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Over-expression of Sub1A, a submergence tolerance gene from rice, confers enhanced hypoxic stress tolerance in transgenic tobacco plants

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Sub1A, an ethylene-response-factor-like (ERE-like) gene, mediates the extinguished submergence tolerance of rice. To gain further insight into the function of Sub1A in other species, we transformed tobacco plants with the gene under the control of the ubiquitin promoter. Compared to the wild-type plants, transgenic plants over-expressing Sub1A exhibited a greater ability to adapt to hypoxia, as evidenced by the highly induced activities of enzymes (pyruvate decarboxylase and alcohol dehydrogenase) regulating ethanolic fermentation. Furthermore, Sub1A upregulated activities of the main antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase and catalase, making the transgenic plants scavenge reactive oxygen species (ROS) more effectively. This was further confirmed by the less accumulation of malondialdehyde, an end product of lipid peroxidation. Taken together, our results suggest that Sub1A promotes plants hypoxic stress tolerance by regulating genes involved in anaerobic metabolism as well as ROS amelioration. In addition, it also suggests that Sub1A can be used potentially to improve hypoxic stress tolerance in plant breeding.

Key words: Hypoxic stress, Sub1A, Tobacco, transgenic plants.

INTRODUCTION

Waterlogging, flooding and submergence are the most hazardous natural disasters constraining serious crop production in many regions of the world (Fukao et al., 2006; Hattori et al., 2011). Too much water hampers plant growth and development, primarily through initiation of hypoxic conditions due to a 10,000-fold reduction in the diffusion of gas (Fukao and Bailey-serres, 2008). When plants are subjected to hypoxia or anoxia conditions, respiration shifts from the aerobic to the anaerobic mode, leading to a crisis in ATP availability due to the inefficient ATP production in ethanolic fermentation (Bailey-Serres and Voesenek, 2008) and this shift typically requires an increased activity of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). In addition to energy starvation, plants under oxygen deficiency conditions suffer from increased ROS production, which poses substantial oxidative damage to the membrane system (Yordanova et al., 2004).

Sub1A is a kind of ethylene-responsive element-binding protein (EREBPs), which compose a superfamily of transcription factors and exit extensively in plants and microorganisms (Okamura et al., 1997; Nakano et al., 2006). EREBPs are characterized by the presence of the highly conserved EREBP DNA-binding domain of about 60 amino acids (Okamura et al., 1997). Plant proteins that contain ethylene-response-factor (ERF) domains are known regulators of abiotic and biotic stress responses (Gutterson and Reuber, 2004; McGrath et al., 2005). Sub1A containing an ethylene-response-factor (ERF) domain, was first cloned from rice and mediated the extinguished submergence tolerance of rice variety flood resistant 13A (F13A) (Xu et al., 2006). When the F13A Sub1A locus was introgressed into the rice variety Swarna via marker-assisted selection, the resultant new variety showed enhanced submergence tolerance. Like-
wise, over-expression of Sub1A in a submergence sensitive O.sativa ssp. japonica conferred increased submergence tolerance, indicating that Sub1A is a primary determinant of submergence tolerance (Fukao et al., 2006). Further study demonstrated that Sub1A functions to restrain ethylene-promoted gibberelllic acid (GA) responsiveness during submergence by increasing accumulation of the GA signal repressors Slender Roce-t1(SLR1) and SLR1 Like-1 (SLRL1) (Fukao and Bailey-serres, 2008). Additionally, Sub1A confers drought tolerance in rice by augmenting ABA responsiveness, and subsequently activating a number of stress inducible genes, including genes involved in ROS scavenging and ethanolic fermentation (Fukao et al., 2006, 2011; Jung et al., 2010).

To further confirm the role of Sub1A in abiotic stress in other important dicotyledonous crops, tobacco plants over-expressing Sub1A were generated for the first time, and the hypoxic stress tolerance of the transgenic plants was further evaluated.

