

## Short Communication

## Enhanced high temperature tolerance in transgenic rice seedlings with elevated levels of alternative oxidase, OsAOX1a

Yuuki Murakami, Kinya Toriyama\*

Laboratory of Environmental Biotechnology, Graduate school of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, Japan

\*E-mail: torikin@bios.tohoku.ac.jp Tel: +81-22-717-8830 Fax: +81-22-717-8834

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**Abstract** The cyanide-insensitive alternative oxidase (AOX) respiratory pathway can reduce the excessive production of reactive oxygen species. Here we showed that transgenic rice seedlings transformed with *OsAOX1a* for rice alternative oxidase under the control of ubiquitin promoter exhibited thermotolerance after acute exposure, 41–45°C for 10 min, or chronic exposure, 37°C for up to 8 days. In contrast, these high temperature stresses resulted in significant growth inhibition in wild-type and transgenic plants with antisense *OsAOX1a*. The enhanced tolerance was significant in shoot growth, suggesting that the increased levels of AOX protein would dissipate the excess reductants produced in the chloroplasts suffering from oxidative damage due to high temperature stress.

**Key words:** Alternative oxidase, high temperature stress, rice, transgenic plant.

Higher plants have two mitochondrial electron transport pathways. One is the cytochrome c (Cyt) pathway leading to ATP synthesis where the terminal oxidase is inhibited by cyanide. Another pathway is the cyanide-insensitive alternative pathway, which is not coupled to ATP synthesis (Lambers 1998; Taiz and Zeiger 2002; Vanlerberghe and Ordog 2002). The terminal oxidase of the alternative pathway is an alternative oxidase (AOX).

The alternative pathway regulates the level of reducing power in the mitochondrial electron transport chain when reactive oxygen species (ROS) accumulation is accelerated by environmental stress. The main function of the alternative pathway is believed to be reducing excessive ROS and maintaining a balance in the redox status of mitochondria (Lambers et al. 1998; Taiz and Zeiger 2002; Maxwell et al. 1999; Vanlerberghe and Ordog 2002). Recently AOX has been also reported to be related to accumulation of reducing equivalents in the chloroplasts in illuminated leaves (Yoshida et al. 2007; Noguchi and Yoshida 2008).

Overexpression of AOX proteins has been shown to confer an enhanced tolerance to low temperature stress due to alleviation of ROS accumulation (Fiorani et al. 2005; Sugie et al. 2006). However, thermotolerance under high temperature has not yet been reported for transgenic plants overproducing AOX proteins.

We previously produced transgenic rice plants that over-expressed sense (Us) and anti-sense (Uan)

constructs of *OsAOX1a* cDNA under the control of the maize ubiquitin promoter (Abe and Toriyama 2003). The Us lines, Us9 and Us25, produced significant levels of OsAOX1a protein in leaves and calli as shown by immunoblot analyses, whereas the Uan line, Uan4, did not contain any detectable OsAOX1a proteins. Transgenic lines homozygous for the introduced gene were selected. We have confirmed that the increased production of OsAOX1a protein was stable in subsequent generations (T4, T5 and T6) of the homozygous Us lines (data not shown).

Seeds from transgenic lines were germinated on filter paper moistened with 5 ml Murashige-Skoog solution (pH 5.6) (Murashige and Skoog 1962) for 3 days. Seedlings were transferred to 2×18 cm glass test tubes containing a 3.5 cm<sup>2</sup> section of polyethylene mesh fabric. The pore size of the mesh is a square with sides 2 mm long. The mesh fabric was positioned 2 cm deep into 15 ml culture solution (Hyponex solution diluted 1:2000) and grown for 2 days. All test plants were grown in the growth chamber at 25°C under a light intensity of 55 μmol m<sup>-2</sup> s<sup>-1</sup> and a photo-period of 16-h light and 8-h dark. The plants were grown for two days before high temperature treatment. The culture solution in the test tubes was replaced with fresh solution at 5-day intervals.

In order to examine recovery from acute exposures to high temperature stresses, the prepared test tubes were

Abbreviations: AOX, alternative oxidase; ROS, reactive oxygen species.  
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sealed with Parafilm and placed in a water bath set at an elevated temperature for 10 min under room light. Two-thirds of the test tubes were submerged so that all parts of plants were exposed to the designated temperature. After the high temperature exposure, the Parafilm was removed and seedlings were moved to a 25°C growth chamber with the same light condition as described and allowed to recover for 8 days. The length of the aerial parts (plant lengths) and root lengths were measured daily. The plant and root lengths are presented by subtracting the values on the day of heat treatment (0 day) from each day's values.

