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Molecular Characterization of *Arabidopsis* and *Brassica juncea* Cu/Zn-Superoxide Dismutases Reveals Their Regulation of Shoot Regeneration

Tze Soo Lim · Thiruvetipuram Rajam Chitra · Boon Hui Tay · Eng Chong Pua · Hao Yu

Abstract Superoxide dismutases (SODs) are ubiquitous metalloenzymes that catalyze the dismutation of superoxide radicals (O_2) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). In this study we characterized an *Arabidopsis thaliana* CuZnSOD (*CSD1*), a close ortholog of a previously identified *Brassica juncea* CuZnSOD (*MSOD1*). *CSD1* and other two homologs *CSD2* and *CSD3* were spatially regulated in *Arabidopsis*, and *CSD1* exhibited distinct expression patterns in response to different stress treatments. To investigate the *in vivo* function of SOD, transgenic *Arabidopsis* plants, expressing sense and antisense *MSOD1* RNAs, were generated and those with altered SOD activity were selected for further characterization. Although SOD transgenic plants exhibited normal phenotypes, the shoot regeneration response in transgenic explants was significantly affected by the modulated SOD activity and the corresponding H_2O_2 levels. Transgenic explants with downregulated SOD activity were poorly regenerative, whereas those with upregulated SOD activity were highly regenerative. These results suggest that shoot regeneration *in vitro* is regulated by the SOD activity.

Keywords *Arabidopsis thaliana* · Cu/Zn-superoxide dismutase · Shoot regeneration · Hydrogen peroxide · Polyamine

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Introduction

In plants, reactive oxygen species (ROS) are generated as byproducts of normal metabolic processes (Bowler and others 1994). At low concentration, ROS fulfill crucial physiologic functions in the plant. Different developmental or environmental signals feed into the ROS network to perturb ROS homeostasis, which allows the regulation of specific developmental, metabolic, and defense pathways (Mittler and others 2004). However, biotic and abiotic stresses can lead to alteration of redox homeostasis, resulting in oxidative stress (Apel and Hirt 2004).

SODs constitute the first line of cellular defense against oxidative stress by catalyzing the dismutation of the harmful O_2^- anion to O_2 and H_2O_2 (Bowler and others 1992). The role of SODs against oxidative or environmental stress has been extensively investigated in transgenic plants overproducing these enzymes (Bowler and others 1991; McKersie and others 1993, 1996; Perl and others 1993; Sen Gupta and others 1993a, b; Trolinder and Allen 1994; Van Camp and others 1994, 1996; Slooten and others 1995; Arisi and others 1998; Tanaka and others 1999; Van Breusegem and others 1999, Tertivanidis and others 2004; Wang and others 2005). However, none of these studies has revealed the effects of SOD activity on plant morphogenesis.

Different kinds of stress, including oxidative stress, have been shown to significantly increase plant regeneration in various species (Lee and others 1999; Immonen and Anttila 1999; Immonen and Robinson 2000; Mundhara and Rashid 2001; Ikeda-Iwai and others 2003). One possible link between oxidative stress and plant morphogenesis could be H_2O_2 that may serve as a signaling molecule to mediate cellular responses during plant morphogenesis since several studies have implicated H_2O_2 in this process (Cui and

others 1999; Luo and others 2001; Papadakis and Roubelakis-Angelakis 2002; Libik and others 2005). From the data obtained to date, it is difficult to distinguish if the alteration of H_2O_2 is the cause or effect of plant morphogenesis *in vitro*.

Although results from the above-mentioned studies have implicated H_2O_2 as a mediator in shoot organogenesis, direct molecular evidence showing its involvement in shoot regeneration has not been reported to date. In a previous study, we have isolated a CuZnSOD from *Brassica juncea* (*MSOD1*; Liu and others 1998). To further understand the function of CuZnSODs, we have examined the expression patterns of the *Arabidopsis* orthologs of *MSOD1* (*CSD1-3*) and generated transgenic *Arabidopsis* plants with altered activity of SODs. Our results showed that *CSD1-3* genes were spatially regulated and *CSD1* was responsive to stress. We found that alteration of SOD activity in *Arabidopsis* changes the endogenous H_2O_2 levels, resulting in a change in shoot regeneration response.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) seeds were surface sterilized by sequential washes of 70% ethanol (v/v) for 1 min, 15% Clorox® (1% sodium hypochlorite) solution for 10 min, and rinsed with sterile distilled water before being sown onto agar plates. The Murashige and Skoog (MS) agar medium contained MS salts (Murashige and Skoog 1962), 3% sucrose, and 0.8% agar, pH 5.7. The plates were incubated at 4°C for 2 days before being placed at room temperature under white light for germination and growth. For growth on soil, seeds were sown on commercial potting compost and stratified at 4°C in a cold room for 4 days before being transferred to a Biotron growth chamber (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) under constant illumination at a light intensity of 50–100 µE m^{-2} s^{-1} and temperature of 22°C. The humidity was maintained at 60%.

