Note

Plant Biotechnology, **20** (4), 339–341 (2003)

Alternation of the Accumulation of Alternative Oxidase by Introducing Sense and Antisense *OsAOX1a* in Rice

Fumitaka ABE[a] and Kinya TORIYAMA*

Laboratory of Plant Breeding and Genetics, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981-8555, Japan

Present address: [a]National Institute of Crop Science, Kannondai, Tsukuba, Ibaraki 305-8518, Japan

**Corresponding author* E-mail address: torikin@bios.tohoku.ac.jp

Received 3 March 2003; accepted 1 July 2003

Abstract

Sense and antisense DNA constructs of rice (*Oryza sativa* L.) alternative oxidase (AOX) gene, *OsAOX1a*, under the control of maize *Ubiquitin* promoter were introduced into rice. Leaves of transgenic plants with sense *OsAOX1a* were shown to produce high levels of AOX protein, which was not usually observed in wild-type leaves. Transgenic plants with decreased levels of AOX protein were identified by the investigation of AOX protein in calli derived from mature seeds with antisense *OsAOX1a*. Transgenic plants with increased and decreased levels of AOX will be useful for studies on the role of AOX.

Key words: Alternative oxidase, antisense, *Oryza sativa* L., transgenic plant.

Abbreviations

AOX, alternative oxidase.

Higher plants have two mitochondrial electron transport pathways from ubiquinone to O_2 (Moore and Siedow, 1991). One is the cytochrome pathway coupled to ATP production where the terminal oxidase is inhibited by cyanide. The other is the cyanide-insensitive alternative pathway, which is not coupled to ATP production. A terminal oxidase of the alternative pathway is an alternative oxidase (AOX), which is encoded by a nuclear gene. It has been suggested that the AOX is involved in reducing the harmful reactive oxygen species under stress conditions (Purvis, 1997; Wagner and Moore, 1997).

Investigation of the role of AOX under stress conditions has been facilitated by the use of transgenic technique. Transgenic plants expressing sense and antisense *AOX* cDNA were successfully produced in tobacco (Vanlerberghe *et al.*, 1994). The studies using these transgenic plants showed that AOX lowers production of mitochondrial reactive oxygen species (Maxwell *et al.*, 1999), that AOX acts as a modulator of a programmed cell death (Vanlerberghe *et al.*, 2002) and that AOX may play a role in the hypersensitive response at the viral infection (Ordog *et al.*, 2002).

In rice, three *AOX* genes, *OsAOX1a*, *OsAOX1b* and *OsAOX1c* have been identified (Ito *et al.*, 1997) and one of which, *OsAOX1a*, has been shown to be expressed at relatively much higher levels than *OsAOX1b* and *OsAOX1c* in leaves, roots (Saika *et al.*, 2002), immature anthers (Abe *et al.*, 1997) and calli (Abe *et al.*, 2002). In the present study, we report the production and selection of transgenic rice plants with either increased or decreased levels of AOX protein by introducing sense or antisense *OsAOX1a* under the control of *maize Ubiquitin* promoter.

OsAOX1a cDNA taken from cultivar Hayayuki (Abe *et al.*, 1997; DDBJ accession No. AB007452) was cloned into a binary vector, which was constructed for expressing a gene under the control of maize *Ubiquitin* promoter based on pBI101 (Yokoi *et al.*, 1998), in either sense or antisense orientation (**Fig. 1A**). Each construct, designated as Us (Ubi pro::sense-*AOX*) and Uan (Ubi pro::antisense-*AOX*), was transferred to *Agrobacterium tumefaciens* EHA101, and then introduced into rice cultivar Yamahoushi by the method of Yokoi *et al.* (1997).

Hygromycin-resistant rice plants, 23 plants for Us and 16 plants for Uan, were regenerated. All of the transformants were of normal appearance, and self-pollinated seeds were obtained. Integration of the transgene and the number of copies were inves-

