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ORIGINAL RESEARCH

Ectopic Expression of a Cold-Responsive CuZn Superoxide Dismutase Gene, *SodCc1*, in Transgenic Rice (*Oryza sativa* L.)

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Abstract A cytosolic antioxidant enzyme gene, *SodCc1*, encoding CuZn superoxide dismutase was characterized from rice. *SodCc1* mRNA was up-regulated by cold (4°C and 12°C) and by abscisic acid (ABA) treatment. Transgenic rice plants of *Ubi:SodCc1* were generated and overexpression of *SodCc1* was confirmed at both transcriptional and translational levels. A stress tolerance test via chlorophyll fluorescence at the seedling stage showed no enhanced tolerance by *Ubi:SodCc1* plants to cold or methyl-viologen-induced oxidative stress, but they were slightly resistant to drought. Our wilting assay demonstrated no improvement in tolerance to either cold or drought, indicating that cytosolic *SodCc1* might not be significantly involved in conferring such tolerances in rice.

Keywords Abiotic stress · CuZn superoxide dismutase · Rice · *SodCc1* gene · Transgenic rice

Environmental stresses, such as cold and drought, are associated with increased production of reactive oxygen species (ROS; Foyer and Mullineaux 1994). ROS, which include the superoxide anion radical ($O^{2.-}$), hydroxyl radical (OH·), and hydrogen peroxide (H_2O_2), are generated from all aerobic cells during metabolic processes (Foyer et al. 1994; Asada 1999). These can react very rapidly with DNA, lipids, and proteins, causing severe cellular damage (van Breusegem et al. 1999). Tolerance to some abiotic stresses is correlated with an increased capacity to scavenge or detoxify ROS (Malan et al. 1990). Enzymes involved in selective detoxification include superoxide dismutase (SOD), which catalyzes superoxides to H_2O_2 and O_2; catalase (CAT), converting H_2O_2 to water; and glutathione reductase (GR) and ascorbate peroxidase (APX), which scavenge H_2O_2 in the ascorbate–GSH cycle (Hammond-Kosack and Jones 1996; Ahmad et al. 2008). Of these, SODs initiate the defense system by removing superoxide (Beyer et al. 1991) and can be classified into three distinct groups by their metal cofactors: CuZn, Mn, and Fe. CuZnSOD is present in the cytosol and chloroplasts, whereas MnSOD and FeSOD are localized to the mitochondria and chloroplasts, respectively (Jackson et al. 1978; Bowler et al. 1992). Several SOD cDNAs have been cloned in plants (Bowler et al. 1992; Sakamoto et al. 1992; Perl et al. 1993; Kaminaka et al. 1997), and transgenics with enhanced SOD activity have been produced and characterized (Tepperman and Dunsmuir 1990; Bowler et al. 1991; Pitcher et al. 1991; McKersie et al. 1993; Perl et al. 1993; Sen Gupta et al. 1993; Kornyeyev et al. 2001; Wang et al. 2005; Kim et al. 2007). For instance, transgenic tobacco, expressing a MnSOD cDNA, is less damaged when exposed to paraquat, an oxygen free-radical-generating herbicide (Bowler et al. 1991). Similarly, transgenic potato plants expressing tomato CuZnSOD are more resistant to paraquat (Perl et al. 1993). Transgenic tobacco and cotton that overexpress chloroplastic CuZnSOD and chloroplast-targeted MnSOD show enhanced photosynthetic rates under chilling stress (Sen Gupta et al. 1993; Kornyeyev et al. 2001). To obtain plants with improved tolerance to oxidative and abiotic stresses, several research groups have expressed multiple antioxidant enzymes, e.g., SOD and APX, simultaneously in transgenic plants and then examined their

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tolerance (Kwon et al. 2002; Tang et al. 2006; Lee et al. 2007a, b).