Genomic DNA Isolation and Southern Analysis

Genomic DNA was isolated using the DNAzol ES kit according to the manufacturer's protocol (Molecular Research Centre, Inc., Cincinnati, OH, USA). For Southern analysis, 10 µg of genomic DNA was digested with different restriction endonucleases and separated on 1% agarose gel by electrophoresis in 1 × Tris-Borate-EDTA buffer. The gel was treated with 0.25 N HCl for 10 min, denatured twice in a solution of 0.5 N NaOH/1.5 M NaCl, each for 15 min

with shaking, rinsed with sterile water, and transferred to the neutralization solution [0.5 M Tris-HCl (pH 7.5) and 3 M NaCl] twice, each for 15 min with shaking. The DNA was transferred overnight onto a positively charged membrane (Roche Diagnostics GmbH, Mannheim, Germany) by capillary action in 10 × SSC [3 M NaCl and 0.3 M sodium citrate (pH 7.0)]. The membrane was crosslinked, prehybridized at 42°C for 2 h in DIG^{TM} Easy Hybridization buffer (Roche Diagnostics GmbH), and hybridized overnight at 42°C in the same buffer containing 25 ng/ml of DIG-labeled double-stranded DNA probe. The membrane was washed in 2 × SSC/0.1% sodium dodecyl sulfate (SDS) twice, each for 5 min at room temperature, followed by 0.5 × SSC/ 0.1% SDS twice, each for 15 min at 68°C, and 0.1 × SSC/ 0.1% SDS for 15 min at 68°C. Detection of the hybridized signal was carried out by capturing the chemiluminescent signal on an X-ray film after CDP-Star^{TM} (Roche Diagnostics GmbH) was applied.

Stress and Chemical Treatments

Dehydration was induced by removing plants from the medium and placing them on a dry filter paper, which was placed in a flow-hood for 30 min. Osmotic stress was imposed by NaCl or PEG (average molecular mass = 6000) treatment. Seedlings were pulled out of agar and placed on filter papers soaked with different concentrations of NaCl or PEG solutions. For the treatment of abscisic acid (ABA), 1-aminocyclopropane-1-carboxylate (ACC), AgNO$_3$, 2-aminoethoxyvinyl glycine (AVG), benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellic acid (GA$_3$), H_2O_2, methyl jasmonic acid (MeJA), putrescine (Put), salicylic acid (SA), spermidine (Spd), and spermine (Spm), different concentrations of the chemicals in appropriate solvent solutions were sprayed on the seedlings. Seedlings were immersed in water of different pHs for pH treatment. For temperature treatment, 2-week-old seedlings in agar plates were incubated at 0°C or 4°C in the dark and at 22°C, 28°C, 30°C, or 37°C under light. For cold treatment, seedlings were thawed at room temperature for 2 h. Control treatments with seedlings soaked or sprayed with solvent solutions or on agar plates under room temperature were also conducted simultaneously. All treatments conducted on Petri dishes were sealed and conducted under light at room temperature for 6 h unless otherwise stated. For phenotypic analysis of stress treatments of transgenic plants, wild-type and transgenic seedlings on the same MS agar plates were subjected to optimum conditions or different concentrations of stress and chemical treatments. Gene expression analyses were repeated at least three times using samples collected separately.

Shoot Regeneration

Shoots were regenerated from *Arabidopsis* root culture as described by Banno and others (2001). Roots of young seedlings were transferred to B5 liquid medium and incubated for 15 days with shaking at 125 revolutions per minute. The roots were cut into approximately 1-cm segments and six such segments were incubated on callus-inducing medium (CIM) containing MS medium (MS salts, Gamborg's B5 vitamins, 1% sucrose, and 0.25% Phytagel as gelling agent) supplemented with 2 μM 2,4-D. After 4 days on CIM, the root segments were transferred to shoot-inducing medium (SIM) containing MS medium supplemented with 0.8 μM indole-3-acetic acid (IAA) and 12.5 μm N^6-Δ^2-isopentyladenine. For each genotype, shoot regeneration experiments were repeated three times with 48 root segments tested in each independent experiment.

RNA Isolation and Real-time PCR

Total RNA from plant tissues was isolated using the RNeasy Kit (Qiagen GmbH, Hilden, Germany) and reverse transcribed by using the Thermoscript^{TM} RT-PCR System (Invitrogen, Carlsbad, CA, USA). Real-time PCR assays were performed in triplicate on a BioRad iCycler iQ Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using tubulin (*TUB2*) as an internal standard. The difference between the cycle threshold (Ct) of the target gene and the Ct of tubulin, $\Delta Ct = Ct_{target\ gene} - Ct_{tubulin}$, was used to obtain the normalized expression of target genes, which corresponds to $2^{-\Delta Ct}$. Each experiment was replicated at least three times using samples collected separately. Primers designed for real-time PCR were as follows: BJS1-P1 (5′-CACTTCCAGGCATTTCATC-3′) and BJS1-P2 (5′-GAAACGGTTCCTGTCACAG-3′) for *MSOD1*, ATS1-P1 (5′-AGGGGTTTCCTGAGATCACAA-3′) and ATS1-P2 (5′-CTTAAGGCCAG-AAACTGTTCC-3′) for *CSD1*, ATS2-P1 (5′-CATTCTCATCTCCTTCTCG-3′) and ATS2-P2 (5′-GATTGGAGACGGTGGAGAT-3′) for *CSD2*, ATS3-P1 (5′-ACTGATGGAAGCTCCTAGAGG-3′) and ATS3-P2 (5′-GCCAGGAGAGAG-TCCTGAGAT-3′) for *CSD3*, and TUB2-P1 (5′-ATCCGTGAAGAGTACCCA-GAT-3′) and TUB2-P2 (5′-AAGAACCATGCACTCATCAGC-3′) for *TUB2*. RT-PCR was repeated at least three times using samples collected separately.

