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Overexpression of an alternative oxidase gene, *OsAOX1a*, improves cold tolerance in *Oryza sativa* L.

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ABSTRACT. Low temperature is a major environmental stress in rice cultivating and production. The alternative oxidase 1 (*AOX1*) gene is potentially important for genetic engineering to increase cold adaptation. However, previous studies related to this effect have mostly focused on the dicot plants *Arabidopsis* and tobacco, whereas functional research on rice is limited. In this study, we cloned a rice predominant cold-response *AOX1* gene, *OsAOX1a*. Transgenic rice plants with overexpression of *OsAOX1a* were obtained. We found that *OsAOX1a* overexpression could strongly enhance the cold growth of seedlings, especially with respect to root extension. However, growth between transgenic and control plants did not differ under normal conditions. Furthermore, the lipid peroxidation and ion leakage rate were determined after cold treatment in transgenic plants. Both factors were reduced by *OsAOX1a*

overexpression, which revealed that *OsAOX1a* could reduce oxidative damage under cold stress. Taken together, our results suggested that overexpressing *OsAOX1a* could improve growth performance of rice under cold stress, which might be closely related to the reduction of reactive oxygen species generation and oxidative damage.

Key words: Alternative oxidase; Rice; Cold; *OsAOX1a*; Oxidative damage

INTRODUCTION

Cold stress is one of the most serious abiotic stresses that limits rice growth and production all over the world (Mukhopadhyay et al., 2004). Cold exposure at the seedling and subsequent vegetative growth stages can lead to poor germination, yellowing leaves, and growth retardation (de Los Reyes et al., 2013). Unpredictable chilling snaps at the reproductive stage causes heading delay and high sterility, resulting in severe yield losses (Jena et al., 2012). Therefore, in rice breeding, screening for cold tolerance gene(s) is particularly important to deal with the current increase in occasional extreme weather events.

The alternative oxidase (AOX) enzyme is a cyanide-resistant terminal oxidase involved in the mitochondrial electron transport pathway (mtETC). It directly couples with the ubiquinol pool and catalyzes the four-electron reduction of oxygen to water. Since the ATP production is by-passed, free energy could be quickly dissipated as heat by AOX. In thermogenic plant species, AOX is enriched in floral tissues and produces heat to facilitate pollination by volatilizing insect attractants (Finnegan et al., 2004). For non-thermogenic plants, various additional physiological functions have also been suggested. Research with several different plant species has revealed that AOX might play an important role in the optimization of respiratory metabolism (Rasmusson et al., 2009). AOX might also oxidize the excess reductants to provide a dissipation mechanism to prevent damage to the photosynthesis process (Fu et al., 2012). The most widely studied function of AOX has been its reduction on reactive oxygen species (ROS) production under stress conditions. The mtETC is a main source of stress ROS generation. Because AOX is able to prevent excessive reduction of the ETC and reduce the member potential, it should also act to dampen ROS generation.

In almost all plant species, AOXs are encoded by a small nuclear multi-gene family that consists of two subfamilies in dicots, *AOX1* and *AOX2*, whereas only *AOX1* exists in monocots. It is well documented that *AOX1*s could be induced by various biotic and abiotic stresses, whereas *AOX2* is usually developmentally expressed and constitutively regulated by environmental factors (Polidoros et al., 2009). Many studies have indicated that the lack of *AOX1* reduces the tolerance or growth ability under stress. Furthermore, other evidence has suggested that overexpression of *AOX1* could increase stress tolerance ability by generating less ROS (Vanlerberghe et al., 2009). However, most of these genetics studies were performed on the dicot plants *Arabidopsis thaliana* and *Nicotiana tabacum* or on species with unique physiological processes (Wang et al., 2011; Zidenga et al., 2012). Therefore, the function of *AOX1* in monocot rice remains largely unknown.

We previously identified two rice *AOX1* genes, *OsAOX1a* and *OsAOX1b*, that were strongly responsive to various environmental stresses (Li et al., 2013). Here, we cloned one

cold-inducible gene, *OsAOX1a*, and overexpressed it in *Oryza sativa.* Clear differences in cold tolerance were observed between wild-type and transgenic plants. Further analysis suggested that the stress response was closely associated with lipid peroxidation and oxidative damage in the plant.