Damage due to chronic exposure to high temperatures was also assessed using plants growing in test tubes. Two days after transplanting into the test tubes, the young plants were moved and cultivated in small plastic houses set at 37°C and 43°C using thermo controllers. These small plastic houses were installed in a 25°C growth chamber with the same light condition as described. These plants during the exposure at 25°C were also cultivated in the same growth chamber. Growth parameters were measured every day for up to 8 days. These experiments were conducted twice by using the Us lines of different generations. Statistic analysis was carried out using Dunnett' test at the 5% level.

As a result, plant lengths and root lengths were nearly identical for all plants after acute exposure at 25°C (Figure 1). Plant lengths in Us9T5 and Us25T6 grown at 25°C for 8 days after the exposure at 41°C for 10 min was  $107.6 \pm 5.9$  and  $109.8 \pm 7.2$ , respectively, whereas plant lengths in WT plants and Uan4T4 were  $55.5 \pm 6.5$  and  $43.1 \pm 11$  (Figure 1A). T4, T5 and T6 in the Us and the Uan lines indicate the different generations for each

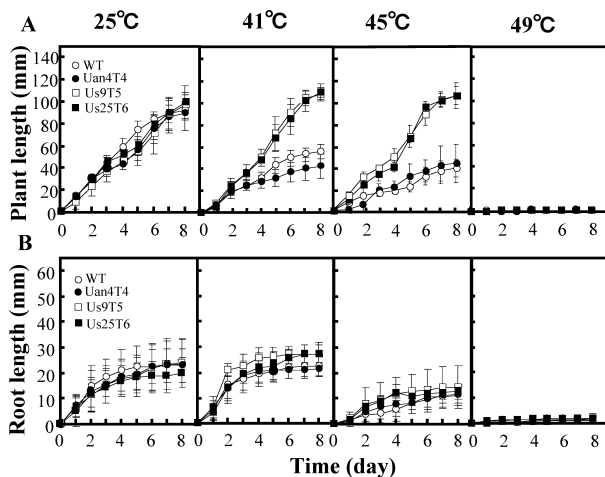


Figure 1. Growth of transgenic lines at 25°C for 8 days after acute exposure to various temperatures for 10 min. A and B show plant lengths and root lengths of overexpressing sense transgenic lines (Us9T5 and Us25T6), anti-sense transgenic line (Uan4T4) and wild-type (WT) plants, respectively (n=10 per line). Scores are presented by subtracting the values on the day of heat treatment (0 day) from each day's values. Error bars indicate the standard deviation (SD).

line. When the treatment consisted of high temperature exposure at 45°C, plant lengths in Us9T5 and Us25T6 were  $104.4 \pm 7.0$  and  $104.1 \pm 7.9$ , respectively, whereas plant lengths in WT plants and Uan4T4 were  $38.6 \pm 8.5$  and  $42.8 \pm 17.4$ , respectively (Figure 1A).

In addition, thermotolerance during chronic exposures to high temperature stress was investigated. The plant lengths in Us9T5 and Us25T6 grown at 37°C for 8 days were  $70.1 \pm 3.2$  and  $69.8 \pm 9.3$ , respectively, whereas the plant lengths in WT plants and Uan4T4 was  $40.2 \pm 8.3$  and  $36.3 \pm 5.4$  (Figure 2A). Furthermore, the observed suppression of growth and development in all Us lines was significantly less than WT plants and the Uan4T4 line during exposure to 37°C for 8 days. Plant lengths were not significantly different in any of the tested plants during exposure to the normal growth temperature (25°C) for 8 days, whereas there was considerable growth inhibition and formation of necrotic areas in all tested plants during exposure to 43°C for 8 days (Figure 2A).

The root lengths at 8th day in Us9T5 and Us25T6 is significantly longer than those in WT plants and Uan4T4 when plants were exposed to acute high temperature at 41°C (Figure 1B) and chronic high temperature at 37°C (Figure 2B). Under the other experimental conditions, the root lengths were not significantly different (Figure 1B, 2B).