Plasmid Construction and Plant Transformation

The SOD cDNA was cloned into pBluescript SK (+/−) phagemid vector (Stratagene, La Jolla, CA, USA) prior to preparation of sense and antisense cDNA fragments. The sense SOD cDNA of 747 bp was amplified using T3 (5′-AATTAACCCTCACTAAAGGG-3′) and SSOD Rev (5′-CGAGCTCGACGA-CTCACTATAGGG-3′; *Sac*I site underlined) primers. After digestion with *Bam*H1 and *Sac*I, the cDNA was inserted in a sense orientation between the CaMV 35S promoter and nopaline synthase terminator in the pBI121 vector predigested with the same enzymes. The construct was named S-*MSOD1*. An antisense SOD cDNA of 587 bp was amplified using T3 and ASSOD Rev (5′-GCTCTAGAGCGTAATACGACTCACTATAGGGC-3′; *Xba*I site underlined) primers, digested with *Xba*I and *Eco*RV, and inserted in an antisense orientation at the *Xba*I and *Sma*I site between the 35S promoter and the GUS coding sequence in pPZP-TE vector. pPZP-TE was a modified binary vector that harbored the expression cassette of the CaMV 35S promoter, GUS, and NOS terminator derived from pBI121, and contained the neomycin phosphotransferase coding sequence under the control of the nopaline synthase promoter.

Both constructs were introduced into the *Agrobacterium tumefaciens* strain (LBA4404) and used to transform wild-type (Col-0) *Arabidopsis thaliana* by the floral-dip method, as described previously (Clough and Bent 1998). Independent transgenic lines were selected based on their kanamycin-resistant phenotype (50 mg/L) and shoots of these independent lines were maintained by tissue culture of root explants.

Measurement of SOD Activity

The levels of SOD activity in plant tissues were measured as described by Madamanchi and others (1994). This assay was based on the reaction whereby riboflavin in the reaction mixture could be photochemically reduced by illumination. Reduction of riboflavin resulted in the transfer of an electron to O_2 to form O_2, which reduced 4-nitro blue tetrazonium chloride to form blue formazan that could be detected spectrophotometrically at the wavelength of 560 nm. The presence of SOD could inhibit blue formazan formation, thereby decreasing absorbance at 560 nm.

The assay was conducted by homogenizing the tissue (0.2 g) using mortar and pestle. The homogenate was mixed with 1.5 ml of the extraction buffer consisting of 50 mM phosphate buffer (pH 7.0) and 0.1 mM EDTA. The mixture was centrifuged at 18,000 *g* for 10 min at 4°C, after which 20 μl of the supernatant was added to the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM 4-nitro blue tetrazonium chloride, and 25 μM riboflavin. The reaction was initiated by adding the enzyme extract containing 0.7–0.2 μg of protein and placing the mixture under two 15-W fluorescent lamps. The reaction
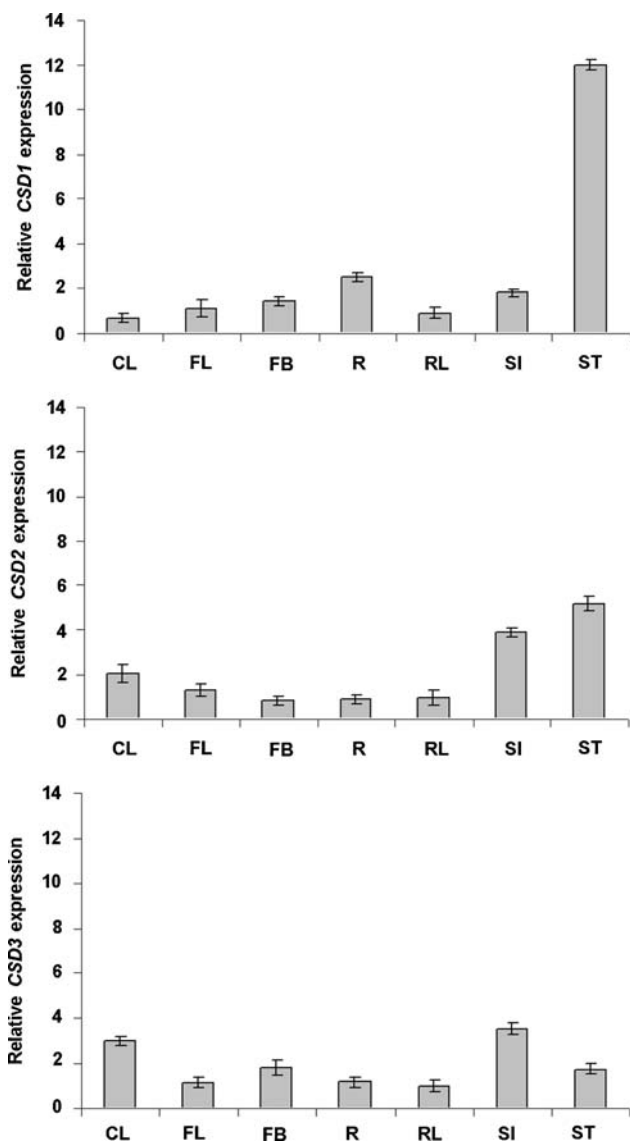
was terminated after 10 min by switching off the light. The mixture was stirred and the color intensity (blue) was measured at 560 nm. The protein concentration in the supernatant was determined by the Bradford assay (Bradford 1976) and the volume of the enzyme extract corresponding to 50% inhibition of the reaction was considered one enzyme unit (Madamanchi and others 1994).