In rice, several SOD genes have been isolated. A mitochondrial MnSOD gene, *SodA1*, and a cytosolic CuZnSOD gene, *SodCc2*, are strongly induced by drought, salinity, and abscisic acid (ABA), while *SodCc1* is not induced by drought or salinity, and expression of a FeSOD gene, *SodB*, is decreased by drought (Sakamoto et al. 1993, 1995; Kaminaka et al. 1997). Interestingly, the plastidic CuZnSOD gene, *SodCp*, is induced by salinity only in the light and after H_2O_2 treatment (Kaminaka et al. 1999). The relationship between SOD activity and stress tolerance has been investigated in rice. High activity of SOD in collaboration with other antioxidant enzymes is important to plant recovery after water stress and for minimizing spikelet sterility that arises from drought stress (Srivalli et al. 2003; Selote and Khanna-Chopra 2004). The activity of antioxidant enzymes, including SOD, is positively correlated with chilling, drought, and salt sensitivity among rice varieties (Dionisio-Sese and Tobita 1998; Guo et al. 2006; Moradi and Ismail 2007). A novel *cis*-element, CORE (coordinate regulatory element for antioxidant defense), responsive to oxidative stress has been identified from the promoter regions of rice antioxidant defense genes, including *SodCc1* (Tsukamoto et al. 2005). Transgenic rice plants expressing pea MnSOD and mangrove CuZnSOD have also been generated and characterized (Wang et al. 2005; Prashanth et al. 2007).

In this study, we investigated whether expression of the cytosolic SOD gene, *SodCc1*, could be induced by various stresses, and we examined its role in conferring abiotic stress tolerance by transgenic rice.

Materials and Methods

Plant Material, Growing Conditions, and Stress Treatments

Germinated seeds of Japonica rice (*Oryza sativa* cv. Dongjin) were hydroponically grown in distilled water for 4 days then for another 4 days in Yoshida solution (Yoshida et al. 1976). Conditions included 16 h (day) at 29°C and 8 h (night) at 21°C. The 8-day-old seedlings were exposed to drought (air drying on filter paper), cold (4°C), salt (250 mM NaCl), or ABA (100 μM ABA) treatments under continuous light for up to 24 h. To cold-treat mature plants, field-grown rice at the pre-anthesis stage was exposed to 12°C under continuous light for 4 days in a growth chamber.

Bacterial Strains and Plasmids

Escherichia coli strain XL-1 Blue MRF' and *Agrobacterium tumefaciens* LBA4404, pGA1611 (Kim et al. 2003), and a disarmed Ti plasmid, pAL4404 (Hoekema et al. 1983), were used for routine cloning and rice transformation experiments.

Generation of Transgenic Rice Plants

Full-length cDNA of *SodCc1* was cloned into the *Hpa*I and *Kpn*I sites of binary vector pGA1611 (Kim et al. 2003) under control of the maize *ubiquitin* promoter. Japonica rice cv. Dongjin was transformed by the *Agrobacterium*-mediated co-cultivation method (Hiei et al. 1994; Lee et al. 1999) and hygromycin-resistant plants were selected on a 40 mg L^{-1} hygromycin-B-containing medium. Regenerated plants were transferred to the greenhouse for growth until harvest.

Southern and Northern Blot Analyses

Genomic DNA was extracted from transgenic and wild-type leaves as described by Chen and Roland (1999). DNA (5 μg), digested with *Hin*dIII for 12 h at 37°C, was separated on a 0.8% agarose gel and transferred to a Hybond-N membrane (Amersham, UK) using a vacuum