MATERIAL AND METHODS

Plant materials and rice transformation

O. sativa seeds were surface sterilized in 70% ethanol for 2 min followed by 50% sodium hypochlorite for 20 min, rinsed seven times in sterile distilled water, and placed on 1/2 Murashige and Skoog medium for 14 days. *OsAOX1a* genes were cloned with the forward primer: 5'-CTATCTAGAATGAGCTCCCGGATGGCCG-3' and the reverse primer: 5'-CTAGGTACCGTGATATCCGATCGGCGCA-3' and were then subcloned into the pCAMBIA 1300 vector. The binary constructs were transformed by *Agrobacterium tumefaciens* strain EHA105 and used to infect rice calli as previously described (Duan et al., 2012). Lines of hygromycin-resistant transgenic plants were selected, and before being transplanted in the greenhouse, pieces from the transgenic plant leaves were sampled for DNA extraction with the DNeasy Plant Mini Kit (Qiagen) according to manufacturer instructions. The primary transgenic rice plants were self-pollinated in a greenhouse, and the resulting seeds (T1) were collected. Rice plants (T2) were cultivated in an experimental field in Hefei of Anhui Province in China under natural growing conditions. The resulting T3 plants were analyzed for phenotypic changes under cold stress.

RNA extraction, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis, and Western blot

Total RNA of the rice seedlings was extracted with the RNeasy Plant Mini Kit (Qiagen) in accordance with manufacturer instructions. Approximately 2 µg total RNA was used to synthesize the 1st-strand cDNA with the Reverse Transcription System (Promega). The cDNA product was diluted two times with deionized water, and used as the template for PCR. SYBR Green-monitored real-time quantitative PCR was performed with an ABI PRISM 7500 real-time PCR system (Applied Biosystems), and each analysis was performed with three biological replicates. The PCR conditions consisted of denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Quantification was performed using the $\Delta\Delta C_T$ method, and the data were normalized through the quantity of the reference gene, *ACTIN*. The dissociation curves were analyzed in all amplifications, and each analysis was performed with three biological replicates. The expression of *OsAOX1a* was determined by qRT-PCR with gene-specific primers (Li et al., 2013) and with Western blot analysis using the anti-FLAG antibody (Cell Signaling).

Copy number identification and Western blot

Total DNA of rice leaves was extracted using the DNeasy Plant Mini Kit, according to manufacturer instructions. To determine the copy number of transgenic events, the TaqMan

assay was performed for quantification of real-time PCR analysis (Yang et al., 2005). The TaqMan Premix (TaKaRa) was used in reactions. FAM was used as the reporter of *hpt* genes, *SPS* was used as the reporter of the internal control, TAMRA was used as the quencher for either the target genes or the internal control, and the primers were synthesized as described previously (Yang et al., 2005). PCRs were carried out in an Applied Biosystems ABI7500 system equipped with a 96-well plate using the following program: 5 min at 95°C for predenaturation, and then 30 cycles each of 10 s at 95°C and 34 s at 60°C.

Electrolyte leakage and malondialdehyde measurement

Leaves were weighed and washed three times in deionized water and were then immersed into 15 mL deionized water at 25°C. After 12 h, the conductivity of the solution was measured using a conductivity meter (initial conductivity). The tube was heated at 100°C for 20 min and cooled in an ice bath, and conductivity was re-measured (final conductivity). The relative ion leakage was calculated according to the following equation: ion leakage = initial conductivity / final conductivity x 100%.

The amount of malondialdehyde (MDA) derived from unsaturated fatty acid peroxidation of the membrane lipids was measured as described previously (Gueta-Dahan et al., 1997). One hundred milligrams plant tissue was homogenized with liquid nitrogen, hydrated in 1 mL 2.5% trichloroacetate (TCA; w/v), and centrifuged at 12,000 *g* at 4°C for 20 min. Then, 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) TCA was added into 100-µL aliquot supernatants. The mixture was incubated for 30 min at 100°C and supernatants were collected. The absorbance values were measured at 532 nm based on 600 nm.