The same experiments were also carried out for Us lines of different generation; Us9T4, and Us25T4. The alleviated degree of growth inhibition in the Us lines was evidently visible, after acute exposures at 41 or 45°C for 10 min (Figure 3A) and after chronic exposures at 37°C for 8 days (Figure 3B). The results were confirmed that

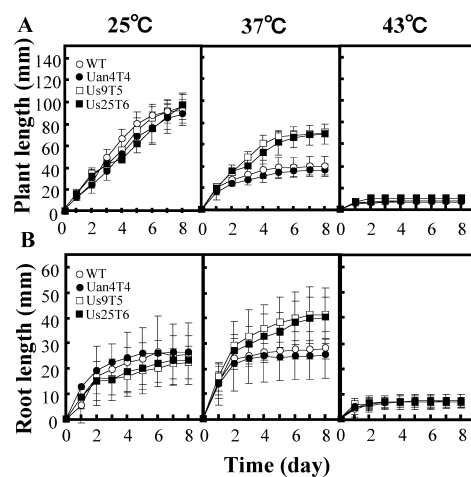


Figure 2. Growth of transgenic lines during chronic exposures at various temperatures for 8 days. A and B show plant lengths and root lengths for overexpressing sense transgenic lines (Us9T5 and Us25T6), anti-sense transgenic line (Uan4T4) and wild-type (WT) plants, respectively (n=10 per line). Scores are presented by subtracting the values on the day of heat treatment (0 day) from each day's values. Error bars indicate the standard deviation (SD).