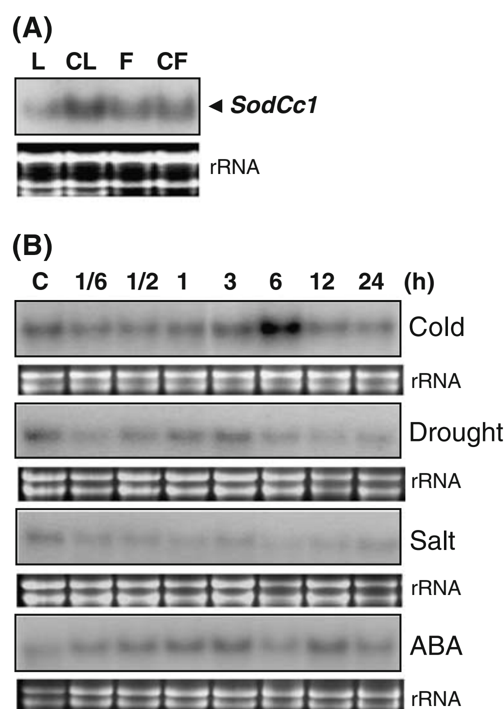

Fig. 1 Northern blot analysis of *SodCc1*. **a** Total RNA from mature leaves (*L*), cold-treated leaves at 12°C for 4 days (*CL*), young florets (*F*), and cold-treated florets at 12°C for 4 days (*CF*) was used for hybridizations with radiolabeled *SodCc1* cDNA probe. **b** Total RNA from whole seedlings treated for indicated times was used for hybridization with radiolabeled *SodCc1* cDNA probe. *C* control; cold, 4°C; drought, air drying; salt, 250 mM NaCl; ABA, 100 μM ABA. EtBr-stained rRNA bands indicate amount of RNA loading

transfer system (Hoefer, USA). Total RNA was isolated with Trizol reagent (Molecular Research Center, USA) from either stress-treated or untreated samples. Total RNA (30 μg) was resolved on a 1.3% formaldehyde agarose gel and blotted onto a nylon membrane (Sambrook et al. 1989). For probe preparation, *SodCc1* cDNA was labeled with (α-^{32}P) dCTP using the random priming method (Feinberg and Vogelstein 1983). After hybridization, the membrane was washed with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature (RT) for 15 min; 1× SSC, 0.1% SDS at RT for 15 min; and 0.1× SSC, 0.1% SDS at RT for 15 min. Hybridization signals were detected with a BAS-1500 image analyzer (Fuji, Japan) and exposed on Hyperfilm™ MP (Amersham).

SOD Native Gel Assay

Mature leaves from individual transgenic lines were ground in liquid nitrogen, and SOD activity of their homogenates was measured via native gel electrophoresis as described by McCord and Fridovich (1969).

Stress Tolerance Test

To assess their tolerance to stress, we examined either chlorophyll fluorescence or the wilting ratio in transgenic plants. A plant efficiency analyzer (Hansatech, UK) was used for measuring fluorescence as an indicator of Photosystem II activity. The youngest extended leaves from 8-day-old seedlings were segmented to 5 cm long and then subjected to the following stress treatments: (1) floating on distilled water at 4°C for 24 h (cold), (2) air drying under 70% relative humidity at 29°C (drought), or (3) floating on a methyl viologen (MV) solution (100 μM) for 12 h in the dark and then for 6 h under white fluorescent lamps at 70 μmol m^{-1} s^{-1} (oxidative stress). Fluorescence signals were measured after 30 min of dark adaptation. The ratio Fv/Fm was calculated to assess functional damage (Genty et al. 1989). The 8-day-old whole plants were tested for wilting according to the following parameters: (1) 4°C for 4 days, then 7 days of recovery (cold); (2) Yoshida solution plus 250 mM NaCl for 2 days, then 7 days of recovery (salt); (3) water retraction for 2 days, then 7 days of