RESULTS AND DISCUSSION

Generation of *OsAOX1a* overexpression plants

The full length of *OsAOX1a* cDNA obtained from cultivar Nipponbare was amplified using gene-specific primers. After sequencing conformation, the coding sequence was subcloned into the binary pCAMBIA 1300 vector, and a FLAG-tagged sequence was in-frame inserted at the 3'-terminal end. A constitutive CaMV35S promoter was used to drive the *OsAOX1a*-FLAG expression. The construct was transformed into *O. sativa* L. ssp *japonica* (cv. Nipponbare), and more than 40 independent transformants were screened for hygromycin resistance. In all transgenic lines, integration of the transgene was confirmed by specific PCR examination of the expression cassette. The number of copies was investigated using real-time PCR in the T1 generation. Based on standard protocols, six lines were recognized as single copies, 27 lines contained medium copy numbers (two to four copies), and 12 lines contained high copy numbers (more than four copies) (Yang et al., 2005). The seeds of single copy T1 generations were collected, and the 3:1 segregation on hygromycin of all six T2 lines further confirmed that they were single-copy transgenic plants. After being self-pollinated, the homozygous T3 seeds were selected for further examination.

To determine the expression of *OsAOX1a* in the transgenic plants, assays at the RNA and protein levels were performed. Total RNA was isolated from 7-day-old seedlings of the six transgenic lines and wild types. qRT-PCR analysis, using *OsAOX1a*-specific primers, was

used to test the relative expression level in each transformant. The housekeeping *ACTIN1* gene was used as the internal control (Caldana et al., 2007). The transcript abundance was enhanced 5.6-, 24.8-, 13.7-, 8.2-, 18.2-, and 27.1-fold, in lines 1 to 6, respectively (Figure 1A). The expression of OsAOX1a-FLAG was further examined by Western blot analysis using the anti-FLAG antibody. Signals of approximately 45 kDa were recognized in the transgenic lines, but not in the wild-type sample (Figure 1B). Taken together, our results suggested that the additional *OsAOX1a* was successfully expressed and translated in the single-copy transgenic rice. In addition, overexpression lines 2 and 6, which showed relatively higher transcript levels, were selected for subsequent stress examinations.

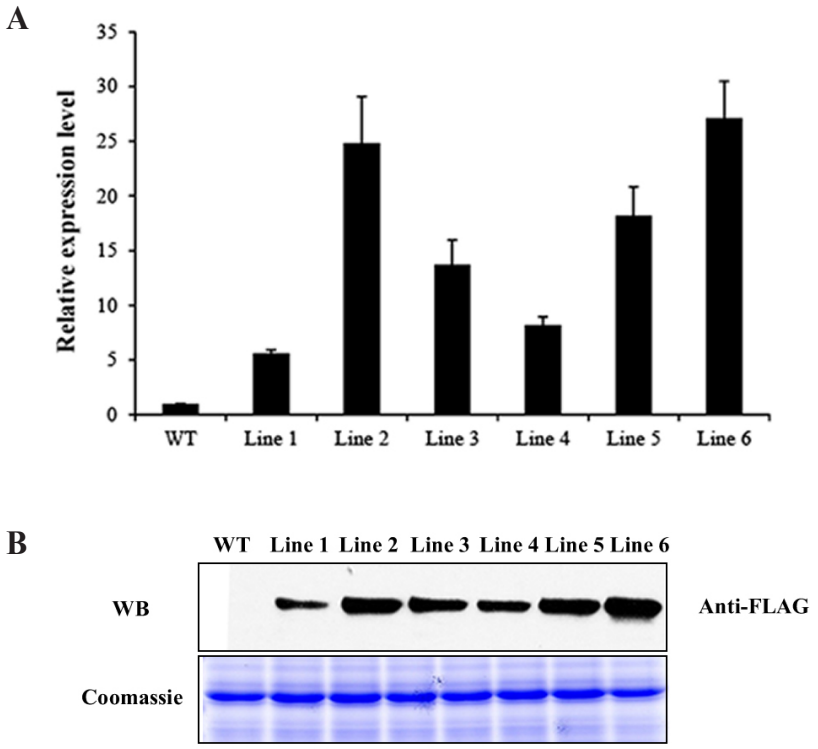

Figure 1. *OsAOX1a* overexpressed in the transgenic plants. **A.** qRT-PCR analysis on the expression level of *OsAOX1a* in wild-type and transgenic plants. WT: wild-type rice plant, line 1-line 6, 6 lines of single copy transgenic plants. ACTIN was used as internal control. Three independent samples were collected from each lines, and the error bars indicate the SD of three biological replicates. **B.** Western blot on the expression of the OsAOX1a-FLAG fusion protein in wild-type and transgenic plants. Each line were loaded 20 µg total protein on two gel at same time. One gel was applied to perform Western blot using anti-FLAG antibody (WB) and another was stained by Coomassie blue (Coomassie).