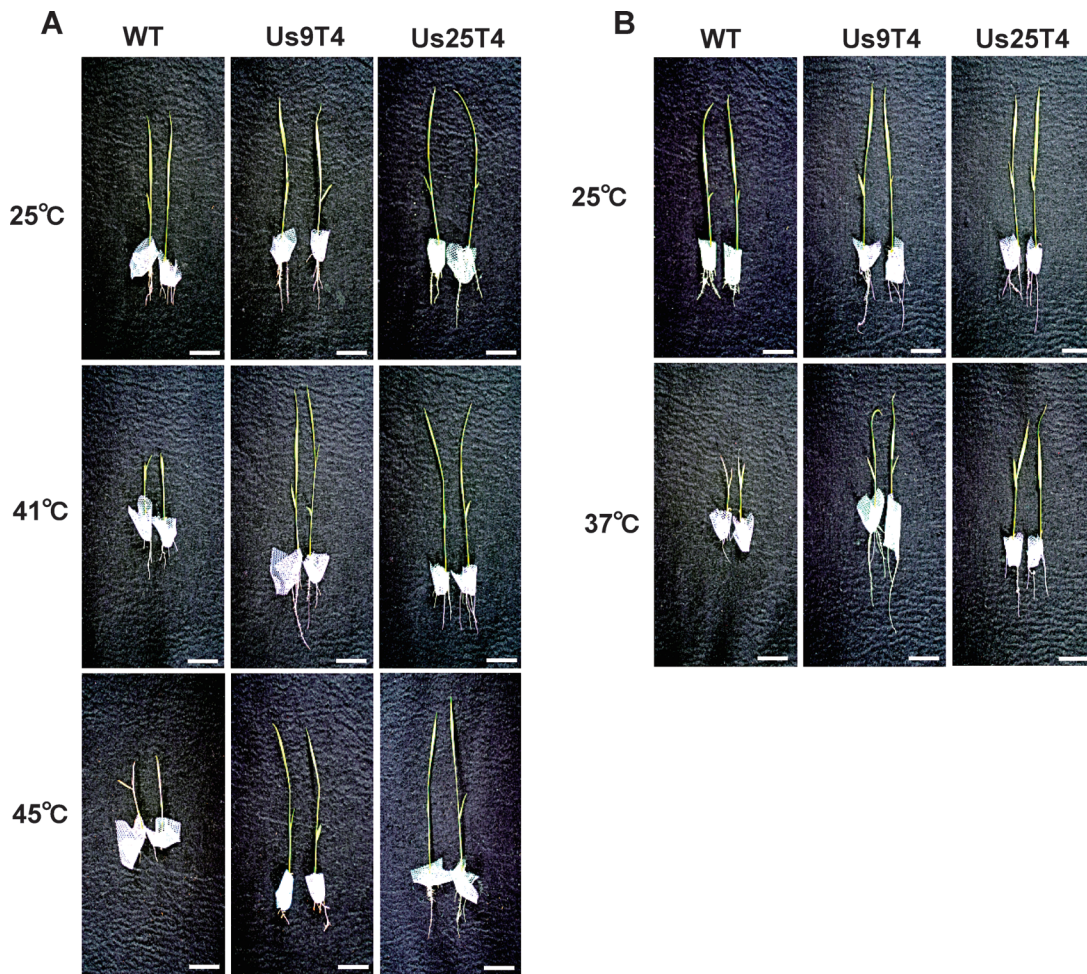


Figure 3. Visible damage after acute (A) and chronic exposure (B) to high temperature stress. Overexpressing sense transgenic lines (Us9T4 and Us25T4) significantly alleviate growth inhibition as compared with wild-type (WT) plants. Scale bar, 2 cm.

the less impaired growth at the 8th day was significantly different in the plant lengths after acute exposure at 41 and 45°C or after chronic exposures at 37°C, while it was significantly different in the root lengths after acute exposure at 41°C or after chronic exposures at 37°C (data not shown for plant and root lengths).

Effects of energy conservation are lower whenever AOX activity is raised because AOX does not cause the formation of a mitochondrial proton gradient as well as other enzymes in mitochondria (Taiz and Zeiger 2002). Hence, we thought that the Us lines with increased levels of AOX protein might grow less than WT plants because the Us lines would be producing less energy under normal growth temperature conditions. Such thinking, however, was an idle fear from these results. The Us lines, as well as the Uan line, grew normal until flowering and set seeds in the same manner as wild-type plants (data not shown).

Accumulation of ROS (e.g.  $H_2O_2$ ) in *Arabidopsis* cell suspension has been reported at moderate high temperature (37°C) or at acute exposure to 44°C (Roman et al. 2006). Such induction of ROS in mitochondria is

likely to occur in our current study and overexpressed OsAOX1a would serve to reduce excessive ROS and maintain a balance in the redox status of mitochondria.

Chloroplasts in the aerial parts of plants also have electron transport chain (ETC) similar to those of mitochondria and are the primary sites of thermal damage and generation of ROS production in plant cells. A moderate high temperature delays growth and development of high temperature-sensitive plants, whereas most tissues of the higher plants are unable to survive extended exposure temperatures above 45°C (Levitt 1980; Taiz and Zeiger 2002). Specially, the damage in photosynthesis is caused by temperature exposure to the range of 35°C to 45°C (Berry and Björkman 1980; Weis and Berry 1988). Indeed, photosynthetic activity of the pretreated intact leaves in WT tobacco and *Arabidopsis* plants, which reflect the activity of photosynthetic ETC, was inhibited by 40 to 45°C treatments for a few minutes (Murakami et al. 2000; Iba 2002). Furthermore, thermal denaturation of proteins in crude preparations from chloroplasts of WT tobacco plants was shown to occur in a similar

temperature range by differential scanning calorimetry (Iba 2002). Our results of growth inhibition after acute exposures between 41°C and 45°C in WT plants are consistent with the range of high temperatures that inhibited photosynthetic activity and caused the denaturation of the proteins (Figure 1A, 3A).

Photosynthesis is not an independent process of the chloroplast but is intricately interwoven with mitochondrial metabolism (Gardeström et al. 2002; Noguchi and Yoshida 2008). Indeed, Yoshida et al. have recently demonstrated the importance of AOX function; AOX plays a role to dissipate the excess reducing equivalents, which are transported from the chloroplasts, and serve in efficient photosynthesis (Yoshida et al. 2007; Noguchi and Yoshida 2008).

In the current study, we demonstrated that transgenic rice plants overexpressing *OsAOX1a* proteins exhibited enhanced high temperature tolerance compared with WT and the *Uan* line. It is noticeable that the less impaired growth rates was significant in the growth of the aerial parts. The elevated levels of AOX protein in the *Us* lines are considered to serve as protecting several heat-sensitive components located in the chloroplasts, thus alleviate the inhibition of shoot growth. To our knowledge, this is a first report demonstrating high temperature tolerance in transgenic plants overexpressing an *AOX* gene. We expect that overexpression *OsAOX1a* proteins can be useful to provide crops with increased yield under high temperature stress.

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