Histochemical Detection of H_2O_2

The levels of H_2O_2 accumulated in cultured tissue were assayed as described previously (Thordal-Christensen and others 1997) with minor modifications. In general, tissues were incubated under gentle shaking for 8 h in 1 mg/ml 3,3-diaminobenzidine-HCl (pH 3.8) in the dark. This was followed by incubation for 10 min in 10 mM H_2O_2, after which tissues were incubated overnight with acetic acid/glycerol/ethanol (1:1:3 v/v/v) solution. The samples were stored in 95% ethanol. The presence of H_2O_2 in the tissue was visualized as reddish brown coloration. At least ten leaves were tested for each line in two experiments and both showed similar results.

Results

Spatial Expression of *CSD1-3* in *Arabidopsis*

In a previous study we isolated a 766-bp full-length cytosolic CuZnSOD cDNA (X95728) from *Brassica juncea* (Liu and others 1998). This cDNA encoded a putative protein MSOD1, which shared 92%, 65%, and 60% amino acid identities with three *Arabidopsis* CuZnSOD

homologs, CSD1 (X60935), CSD2 (AF061519), and CSD3 (AF061520), respectively (Figure 1; Kliebenstein and others 1998). It has been suggested that CSD1 was a general stress-response enzyme because its expression was induced in response to different light regimes, ozone fumigation, and ultraviolet-B irradiation (Kliebenstein and others 1998).

To extend these findings, we investigated in detail whether *SOD* transcripts were spatially regulated in different organs. The expression of *CSD1-3* was examined by real-time PCR from total RNA isolated from various organs of wild-type *Arabidopsis* plants. *CSD1* transcripts were detected in all organs, among which its expression levels varied greatly (Figure 2). Although its gene expression was significantly high in stems, it was low in other organs. *CSD2* expression was high in siliques and stems, whereas *CSD3* transcripts were relatively high in siliques and cauline leaves (Figure 2). Both genes exhibited low and variable transcript levels in other organs.

CSD1 Expression in Response to Stress Treatment

We further investigated in detail *CSD1* expression in response to various external stimuli by subjecting *Arabidopsis* seedlings to various treatments such as exogenous phytohormones (Figure 3A), temperature (Figure 3B), osmotic stress (Figure 3C), PAs (Figure 3D), ethylene inhibitors (Figure 3E), salicylic acid (SA), H_2O_2, pH, and dehydration (Figure 3F). For each treatment, the mean value of *CSD1* expression in untreated samples was set to 1, and mean values from 0.8 to 1.2 were regarded as unaffected. After responsive treatments, the *CSD1* transcripts were upregulated in response to ACC (Figure 3A,

Fig. 1 Amino acid comparison between MSOD1, CSD1, CSD2, and CSD3. Identical residues are marked with asterisks, whereas conserved and semiconserved substitutions are denoted by a colon (:) and a period (.), respectively. The His-45, His-47, and His-119 amino acid motifs for Cu^{2+} binding are boxed, whereas His-62, His-70, and Asp-82 amino acid motifs for binding Zn^{2+} are marked by arrows

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CSD1    ------------------------------------------------------------
CSD2    MAATNTILAFSSPSRLLIPPSSNPSTLRSSFSGVSLNNNNLHRLQSVSFAVKAPSKALTV 60
CSD3    -------------------------------------------------AP------ 2