Overexpression of *OsAOX1a* enhanced growth performance under cold stress

AOX1 has previously been demonstrated to alleviate cellular damage under various environmental stresses. A large number of recent studies have focused on its role in response

to salt stress, drought, or bacterial infection, as well as other biotic stresses. *AOX* was 1st identified in the floral part of the thermogenic plant *Araceae* as a temperature-regulating protein (Ito et al., 2011); however, functional studies on cold tolerance are rare relative to those on other abiotic stresses. The little evidence that is available has been obtained using the model dicot species *Arabidopsis*. Overexpressing *AtAOX1* produced better shoot growth at 12°C, whereas shoot growth was significantly inhibited in knockdown plants compared to wild types (Fiorani et al., 2005). In addition, biochemical and molecular changes were also observed in tobacco plants with suppressed or constitutively expressed *NtAOX1a*, although growth performance was not mentioned (Wang et al., 2011). On the other hand, increasing evidence from different plant species has indicated that the expression of *AOX1* or the activity of AOX might be induced by low temperatures, as well as by drought or other abiotic stresses. Therefore, the identification and determination of rice AOX1 function under cold stress is becoming increasingly relevant.

Four *AOX1* genes are distributed across the rice genome. Only three were cloned as the *OsAOX1d* transcript has not yet been identified. It has been reported that *OsAOX1a* is the predominant gene of the rice *AOX1* gene family (Abe and Toriyama, 2003). Stress-induced *OsAOX1a* expression has been well documented. Abiotic stress, including drought, salt, and low- and high-temperatures, could all significantly upregulate *OsAOX1a*, whereas abscisic acid exposure did not (Ohtsu et al., 2002). Moreover, transgenic rice harboring constitutively expressed *OsAOX1a* exhibited great improvement with respect to high temperature tolerance (Murakami and Toriyama, 2008). However, the underlying physiological mechanism for this increased tolerance has not yet been identified.

In the present study, the cold tolerance of *OsAOX1a* overexpression lines was examined under light conditions. After germination for 2 days, young seedlings were cultured at 4°C for 10 days. As shown in Figure 2A, the growth of wild-type plants was almost fully inhibited, while two transgenic *OsAOX1a* lines could maintain slow growth both with respect to roots and sheaths. The growth of the three types of plant was similar under normal conditions (30°C). The statistical analysis suggested that the sheath lengths of transgenic lines were slightly larger than (line 2) or the same as (line 6) that of the wild type (Figure 2B). However, root growth was significantly enhanced by *OsAOX1a* expression under cold, which were 3.71- and 4.61-fold compared to the wild type. It is interesting to note that the main functional site of the *Arabidopsis* AOX gene, *AtAOX1*, is the green tissue and not the root (Fiorani et al., 2005). A previous study demonstrated that the cold-induced *OsAOX1a* expression in leaves was more significant than that in roots, which might explain the functional differentiation between *OsAOX1a* and *AtAOX1a*.

Overexpression of *OsAOX1a* results in less oxidative damage under cold stress

Oxidative damage is the primary contributor to cold injuries in plants (de Los Reyes et al., 2013). Therefore, the MDA content and ion leakage were measured as indictors of lipid peroxidation and direct cell damage. Before cold treatment, both of the transgenic lines had reduced MDA levels compared to wild-type rice (Figure 3A), which suggested that *OsAOX1a* might dampen the ROS generation during normal growth as well. After 5 days of cold treatment, MDA contents were significantly increased in the wild type. The *OsAOX1a* overexpression lines, however, exhibited either less accumulation (line 1) or even a slight decrease (line

6) of MDA. The rates of ion leakage were also determined under cold treatment. Consistent with the lipid peroxidation results, *OsAOX1a* overexpression resulted in less leakage under cold stress (Figure 3B), which suggested less cold injury of the cell membrane.

