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ABA-Independent Expression of Rice Alternative Oxidase Genes under Environmental Stresses

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Abstract

Transcript levels of alternative oxidase genes (*AOX1a* and *AOX1b*) of rice were studied under various stresses using a Northern hybridization analysis. The steady-state mRNA levels of these genes increased under low temperature, high salt and drought conditions, but not in the presence of exogenous ABA. Two DRE/CRT-like sequences are also found in the promoter region of *AOX1a*.

Keywords: ABA, alternative respiration, AOX, environmental stresses

Abbreviations

AOX, alternative oxidase; ABA, abscisic acid: DRE/CRT, dehydration responsive element/C-repeat; ROS, reactive oxygen species; P5CS, Δ^1-pyrroline-5-carboxylate synthetase.

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

Many studies have been performed on the alternative pathway and the alternative oxidase (AOX), the terminal oxidase of this pathway, to elucidate their functions. In some plants, the alternative pathway is induced by several conditions, *e.g.*, by treatment with inhibitors of the cytochrome pathway, such as antimycin A (Vanlerberghe and McIntosh, 1994; Saisho *et al.*, 1997), by treatment with salicylic acid (Kapulnik *et al.*, 1992; Rhoads and McIntosh, 1993) and by wounding (Hiser and McIntosh, 1990). Likewise, when several plant species are placed under low temperature, the capacities of the alternative pathway are enhanced (McCaig and Hill, 1977; Stewart *et al.*, 1990; Vanlerberghe and McIntosh, 1992). In maize and tobacco, the amount of AOX protein is increased under low temperature (Stewart *et al.*, 1990; Vanlerberghe and McIntosh, 1992). In rice, steady-state mRNA levels of the two *AOX* genes, *AOX1a* and *AOX1b*, are increased by low temperature (Ito *et al.*, 1997). These facts suggest that the alternative pathway of higher plants is involved in responses to environmental stresses. In this paper, we investigated the expressions of *AOX1a* and *AOX1b* in rice under low temperature, high salt and drought conditions by Northern hybridization analysis.

Rice (*Oryza sativa* L. cv Nipponbare) was grown under constant light at 28℃ for 5 days with water and subsequently exposed to the following conditions. For the high salt condition and ABA treatment, the water was replaced with 250 mM NaCl solution and 1 mM ABA solution, respectively. For the drought condition, water was completely removed. For the low temperature condition, rice was transferred in darkness and grown at 4℃. The control rice for the low temperature condition was grown in darkness at 28℃ and the control for the other conditions was grown in light at 28℃. The leaves and the roots of rice were collected separately just before the treatments started (0 h), and 6, 12, 24 and 48 h after the treatments started. Rice total RNAs from leaves and roots were extracted by the method of Kawakami and Watanabe (1988).

The steady-state mRNA levels of *AOX1a* and

AOX1b under low temperature, high salt and drought conditions were analyzed by Northern hybridization using the 3'-untranslated region of each gene as a probe (see Ito *et al.*, 1997). Specific probes for *AOX1a* and *AOX1b* were made by PCR using primers 5'-GATGTTTGTCTACTGCCGAGGATTT-3' and 5'-ATGTAGTATATATAACTCAGCTGCC-3' and primers 5'-TCATCATTCATCAACGGGCGATGC-3' and 5'-TGTGCACGGGTCAGCCAACGGCCA-3', respectively. Northern hybridization was performed with a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany). Growth of rice treated with low temperature conditions (4℃), high salt solution (250 mM NaCl) and drought for 48 h was clearly delayed compared with the control plants (data not shown). This indicates that our treatments damaged the rice. The steady-state mRNA levels of *AOX1a* and *AOX1b* increased both in the leaves and the roots of rice as a result of the three treatments (**Fig. 1**). Our results obtained with leaves under low temperature were coincident with those of Ito *et al.* (1997). Although the *AOX1b* signals were relatively weak compared with the *AOX1a* signals, the qualitative signal patterns of *AOX1a* and *AOX1b* were similar to each other. The results obtained for the leaves and the roots were slightly different (**Fig. 1**). When rice was treated with low temperature, the mRNAs of *AOX1a* and *AOX1b* in the roots increased more rapidly and drastically than that in the leaves. When rice was treated with high salt and drought conditions, the mRNAs of both genes in the leaves increased with time. On the other hand, the amounts of mRNA in the roots treated with high salt condition were greatest 6 h after the treatment started and then decreased gradually. In dehydrated roots, the signal intensity slightly increased in the first 6 h and then leveled off.

