesting subject for future investigation would be to determine whether the single amino acid substitution affects low temperature tolerance of anthers at the booting stage.

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