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Full Length Research Paper

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-

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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 gibberellic acid (GA) responsiveness during submergence by increasing accumulation of the GA signal repressors Slender Roce-1(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_4 seeds were used in the hypoxic stress analysis. The probes used for Northern blot were the full-length cDNAs of *Sub1A* labeled with α-[^{32}P]-dCTP via PCR from plasmid pBin438. Hybridization was carried out using standard procedures (Sambrook et al., 1989) with 10 about 10 µg 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 been 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 µL L^{-1} (equals to approximately 80% air saturation) for normoxic treatment or 0.05 µL L^{-1} (0.6% air saturation) for hypoxic treatment, by continuously bubbling the solution with 100% (v/v) O_2 or 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 m of 80% (v/v) ethanol solution on ice and then centrifuged at 16,000 *g* for 20 min at 4℃. 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℃ 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 spectro-photometrically 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_2O_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.

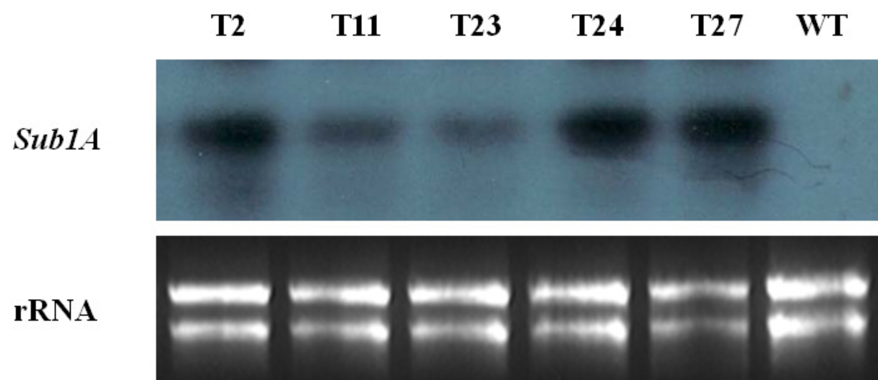

Figure 1. RNA blotting of *Sub1A* in the tobacco plants. Total RNA was extracted from four-leaf stage tobacco roots stressed by hypoxia for 48 h, and hybridized with α-[^{32}P]-labeled probe. About 10 µg RNA was used in the blot and rRNA was used as loading control. WT, wild-type plants; T2, T11, T23, T24 and T27 are five independent *Sub1A* over-expressing lines.

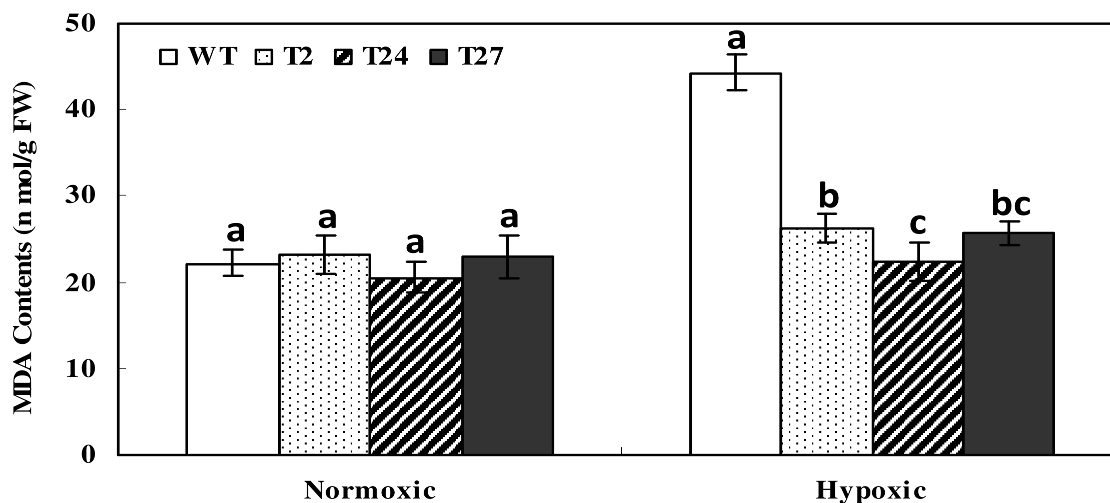

Figure 2 MDA contents in the tobacco roots. Four-leaf stage tobacco seedlings were exposed to hypoxia for 48 h, and MDA contents were determined. WT, wild-type plants; T2, T24 and T27 are three independent *Sub1A* over-expressing lines. Values at the same treatment having the same letter are not significantly different at $P \leq 0.05$, 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.

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.