MSOD1   ---MGKGVRVLNSSEGVKGTIFFTQEGNGTTTVTGTVSGLKPGLHGFHVHALGDTTNGCM 57
CSD1    ---MAKGVAVLNSSEGVTGTIFFTQEGDGVTTVSGTVSGLKPGLHGFHVHALGDTTNGCM 57
CSD2    VSAAKKAVAVLKGTSDVEGVVTLTQDDSGPTTVNVRITGLTPGPHGFHLHEFGDTTNGCI 120
CSD3    -RGNLRAVALIAGDNNVRGCLQFVQDISGTTHVTGKISGLSPGFHGFHIHSFGDTTNGCI 61
             :.*  ::  . ..* * * : :.*: .* * *.  .:**.** ****:* :*******:

MSOD1   STGPHFNPEGKTHGAPEDANRHAGDLGNITVGDDGTATFTITDSQIPLDGPNSIVGRAVV 117
CSD1    STGPHFNPDGKTHGAPEDANRHAGDLGNITVGDDGTATFTITDCQIPLTGPNSIVGRAVV 117
CSD2    STGPHFNPNNMTHGAPEDECRHAGDLGNINANADGVAETTIVDNQIPLTGPNSVVGRAFV 180
CSD3    STGPHFNPLNRVHGPPNEEERHAGDLGNILAGSNGVAEILIKDKHIPLSGQYSILGRAVV 121
        ******** . .**.*:: ********* .. :*.*  * * :*** *  *:::***.*

MSOD1   VHAEPDDLGKGGHELSLTTGNAGGRVACGIIGLQG------ 152
CSD1    VHADPDDLGKGGHELSLATGNAGGRVACGIIGLQG------ 152
CSD2    VHELKDDLGKGGHELSLTTGNAGGRLACGVIGL-------- 213
CSD3    VHADPDDLGKGGHKLSKSTGNAGSRVGCGIIGLQSSADAKL 162
        **  ********:** :*****.*:..**:*** 
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Fig. 2 Spatial expression of *CSD1-3* in different *Arabidopsis* organs. Transcript levels were determined by real-time PCR and are shown relative to expression of tubulin (*TUB2*) in each sample. Values are mean ± SE from three replicates. CL, cauline leaves; FL, flowers; FB, flower buds; R, roots; RL, rosette leaves; SI, siliques; ST, stems

Generation and Characterization of Transgenic *Arabidopsis* Plants

To investigate the *in vivo* role of SOD in plants, SOD activity was modulated by expressing sense and antisense *MSOD1* cDNAs under the control of 35S promoter in transgenic *Arabidopsis* plants (Figure 4A). The *MSOD1* cDNA fragment used for antisense construct in this study shares high similarity with *CSD1-3* (Figure 1). Independent T1 lines for each construct were selected based on their kanamycin-resistant phenotype. Southern blot analyses found that almost all the lines contained multiple insertions (Figure 4B). Thus, shoots of these independent lines at the T1 generation were maintained by tissue culture of root explants and used for further studies.

Real-time PCR analysis revealed that the *MSOD1* transcripts were detectable in all sense transformants (Figure 4C), whereas the expression of endogenous *CSD1-3* genes was not significantly affected in these sense transformants (data not shown). Among several independent antisense transformants, *CSD1* transcripts were reduced only in AS1 and AS2 transformants (Figure 4D). *CSD2* transcripts remained unaffected in AS1 but decreased in AS2, whereas *CSD3* transcripts were significantly downregulated in both antisense transformants (Figure 4D).

The sense transformants (S1-S5) and the two antisense transformants (AS1 and AS2) were selected for enzyme activity studies (Figure 4E). It was observed that some of the transgenic plants (S4, S5, AS1, and AS2) showed enzyme activity related to the SOD mRNA expression, whereas the enzyme activities of three sense lines (Figure 4C, S1–S3) displayed the opposite trend, indicating that overexpression of *MSOD1* was post-transcriptionally regulated in these lines.

Transgenic plants of S4, S5, AS1, and AS2, designated S-*MSOD4*, S-*MSOD5*, AS-*MSOD1*, and AS-*MSOD2*, respectively, were selected for further shoot regeneration studies. Two sense plants (S-*MSOD4* and S-*MSOD5*) had high expression of *MSOD1* mRNA with an increased SOD activity, whereas two antisense plants possessed decreased expression of endogenous *CSD* genes with reduced SOD activity.

To investigate whether the capacity of H_2O_2 production in transgenic plants corresponded to altered activity of SOD, the levels of H_2O_2 production in leaves of wild-type, AS-*MSOD1*, and S-*MSOD4* plants were examined. The results showed that brown coloration was hardly detectable in an untreated AS-*MSOD1* leaf (Figure 5), but a postincubation with 10 mM H_2O_2 for 10 min increased the brown coloration. In a wild-type leaf, the brown coloration was weakly detected and greatly increased by a postincubation with H_2O_2. In particular, there was a significantly

100–500 μM), 2,4-D (Figure 3A, 0.5–12.5 μM), MeJA (Figure 3A, 10–500 μM), PEG (Figure 3C, 10% and 40%), Spd (Figure 3D, 10 and 50 mM), Spm (Figure 3D, 10 and 50 mM), $AgNO_3$ (Figure 3E, 25–100 μM), AVG (Figure 3E, 5–50 μM), SA (Figure 3F, 0.1–0.2 mM), H_2O_2 (Figure 3F, 1–100 mM), and pH (Figure 3F, 4.8-8). In contrast, *CSD1* transcripts were downregulated in response to ABA (Figure 3A, 100 μM), GA_3 (Figure 3A, 7.5 μM), high temperature (Figure 3B, 22–42°C), and NaCl (Figure 3C, 100–400 mM). BA (Figure 3A), cold treatment (Figure 3B), and dehydration (Figure 3F) treatments did not significantly affect *CSD1* expression.