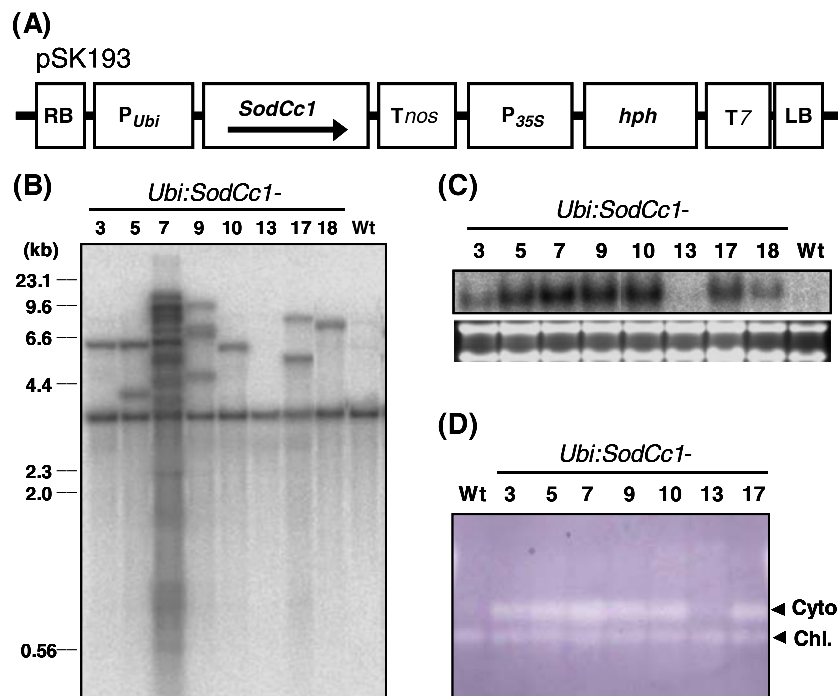

Fig. 2 Generation and analysis of *SodCc1*-transgenic plants. **a** Construction of *SodCc1*-overexpression (*Ox*) binary vector, pSK193, for rice transformation. Full-length *SodCc1* cDNA was fused under maize *ubiquitin* promoter of pGA1611. P_{Ubi} maize *ubiquitin* promoter, P_{35S} CaMV 35S promoter, *Tnos* terminator sequence of *nopaline synthase* gene, *T7* terminator sequence of transcript *7*, *hph* hygromycin phosphotransferase gene, *RB* and *LB* right and left border sequences, respectively, of Ti plasmid from *A. tumefaciens*. **b** Southern blot analysis of *SodCc1* transgenic plants. Genomic DNA (5 μg) from transgenic lines was digested with *Hin*dIII and hybridized with radiolabeled *SodCc1* cDNA probe. Positions and sizes (kbp) of *Hin*dIII-digested λ DNA fragments are indicated. **c** Northern blot analysis of *SodCc1* transgenic plants. Total RNA (30 μg) from mature flag leaves was hybridized with radiolabeled *SodCc1* cDNA probe. EtBr-stained rRNA bands in *lower panel* indicate amount of RNA loading. **d** SOD native gel assay of *SodCc1* transgenic plants. Total protein from mature leaves of transgenic lines was extracted, 20 μg of protein was separated on native PAGE, and SOD was detected by activity staining. *Cyto.* spot of cytosolic SOD activity, *Chl.* spot of chloroplastic SOD activity. *Numbers* and *Wt* indicate transgenic lines and wild type, respectively

recovery (drought); or (4) 45°C for 2 h, then 7 days of recovery (heat).

Results and Discussion

Expression Analysis of *SodCc1*

In rice, SOD activity is closely correlated with stress tolerance (Dionisio-Sese and Tobita 1998; Guo et al. 2006; Moradi and Ismail 2007). Therefore, determining the role of the rice SOD gene during abiotic stress is important to our understanding of that tolerance mechanism. Nevertheless, few reports have been made of such functioning in transgenic plants. Therefore, we first searched for and identified an expressed sequence tag (EST) homologous to a SOD gene from a rice cDNA library of immature seed coats (Lee et al. 2005). Sequence analysis confirmed that this EST was identical to the rice cytosolic CuZnSOD gene, *SodCc1*. To investigate its expression patterns, Northern blot analyses were performed with mature leaves, florets at the pre-anthesis stage, and whole seedlings. *SodCc1* was weakly expressed in the leaves compared with the flowers (Fig. 1a). This expression was up-regulated by cold stress (12°C) in both leaves and florets, although the level of induction was much higher in the former. We also examined expression in seedlings under cold (4°C), drought, salinity, and ABA stress (Fig. 1b). *SodCc1* transcripts were induced transiently by the low temperature and gradually by ABA, but not by salt or drought. This pattern is consistent with that reported by Kaminaka et al. (1999).