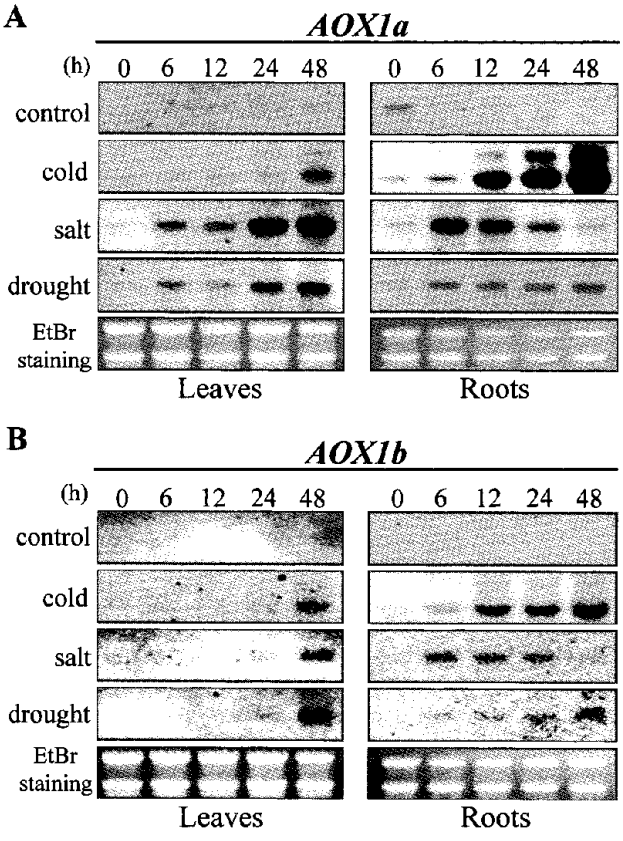

Fig. 1 Northern hybridization analysis of mRNA of *AOX1a* (A) and *AOX1b* (B) in the leaves and the roots of rice under low temperature, high salt and drought conditions. Low temperature, high salt and drought conditions are indicated as cold, salt and drought, respectively, in the figure. The results of the controls for high salt and drought stresses are shown. The results of the controls for low temperature were almost the same. The number above each lane indicates the number of hours after the initiation of treatment prior to the isolation of total RNA. 3 μg of total RNA from rice under high salt and drought conditions was loaded onto each lane. 5 μg and 10 μg of total RNA from rice under low temperature was loaded onto each lane for *AOX1a* and *AOX1b*, respectively. Equal loadings of total RNA were checked by ethidium bromide (EtBr) staining.

Many genes that are induced under environmental stresses such as low temperature, high salt and drought stresses are also induced by exogenous abscisic acid (ABA) (reviewed by Shinozaki and Yamaguchi-Shinozaki, 1997). In many cases, higher plants under such stresses produce ABA endogenously and this ABA induces the expression of various genes allowing higher plants to tolerate these stresses (Mansfield, 1987). Therefore, it was of interest to examine whether the expressions of *AOX1a* and *AOX1b* in rice respond to treatment with exogenous ABA.

To determine the effect of ABA on the *AOX* genes, we first confirmed the result of Igarashi *et al.* (1997) that 1 mM exogenous ABA elevated the steady-state mRNA level of the rice Δ^1-pyrroline-5-carboxylate synthetase gene (*OsP5CS*) (**Fig. 2A**). However, the same treatment caused no change in the steady-state mRNA level of *AOX1a* (**Fig. 2B**) or *AOX1b* (data not shown).

The preceding results showed that the steady-state mRNA levels of the rice *AOX* genes, *AOX1a* and *AOX1b*, were increased by environmental stresses such as low temperature, high salt and drought stresses (**Fig. 1**). The responses of the leaves and the roots to the various treatments were slightly different (**Fig. 1**). This probably reflects a difference in sensitivity to the stresses between the leaves and the roots (*e.g.*, only the roots were

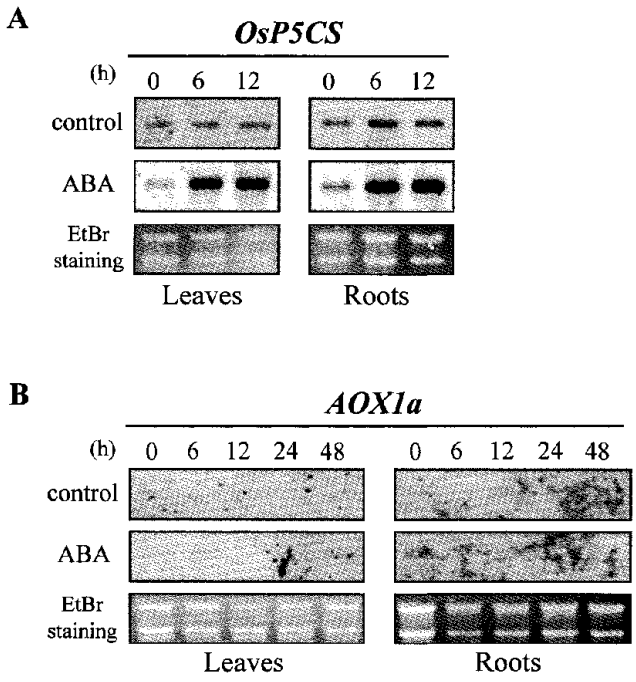

Fig. 2 Northern hybridization analysis of mRNA of *OsP5CS* (A) and *AOX1a* (B) in the leaves and roots of rice exposed to 1 mM exogenous ABA (ABA) or water (control). The entire coding region was used as a probe for *OsP5CS*. 5 μg of total RNA was loaded onto each lane. Other details are the same as in **Fig. 1**.