◄ **Fig. 3** *CSD1* expression in *Arabidopsis* seedlings in response to various treatments. **A** Phytohormones. **B** Temperature. **C** Osmotic stress. **D** Polyamines. **E** Ethylene inhibitors. **F** SA, H_2O_2, pH, and dehydration (Deh). Seedlings were treated with various solutions for 6 h or left on plates at room temperature without treatment (RT). The control for dehydration (Ctrl) was seedlings placed on filter papers in a covered Petri dish in the flow-hood. Transcript levels were determined by real-time PCR and are shown relative to expression of *TUB2* in each sample. Values are mean ± SE from three replicates

enhanced brown coloration in an untreated leaf of S-*MSOD1* (Figure 5). These results demonstrated that, as expected, the SOD activity may regulate, in part, the endogenous H_2O_2 levels in *Arabidopsis*.

Effects of SOD Activity on Shoot Regeneration

Transgenic plants with different levels of SOD activity grew normally and were phenotypically indistinguishable from wild-type plants throughout plant growth and development (data not shown). To examine if SOD activity could affect plant development under different stresses, we treated these transgenic plants under different external stimuli and found that these transgenic seedlings displayed similar phenotypes as wild-type plants (data not shown).

To investigate whether the capacity of shoot regeneration from root explants was affected by upregulation and downregulation of SOD activity, the shoot regeneration response of wild-type and transgenic plants was examined after 20 days in culture (Figure 6A). S-*MSOD4* and S-*MSOD5* were more regenerative (80% and 76%) in culture compared with wild-type (60%) and the control pBI121explants (58%), whereas AS-*MSOD1* and AS-*MSOD2* were poorly regenerative with only 37% and 35% explants forming shoots, respectively (Figure 6A).

The changes in shoot regeneration capacity could be associated with the levels of polyamines (PAs), as shown in other studies (Monteiro and others 2002; Hunter and Burritt 2005). To explore this possibility, the levels of free PAs in cultured explants of wild-type, S-*MSOD4*, and AS-*MSOD1* were analyzed. However, the measured polyamine concentrations (data not shown) did not conclusively link the regenerative capacity of the SOD transgenic lines with altered PAs content.

Discussion

Gene expression analyses have revealed the various levels of *CSD1-3* expression in different *Arabidopsis* organs, indicating that they are developmentally regulated. Some previous studies have associated elevated SOD activity with increased tolerance against oxidative stress (Kliebenstein and others 1998; Walz and others 2002; Posmyk

and others 2005; Verma and Mishra 2005; Misra and Gupta 2006; Zhang and others 2006). Our study showed that *CSD1* gene expression was upregulated in response to ACC, 2,4-D, MeJA, Put, Spd, Spm, $AgNO_3$, AVG, SA, H_2O_2, and pH, whereas it was downregulated in response to ABA, GA_3, high temperatures, and NaCl. The upregulation of *CSD1* by H_2O_2 is in agreement with the finding that SOD activity was increased by H_2O_2 treatment in maize plants (Zhang and others 2006). Conversely, the downregulated or unaltered expression of *CSD1* mRNA in response to ABA, cold, and dehydration is in contrast to previous findings where SOD activity was observed to increase under the same treatments (Walz and others 2002; Posmyk and others 2005; Zhang and others 2006). Because *CSD1* is only one of the homologs contributing to SOD activity, the difference may indicate the post-transcriptional regulation of *CSD1* expression or distinct expression of different SOD genes in response to stress. It is interesting to point out that some stress treatments may indirectly affect *CSD1* expression by regulating the catabolic product of SODs such as H_2O_2, because H_2O_2 can be derived from different metabolic pathways and exerts a promotive effect on *CSD1* expression (Figure 3F).