Generation of Transgenic Rice Overexpressing *SodCc1* cDNA

To study the role of *SodCc1* in conferring stress tolerance, we produced transgenic rice plants ectopically expressing that gene. For this, a full-length *SodCc1* cDNA was fused under the maize ubiquitin promoter (*Ubi*) of binary vector pGA1611 (Kim et al. 2003), generating pSK193 (Fig. 2a). After *Agrobacterium*-mediated co-cultivation of scutellum calli, we obtained eight hygromycin-resistant plantlets. These were investigated via Southern blots for the presence of the transgene using *SodCc1* cDNA as a probe. We confirmed seven independent *Ubi:SodCc1* lines (*Ubi:SodCc1*-3, -5, -7, -9, -10, -17, and -18) with one false transgenic (*Ubi:SodCc1*-13; Fig. 2b). Those lines contained one to several copies of the transgene in addition to the endogenous gene. We also examined whether those transgenic plants had changed phenotype, but found no significant alterations in morphology during two successive generations under greenhouse and field conditions.

Ectopic Expression of *SodCc1* in Transgenic Rice

To assess the expression of *SodCc1* in transgenic lines, total RNA isolated from their flag leaves was subjected to Northern blot analysis. All true transgenic lines of *Ubi:SodCc1* showed a high level of expression (Fig. 2c). *SodCc1* mRNA expression was greatest in lines 5, 7, 9, 10, and 17. We also used a SOD native gel assay to determine that enzymatic activity was changed in the *Ubi:SodCc1* plants. All true transgenics exhibited strong cytosolic SOD activity (Fig. 2d), whereas chloroplastic enzymatic activities were similar among the transgenic lines and wild-type (Wt) control. These results indicated that

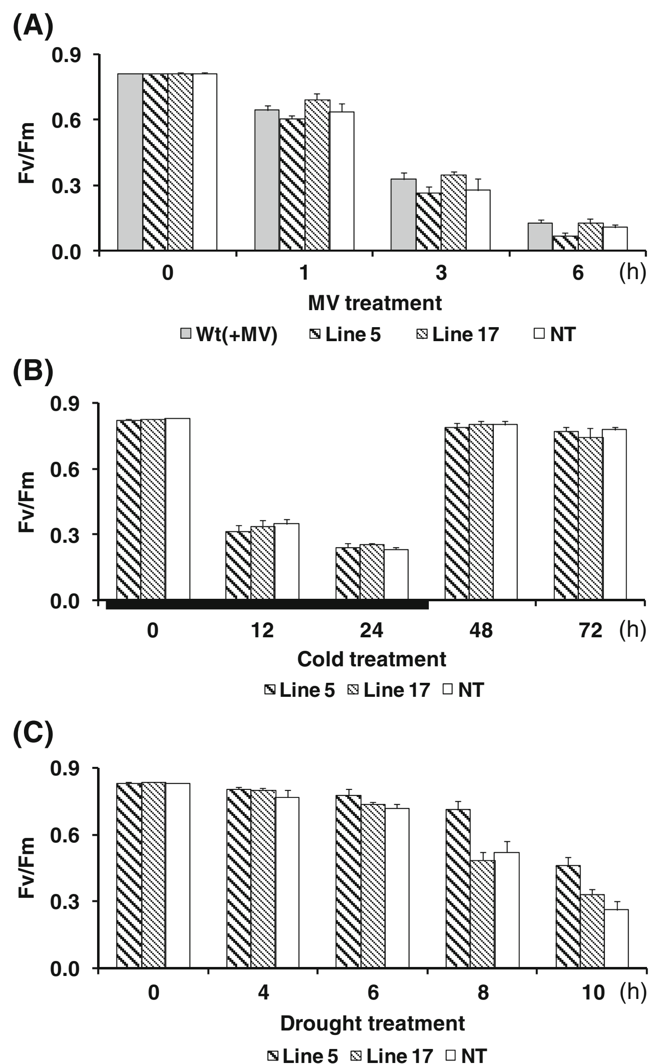

Fig. 3 Measurement of stress tolerance by *SodCc1* transgenic plants via chlorophyll fluorescence. Changes in fluorescence of youngest leaf from lines 5 and 7 were monitored during indicated times for treatment with 100 μM MV **(a)**, 4°C **(b)**, or drought **(c)**. Cold-stressed plants were treated for only 24 h (indicated as *bar* below *x*-axis), then recovered up to 48 h under normal growing conditions. Standard deviations indicated as *vertical bars* are from experiments repeated five times

SodCc1 was highly expressed in *Ubi:SodCc1* plants at both mRNA and protein levels. Based on seed availability and transcript amounts, we selected transgene-homozygotes with their segregating non-transgenic lines (NT). Lines 5 and 17 were then tested for their degree of stress tolerance.