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

Values at the same treatment having the same letter are not significantly different at $P \leq 0.05$ 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).

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 photoxidative damage 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 accu- mulation 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. Similarly, Fukao et al. (2011) showed that *Sub1A* diminishes the ROS accumulation via increasing the abundance of transcripts encoding ROS scavenging enzymes.

Through transcriptome analysis, Jung et al. (2010) showed that *Sub1A* influences multiple tolerance-associated pathways governing anaerobic respiration, antioxidant systems and hormone responses. In this research, through a transgenic approach we have further demonstrated that the over-expression of *Sub1A* contributes to the enhanced hypoxic stress tolerance. Therefore, *Sub1A* is also likely to be applicable to other economically important crops for breeding new varieties with enhanced tolerance to hypoxia.

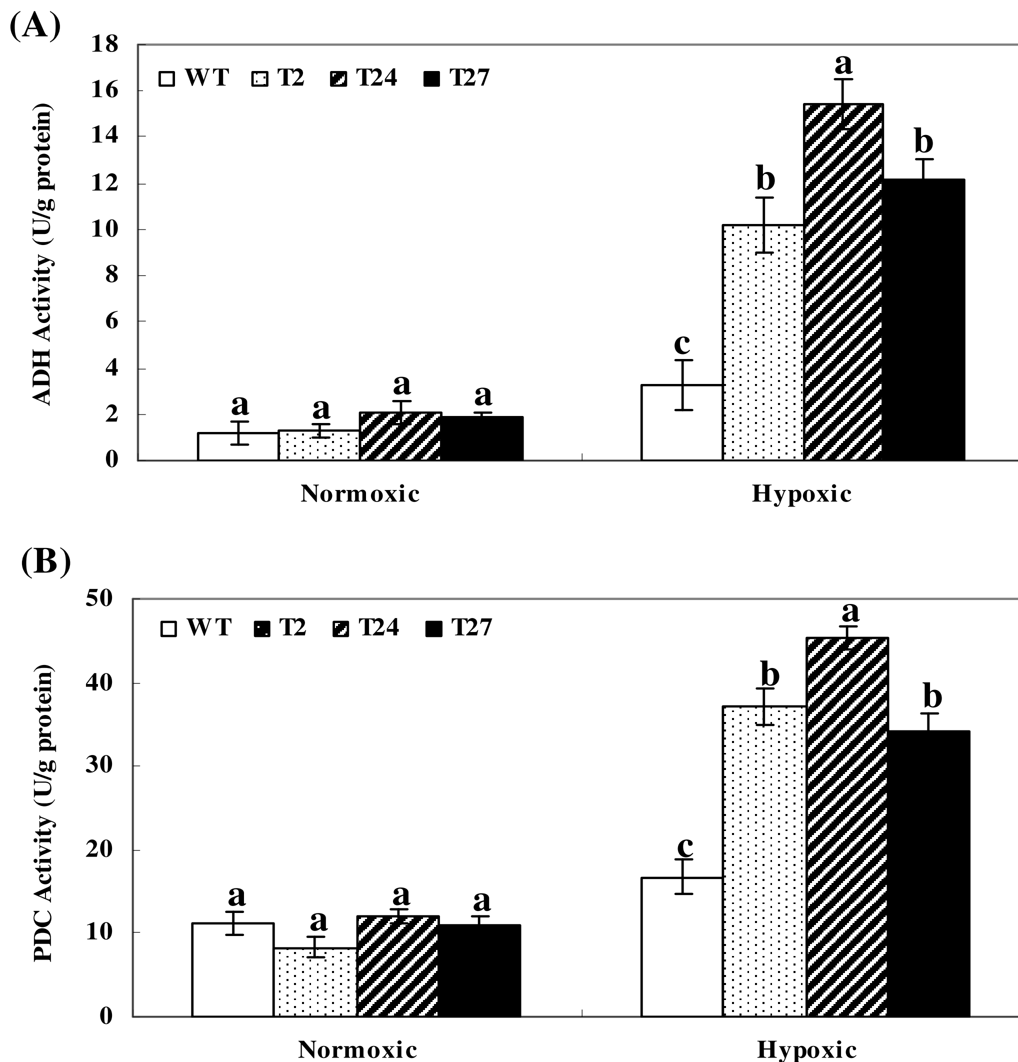

Figure 3 Activities of ADH and PDC in tobacco roots. Four-leaf stage tobacco seedlings were exposed to hypoxia for 48 h, and crude protein was isolated. Units for enzyme activity are U/g protein. WT, wild-type plants; T2, T24 and T27 are three independent *Sub1A* over-expressing lines. (A) ADH activity;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

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