Despite the changes of gene expression in response to stress, up- and/or downregulation of SOD activity on its own was insufficient to confer stress tolerance to plants because the phenotypes that resulted from different treatments were similar between wild-type and transgenic plants. These results are similar to some other previous reports (Tepperman and Dunsmuir 1990; Pitcher and others 1991; Payton and others 1997). It has been reported that SOD protective effects depend on the superoxide radical/ H_2O_2 ratio (Bowler and others 1991). This critical ratio is influenced by many factors, including the type of SOD, its subcellular localization, the level of overproduction, H_2O_2-scavenging capability of different subcellular compartments, and type of cells. In addition, the protection afforded by the SOD transgene may be affected by the variety, age, and growth conditions of the host plant and by the type of method used to detect oxidative damage (Slooten and others 1995). Furthermore, it is possible that high levels of SOD activity must be accompanied by concurrent elevation of "downstream" enzymes such as catalases and peroxidases, which quickly scavenge the resulting H_2O_2 (Williamson and Scandalios 1993; Slooten and others 1995).
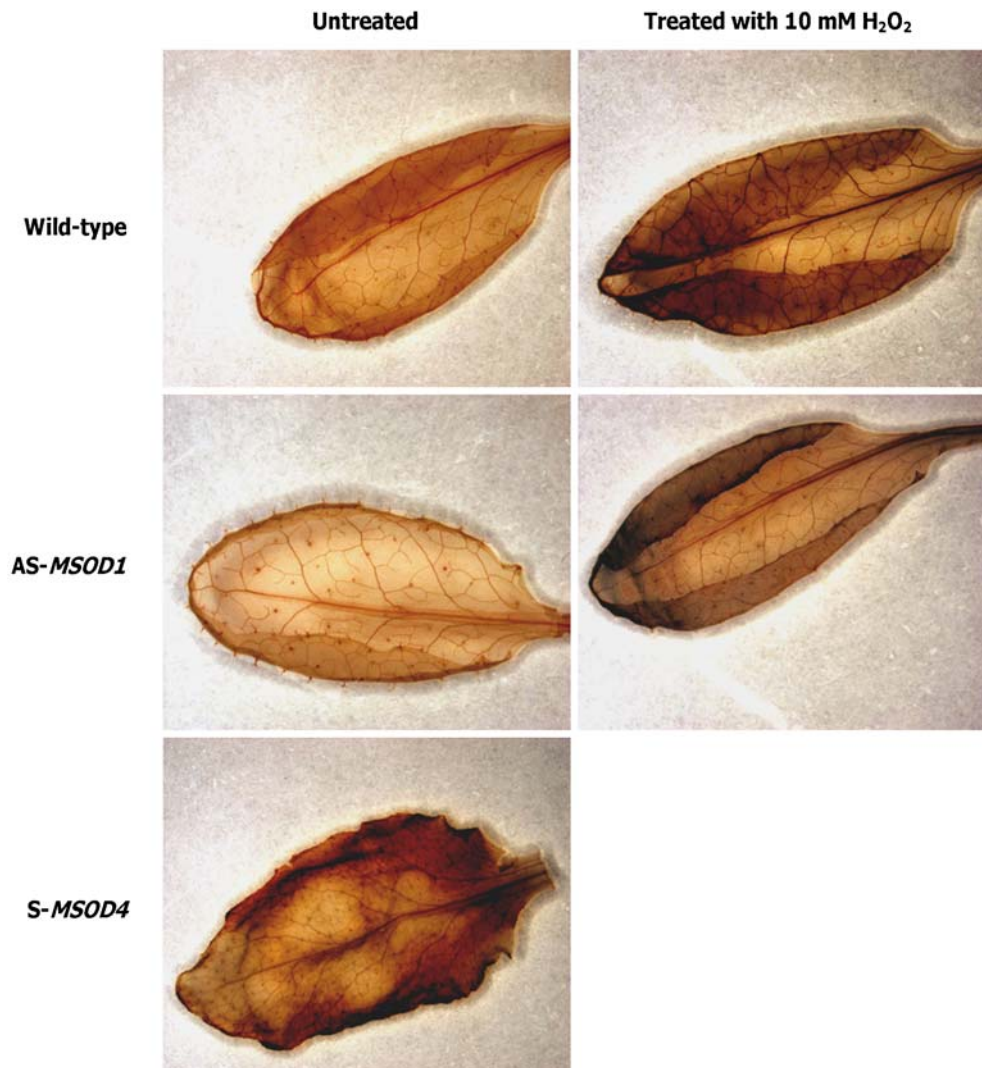
Plant regeneration from cultured cells and tissues via either organogenesis or somatic embryogenesis is a multifaceted process. For the last several decades, much effort has been dedicated to this area to unravel the regulatory mechanism. In general, major factors that have been subjected to intensive investigation include hormones, physiology of cultured tissues, and the genetic background
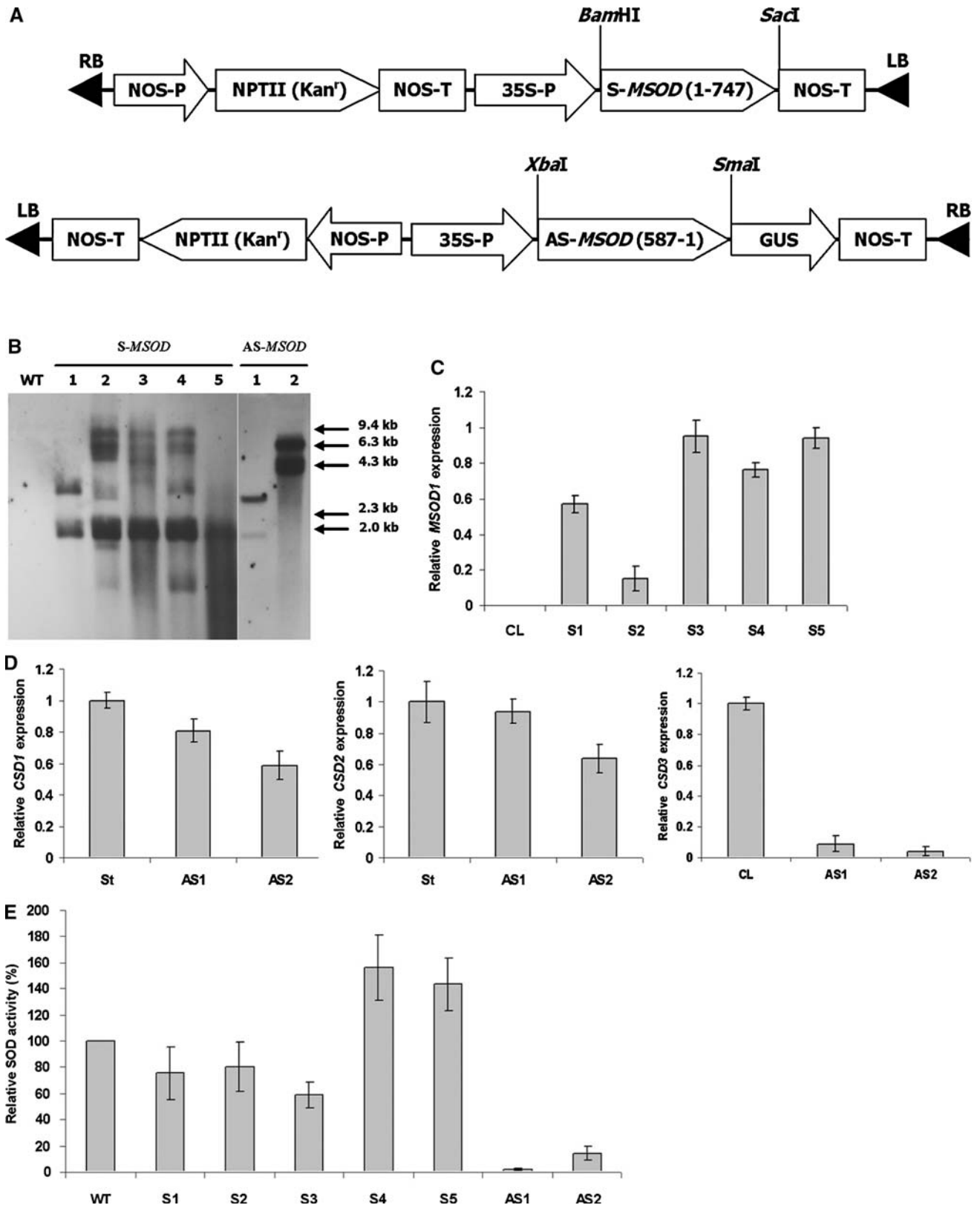