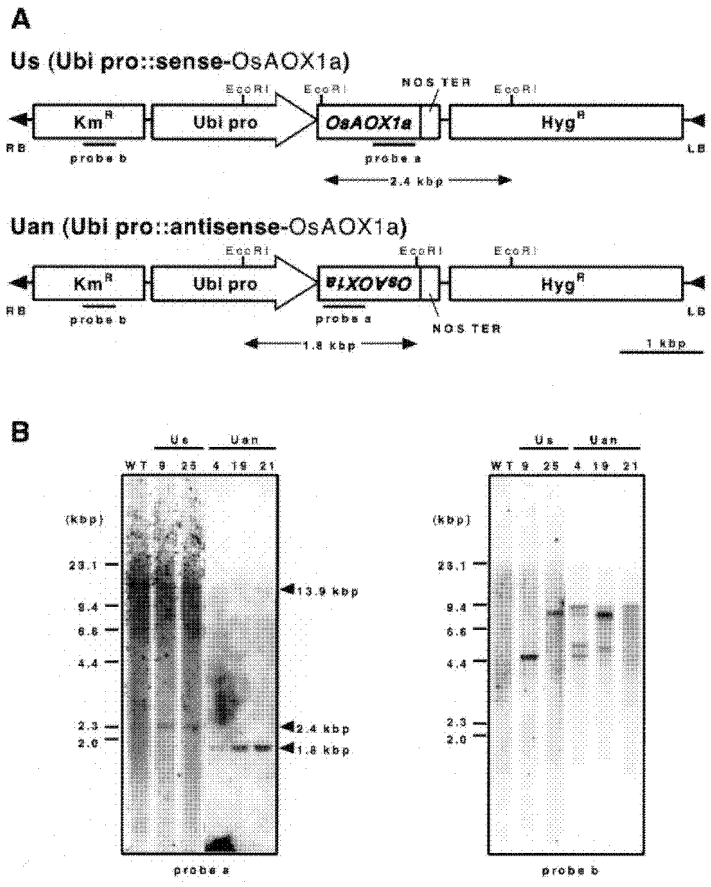

Fig. 1 T-DNA region of transformation vectors (A) and DNA gel blot analysis of transformants (B). A: Schematic diagram of the T-DNA region of the transformation vectors, Ubi pro::sense-*OsAOX1a* (Us) and Ubi pro::antisense-*OsAOX1a* (Uan). Abbreviations: RB and LB, right and left borders of T-DNA; Km^R, gene for kanamycin resistance; Hyg^R, gene for hygromycin resistance; Ubi pro, ubiquitin promoter from maize; NOS TER, terminator of nopaline synthase. The regions used as probes and *Eco*RI restriction sites are indicated. B: Genomic DNA (1 μg) from transformants (Us and Uan) and a wild-type plant (WT) was digested with *Eco*RI, and the blot was probed with probe a (left) or probe b (right). The fragment sizes of a transgene containing *OsAOX1a* cDNA (Us, 2.4 kbp; Uan, 1.8 kbp) and an endogenous *OsAOX1a* (13.9 kbp) are indicated by arrowheads. The molecular markers are indicated in kbp on the left.

tigated by DNA gel blot analysis (**Fig. 1B**). Bands of expected size, which are 2.4 kbp in Us and 1.8 kbp in Uan, were detected in each plant, demonstrating the integration of the transgene. The number of copies varied from one to four.

To examine the amounts of AOX protein, we performed SDS-gel blot analysis. Crude mitochondrial proteins, which were extracted from leaves as described in Abe *et al.* (1997), were subjected to SDS-PAGE and AOX protein was detected with monoclonal antibody against AOX of *Sauromatum guttatum* (Elthon *et al.*, 1989). No AOX band was detected in leaves of a wild-type (non-transformed) plant, whereas a 32 kDa AOX band was detected in all of the Us transformants (**Fig. 2A**). Since we were unable to detect AOX protein in leaves of wild-type plants, it was considered to be difficult to identify Uan transformants in which levels of AOX protein were reduced below those of wild-type plants. On the other hand, we found that a large amount of AOX protein was detectable in calli of wild-type plants. Higher levels of AOX accumulation in calli would be attributed to higher mitochondrial activities and induction of AOX gene expression by the stressful culture condition of calli. Therefore, to screen antisense plants with reduced levels of AOX protein, we induced calli from T_1 seeds of each transgenic plant and compared AOX protein from wild-type and Uan transformants. Three Uan transformants, Uan4, Uan19 and Uan21, were found to have undetectable AOX protein (**Fig. 2B**), although the other 13 lines had high levels of AOX protein similar to that of wild-type (examples, Uan9 and Uan22, were shown in **Fig. 2B**). A

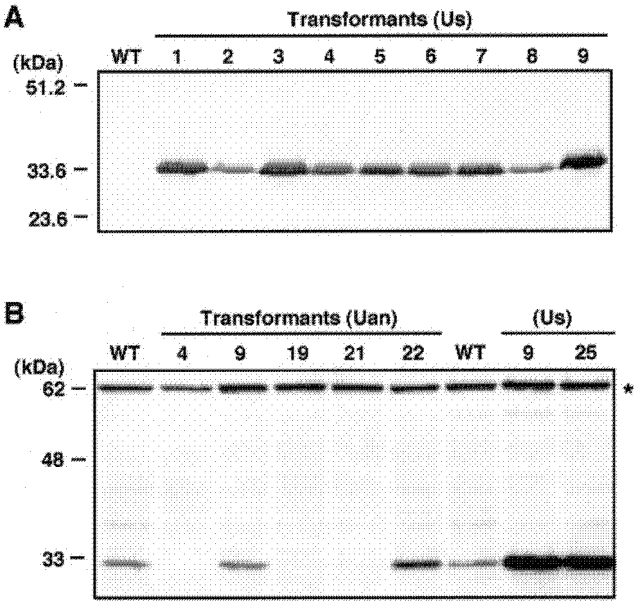

Fig. 2 Detection of AOX protein in leaves (A) and calli (B) of the transformants. A: Crude mitochondrial proteins (20 μg) extracted from leaves of the Us transformants and a wild-type plant (WT) were subjected to SDS-PAGE. The electroblotted membrane was reacted with a monoclonal antibody against AOX of *Sauromatum gutattaum*. The molecular markers are indicated in kDa on the left. B: Crude mitochondrial proteins (30 μg) extracted from calli of the transformants (Us and Uan) and a wild-type plant (WT) were subjected to SDS-gel blots. An asterisk at the right indicates a non-specific band that is cross-reacted with the secondary antibody. The molecular markers are indicated in kDa on the left.

common band of 62 kDa, which was revealed to be cross-reacted band with secondary antibody, showed that the same amount of callus protein was loaded in each lane. In contrast, Us transformants, Us9 and Us25, showed considerable increase of AOX protein in calli (**Fig. 2B**). Thus, transgenic lines with increased levels of AOX protein, Us9 and Us25, and decreased levels of AOX protein, Uan4, Uan19 and Uan21, were identified.

Transgenic rice plants produced in the present study will be useful for studies of the role of AOX under various stress conditions. Progenies homozygous for the introduced genes are now under propagation for further studies.

Acknowledgement

This work was supported in part by a grant for Recombinant Plant Project from the Ministry of Agriculture, Forestry and Fisheries, Japan.

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