MATERIALS AND METHODS

Regeneration of Ubi: Sub1A over-expressing tobacco plants

Primers were designed according to the sequence of DQ011598, and used to clone Sub1A from flood resistant rice F13A via the RT-PCR method. The Sub1A full-length cDNA driven by maize Ubiquitin1 promoter was inserted into the binary construct pBin438 containing kanamycin resistant gene NPTII. This construct was confirmed by DNA sequencing and transformed into A.tumefaciens strain LBA4404. Thereafter, it was introduced into tobacco (Nicotiana tabacum cv Wisconsin 38) using the Agrobacterium-mediated transformation. The infected tobacco leaf discs were cultivated on MS medium containing 1 mg 6-benzylaminopurine (6 BA L\(^{-1}\)), 0.1 mg 1-naphthaleneacetic acid (NAA L\(^{-1}\)) and 100 mg L\(^{-1}\) kanamycin, and the regenerated T\(_0\) plantlets were transplanted into a greenhouse. T\(_1\) seeds were harvested, and then further selected by kanamycin resistance. The subsequent generation was selected by the same method, and homozygous T\(_2\) seeds were used in the hypoxic stress analysis. The probes used for Northern blot were the full-length cDNAs of Sub1A labeled with \(\alpha\)-[\(^{32}\)P]-dCTP via PCR from plasmid pBin438. Hybridization was carried out using standard procedures (Sambrook et al., 1989) with 10 about 10 \(\mu\)g RNA.

Plant materials and hypoxic treatment

Hypoxia treatment was performed as described by Kreuzwieser et al. (2009) with the following modifications; seeds of the characterized transgenic and wild-type tobacco plants were allowed to germinate on solid medium, and grown to four-leaf stage before being transferred onto a floating platform with roots dipped into half-Hoagland solution in plastic containers. \(O_2\) concentration in the solution was adjusted to a constant level of 7.5 \(\mu\)L L\(^{-1}\) (equals to approximately 80% air saturation) for nomoxic treatment or 0.05 \(\mu\)L L\(^{-1}\) (0.6% air saturation) for hypoxic treatment, by continuously bubbling the solution with 100% (v/v) \(O_2\) for 3% (v/v) \(O_2\), respectively, at a flow rate about 1 L min\(^{-1}\). Throughout the treatment, \(O_2\) concentration was monitored by an oxygen meter (Microx TX2; PreSens). Root sections were harvested 48 h after treatment, and stored in liquid nitrogen for further analysis.

Measurement of malondialdehyde content

Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) method described by Hodges et al. (1999) with some modifications. Briefly, 100 mg roots were homogenized in 1 ml of 80% (v/v) ethanol solution on ice and then centrifuged at 16,000 \(g\) for 20 min at 4°C. The resulting supernatants were mixed with 0.5 ml of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) TBA, and incubated at 95°C for 30 min. Following cooling in an ice bath and centrifugation at 10,000 \(g\) for 10 min, the absorbance of the supernatants was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm. The MDA concentration was calculated from the extinction coefficient (155 mM\(^{-1}\) cm\(^{-1}\)).

Enzyme activity assay

Crude protein was extracted in ice-cold extraction buffer containing 50 mM Tris-HCl, 5 mM MgCl\(_2\), 5 mM mercaptoethanol, 15% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA and 0.1 mM pefabloc proteinase inhibitor. Activities of LDH and PDC were measured spectrophotometrically by monitoring the oxidation of NADH at 340 nm (Yin et al., 2009). Phosphate buffer was used to extract protein for measurement of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activity using a sample of 200 mg root tissue as suggested by Knörzer et al. (1996). SOD activity was assayed by the photochemical nitroblue tetrazolium (NBT) method, and activities of APX and CAT were determined by tracking the consumption of H\(_2\)O\(_2\) (Knörzer et al., 1996).