Abiotic Stress Tolerance in *Ubi:SodCc1* Plants

Many environmental stresses increase ROS production. Because such damage can lead to cell death, this catastrophic event should be minimized to ensure cell survival. Others have previously reported several transgenic species with antioxidant enzymes, including chloroplastic or cytosolic isozymes of CuZnSOD, that have enhanced tolerance (Sen Gupta et al. 1993; Kornyeyev et al. 2001; Badawi et al. 2004; Murgia et al. 2004; Tang et al. 2006; Lee et al. 2007a).

To examine stress tolerance in our *Ubi:SodCc1* plants, we conducted both chlorophyll fluorescence assays and wilting analyses at the seedling stage. After 6 h of MV treatment, Fv/Fm for the Wt and NT was reduced to 0.13± 0.01 and 0.11±0.01, respectively (Fig. 3a). Values from lines 5 and 17 declined similarly to those of Wt and NT, indicating that *SodCc1* overexpression had no enhancing effect on MV-induced oxidative stress. This differed from results reported by Prashanth et al. (2007), perhaps because of the origin of the CuZnSOD gene used in respective studies. That is, Prashanth et al. (2007) had utilized cytosolic CuZnSOD from halophytic mangrove plants with strong SOD activity (Cheeseman et al. 1997). Likewise, the lack of influence on MV tolerance might have been because *SodCc1* is expressed in the cytosol instead of the chloroplast, the latter being particularly sensitive to ROS (Foyer et al. 1994). Thus, those photosystems could be easily inactivated by MV-induced oxidative stress (Choi et al. 2001). Over the course of our cold stress treatment, Fv/Fm values for lines 5 and 17 were reduced to 0.32–0.34 after 12 h, then further declined to 0.24–0.25 at 24 h (Fig. 3b). During the recovery period, Fv/Fm was maintained at 0.75–0.77, a range similar to that for NT. For dehydration stress of up to 10 h, the Fv/Fm of NT was reduced to 0.26±0.04 compared with the rather higher values of 0.46±0.04 (line 5, 70% more than for NT) and 0.33±0.02 (line 17; Fig. 3c). Tolerance was quantified by calculating the wilting ratio following cold, drought, salt, or heat stress applications. Ratios for both transformed lines were not changed significantly from that of NT (data not shown).

Based on our determination of cold-stress-responsive expression by the cytosolic CuZnSOD enzyme gene, we generated *SodCc1*-overexpression plants and examined their tolerance to abiotic stress. Transgenic lines 5 and 17 had greater chlorophyll fluorescence than the wild-type or NT after drought treatment (Fig. 3c), suggesting that such overexpression was a factor in maintaining the photosynthetic rate, probably because of enhanced ROS scavenging activity. Similarly, Prashanth et al. (2007) have reported increased drought tolerance by transgenic rice plants that overexpress the cytosolic CuZnSOD gene from mangrove. However, our wilting ratio analysis revealed that the drought tolerance by transgenics did not differ from that of NT seedlings, suggesting that enhanced cytosolic SodCc1 activity was not adequate to confer whole plant tolerance.

By contrast, many researchers have reported that SOD-transgenic organisms, including animals and bacteria, show no improvement in their degree of stress tolerance (Scott et al. 1987; Tepperman and Dunsmuir 1990; Pitcher et al. 1991; Reveillaud et al. 1991; Orr and Sohal 1993; Payon et al. 1997). Therefore, overexpression of a single antioxidant enzyme may not be sufficient to achieve this tolerance due to the complexity of the ROS scavenging system and the presence of physiologically functional, multiple antioxidant enzymes.

An antioxidant defense system that includes SOD is important for recovery after water stress and for maintaining the fertility of rice spikelets at the mature stage (Srivalli et al. 2003; Selote and Khanna-Chopra 2004). Because *SodCc1* was highly up-regulated in our cold-treated mature leaves, unlike in whole cold-treated seedlings, the stress tolerance of these transgenic lines will be further examined at various developmental stages.

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