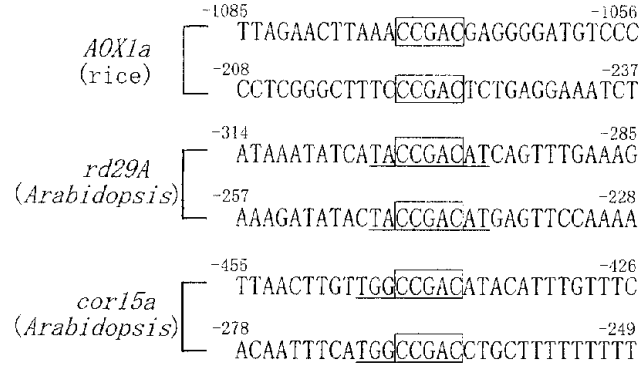

Fig. 3 Partial nucleotide sequences of the promoter regions of *AOX1a* (rice), *rd29A* (*Arabidopsis*; Yamaguchi-Shinozaki and Shinozaki, 1994) and *cor15a* (*Arabidopsis*; Baker *et al.*, 1994) that include the core sequence of DRE/CRT (boxes). DRE (TACCGACAT) and CRT (TGGCCGAC) are shown by underlines. Numbers give the position of the first nucleotide in the sequence with respect to the initiation codon.

directly exposed to high salt stress) and/or a difference between the leaves and the roots in the mechanism that regulates the expression of *AOX1a* and *AOX1b* under the stresses examined in this study. In higher plants, reactive oxygen species (ROS) are generated under low temperature, high salt and drought stresses (Kalir *et al.*, 1981). These ROS damage plant cells. In cultured tobacco cells overexpressing AOX, the production of ROS was significantly lowered (Maxwell *et al.*, 1999). Thus, our results raise the possibility that rice induces the expression of *AOX1a* and *AOX1b* to reduce the production of ROS under low temperature, high salt and drought. Further investigations will be needed to confirm this hypothesis. We are currently investigating whether the production of ROS is actually suppressed in transgenic rice that overproduces AOX1a or AOX1b proteins, and whether this transgenic rice has an improved tolerance for the above stresses. It should be noted, however, that even if AOX1a and AOX1b function to reduce the production of ROS, they may have other functions as well. For example, 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 (Mackenzie and McIntosh, 1999).

Many higher plants produce the plant hormone ABA in response to environmental stresses and this ABA induces the expression of various genes allowing plants to tolerate these stresses (Mansfield, 1987). However, our results (**Fig. 2**) show that the expression of *AOX1a* and *AOX1b* of rice under low temperature, high salt and drought stresses is regulated by an ABA-independent pathway. The *Arabidopsis rd29A* gene is rapidly induced by low temperature, high salt and drought, but in an ABA-independent manner (Yamaguchi-Shinozaki and Shinozaki, 1994). The promoter region of the *rd29A* gene has the nucleotide sequence TACCGACAT, which is a dehydration-responsive element (DRE) that regulates the ABA-independent expression of the *rd29A* gene (**Fig. 3**; Yamaguchi-Shinozaki and Shinozaki, 1994). On the other hand, a similar motif, TGGCCGAC, designated the C-repeat (CRT), was reported in the promoter regions of the cold-inducible *Arabidopsis cor15a* gene (**Fig. 3**; Baker *et al.*, 1994). Interestingly, two DRE/CRT core sequences (CCGAC) were observed in the promoter region of *AOX1a* as shown in **Fig. 3**. Thus, there is a possibility that the expression of *AOX1a* under low temperature, high salt and drought stresses is regulated by the DRE/CRT-like motifs. However, the promoter region of *AOX1b* does not have the DRE/CRT core sequence (data not shown). This is somewhat puzzling because the qualitative expression patterns of *AOX1a* and *AOX1b* were similar under the above stresses. This raises the

possibility that the promoter region of *AOX1b* has a novel *cis*-acting element that regulates it in an ABA-independent manner. Further investigations will be needed to better understand the regulation of expression of *AOX1a* and *AOX1b* under low temperature, high salt and drought stresses.

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