RESULTS

Characterization of Ubi: Sub1A transgenic tobacco plants

Transgenic tobacco plants over-expressing Sub1A were generated by infecting leaf discs with engineered A. tumefaciens harboring a binary vector pBin438. Five out of 12 kanamycin-resistant plants were selected randomly for RNA blotting analysis. As shown in Figure 1, Sub1A was stably expressed in all the transgenic plants, and no signal was detected in the wild-type plants. However, the independent transgenic lines manifested varying expression levels with lines of T2, T24 and T27 been the highest.

The different integration sites of Sub1A in the tobacco genome might contribute to the various expression levels of the transgene. In the subsequent assay, transgenic plants highly expressing Sub1A were selected and used for hypoxic treatment.

Sub1A enhances oxidative stress tolerance

Plants grown under low oxygen conditions usually increase the accumulation of ROS, causing subsequent oxidative damage and lipid peroxidation. Thus, MDA, an end product of lipid peroxidation, was quantified in roots of hypoxic treated tobacco plants. As shown in Figure 2, MDA contents in the wild-type and transgenic seedlings were low and indistinguishable under normoxic conditions.
Figure 1. RNA blotting of Sub1A in the tobacco plants. Total RNA was extracted from four-leaf stage tobacco roots stressed by hypoxia for 48 h, and hybridized with α-[32P]-labeled probe. About 10 µg RNA was used in the blot and rRNA was used as loading control. WT, wild-type plants; T2, T11, T23, T24 and T27 are five independent Sub1A over-expressing lines.

Figure 2 MDA contents in the tobacco roots. Four-leaf stage tobacco seedlings were exposed to hypoxia for 48 h, and MDA contents were determined. WT, wild-type plants; T2, T24 and T27 are three independent Sub1A over-expressing lines. Values at the same treatment having the same letter are not significantly different at P ≤ 0.05, by LSD test. Values shown are means ± SE of three replicates.

When exposed to hypoxic stress for 48 h, the wild-type plants displayed a 2-fold increase in MDA content. However, MDA content in the transgenic plants remain largely unaffected, indicating that the transgenics suffer less from oxidative stress and the over-expression of Sub1A restrains accumulation of MDA triggered by hypoxia.

Sub1A upregulates activities of antioxidant enzymes

The abundance of intercellular ROS is tightly regulated through antioxidant enzymes. Among them, SOD, APX and CAT are major enzymes that detoxify superoxide and hydrogen peroxide under stress conditions. Table 1 presents the elevated activities of the main antioxidant enzymes in tobacco roots in response to hypoxic stress. There were no significant differences in SOD activity between the transgenic lines and the wild-type plants grown under normoxic conditions. In contrast, exposure to hypoxia led to a significant increase in SOD activity in roots of T2, T24 and T27 lines (with a 3.1-, 5.4-, and 4.2-fold increase, respectively) compared with that of wild-type (only a 1.4-fold increase). A similar pattern was observed for the activities of APX and CAT under hypoxic conditions. These results demonstrate that Sub1A upregulates antioxidant enzyme activity, and thus contribute to lessening oxidative stress.
DISCUSSION

Hypoxic stress caused by waterlogging, flooding or submergence seriously constraints plant growth and development, primarily through inhibition of aerobic energy-generating system and the subsequent generation of ROS (Yordanova et al., 2004; Fukao et al., 2006). Accordingly, plants have evolved a set of adaptation mechanisms, including respiration adjustment and enhanced expression of ROS scavenging enzymes, to cope with hypoxic stress. Here, we reported the enhanced hypoxic stress tolerance of tobacco plants over-expressing Sub1A, a submergence tolerance gene.

Fermentative metabolism provides an adaptation strategy which allows glycolysis to continue under low oxygen conditions (Maricle et al., 2006). The highly induced activity of ADH and PDC (enzymes involved in fermentative glycolysis) in the Sub1A overexpressor indicates a capacity of quick acclimation response to the changing environment (Figure 3) consistent with what has been observed in Sub1A introgression rice (Fukao et al., 2006). These results also agree with those of Ismond et al. (2003) that Arabidopsis plants over-expressing either PDC1 or PDC2 had improved hypoxic survival. In addition, adh and pdc loss-of-function mutants in rice and Arabidopsis succumbed rapidly to low oxygen stress (Rahman et al., 2001; Kürsteiner et al., 2003), confirming the vital role of fermentative metabolism in plants response to hypoxia.