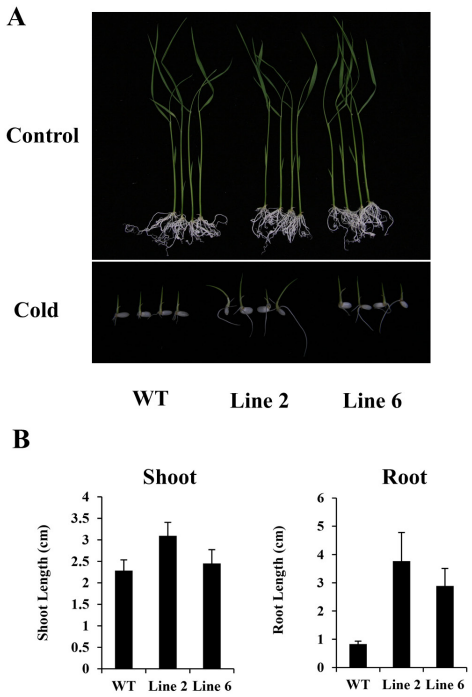

Figure 2. *OsAOX1a* overexpression plants have better cold growth performance. **A.** Upper: 7-day growth under normal condition (30°C); down: 10-day growth under cold stress (4°C). **B.** Quantitive analysis of shoot (left) and root (right) length of cold growth, N = 30. WT = wild-type; line 2 and line 6, two *OsAOX1a* transgenic lines.

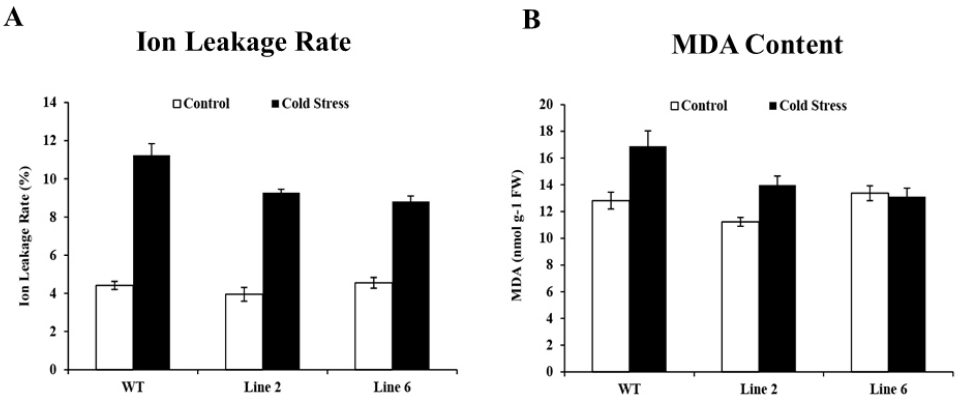

Figure 3. *OsAOX1a* overexpression plants were less damaged by cold. Ion leakage rate (**A**) and malondialdehyde (MDA) (**B**) content were determined separately before and after cold treatment. White columns, no-stress treatment; black columns, cold stress. The error bars represent the standard deviation of three individual experiments.

Both of the indicators, the MDA level and ion leakage, revealed that *OsAOX1a* could reduce oxidative damage *in vivo*. Our results are consistent with a previous study in *Arabidopsis*, but not tobacco. The mitigating effects of *AtAOX1a* and *OsAOX1a* suggested that the cold tolerance of *AOX1* might closely correspond to its role as an ROS suppressor. Furthermore, the short time (hours) of the cold treatment did not affect lipid peroxidation rates between wild-type and *NtAOX1* overexpressing plants. This result does not conflict with the primary role of *AOX1* as an ROS scavenger under long periods of cold growth (Wang et al., 2011). After all, the ROS accumulation mechanism and mitochondrial respiration response are distinct between transient and steady stress. Moreover, the expression of single high temperature protection heat shock protein could lead to a general induction of other heat shock proteins (Rhoads et al., 2005), which suggested that the mitochondrial retrograde regulation pathway might play an important role in temperature adaption. AOX activity could affect mitochondrial function and might also regulate mitochondrial stress singling in response to temperature stress.

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