Yordanova et al. (2004) have shown that root oxygen deficiency posed photodamaging to barley via an increased generation of ROS, which elevates the extent of lipid peroxidation and consequently leads to the formation of MDA. The significant higher MDA production in the wild-type plants indicates the excessive accumulation of ROS and the resulting greater degree of lipid peroxidation. And this is largely due to the relatively lower activity of ROS scavenging enzymes in the wild-type plants encountering hypoxic stress (Table 1). In contrast, the transgenic plants were able to sustain high activities of SOD, APX and CAT, facilitating an effective detoxification of ROS, and thereby protecting the plant from severe oxidative damage. The higher activities of ROS scavenging enzymes might come from their increased transcripts.

Values at the same treatment having the same letter are not significantly different at P≤0.05 by LSD test. Values shown are means ± SE (n=4). WT, wild-type plants; T2, T24 and T27 are three independent Sub1A over-expressing lines.

Sub1A activates ethanolic fermentation

Plants grown under conditions of oxygen deprivation tend to generate metabolic energy by ethanolic fermentation, which requires ADH and PDC as key regulating enzymes. To investigate the role of Sub1A in ethanolic fermentation during hypoxia, activities of ADH and PDC were evaluated in roots of the wild-type and transgenic lines (Figure 3). ADH and PDC exhibited a basal and comparable level of activity in both the wild-type and the transgenic lines under normoxic conditions, and an increased level in response to hypoxic stress. However, the increase in activity was more pronounced in the transgenics than in the wild-type. ADH activity showed only a 1.8-fold increase in the wild-type, but a 5.4- to 6.8-fold increase in the transgenics depending on the lines (Figure 3A). Like ADH, PDC activity displayed a greater increase in the transgenic plants than in the wild-type under hypoxic stress (Figure 3B).

Table 1. Activities of main ROS scavenging enzymes (SOD, APX and CAT) in tobacco roots. Four-leaf stage tobacco seedlings were exposed to hypoxia for 48 h, and crude protein was isolated. Units for enzyme activity are U/mg protein.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>WT</th>
<th>T2</th>
<th>T24</th>
<th>T27</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>Normoxic</td>
<td>5.7±0.4a</td>
<td>6.1±0.5a</td>
<td>5.5±0.2a</td>
<td>6.0±0.1a</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>8.0±0.4c</td>
<td>19.9±1.1b</td>
<td>29.8±1.9a</td>
<td>25.4±1.7a</td>
</tr>
<tr>
<td>APX</td>
<td>Normoxic</td>
<td>11.2±0.7a</td>
<td>13.0±0.6a</td>
<td>12.4±0.5a</td>
<td>12.1±0.3a</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>22.2±1.4c</td>
<td>39.7±2.4ab</td>
<td>34.2±1.6b</td>
<td>44.7±1.9a</td>
</tr>
<tr>
<td>CAT</td>
<td>Normoxic</td>
<td>10.3±1.3a</td>
<td>9.2±0.8a</td>
<td>11.1±1.4a</td>
<td>9.8±1.2a</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>16.9±1.7c</td>
<td>42.4±2.1a</td>
<td>36.3±1.4ab</td>
<td>35.0±1.8b</td>
</tr>
</tbody>
</table>

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**Figure 3** Activities of ADH and PDC in tobacco roots. Four-leaf stage tobacco seedlings were exposed to hypoxia for 48 h, and crude protein was isolated. Units for enzyme activity are U/g protein. WT, wild-type plants; T2, T24 and T27 are three independent Sub1A over-expressing lines. (A) ADH activity; (B) PDC activity. Values at the same treatment having the same letter are not significantly different at P ≤ 0.05, by LSD test. Values shown are means ± SE of three replicates.

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