◄ **Fig. 4** Generation and molecular characterization of sense and antisense transgenic plants. **A** Schematic representation of chimeric genes used for plant transformation. The numbers in the constructs indicate the position of nucleotides relative to the putative transcriptional start site (+1). **B** Southern analysis of transgenic *Arabidopsis* plants containing MSOD constructs. Genomic DNA (10 µg) isolated from whole seedlings of wild-type (WT; nontransformed control), sense (S-*MSOD*), and antisense (AS-*MSOD*) transgenic plants (labeled accordingly) were digested with *Hin*dIII and hybridized with an 818-bp probe prepared from the CaMV 35S promoter. Lambda DNA digested with *Eco*RI/*Hin*dIII was used as a molecular weight marker (right). **C** Expression of *SOD* mRNAs in sense (S1-S5) and **D** antisense (AS1–AS2) transformants. Stems (St) and cauline leaves (CL) of wild-type plants were used as controls. Transcript levels were determined by real-time PCR and are shown relative to expression of *TUB2* in each sample. Values are mean ± SE from three replicates. **E** Relative SOD enzyme activity in crude extracts of young seedlings with 3-4 rosette leaves of selected transgenic plants. Values are mean ± SE from three measurements

of donors. Despite the wealth of data available on these subjects, the underlying mechanisms that regulate plant morphogenesis *in vitro* are not yet clearly understood.

H_2O_2 has been implicated as a potential mediator between oxidative stress and plant morphogenesis *in vitro* (Cui and others 1999; Luo and others 2001; Papadakis and Roubelakis-Angelakis 2002; Pua and Gong 2004). However, it is not clear if H_2O_2 is the cause or the effect of plant morphogenesis. To elucidate the exact role of H_2O_2 in shoot regeneration, in this study we have directly modulated H_2O_2 levels through upregulation and downregulation of SOD activity.

Transgenic explants with increased SOD activities and H_2O_2 production were highly regenerative, whereas explants with decreased SOD activities and H_2O_2 production were poorly regenerative. It has been found that different concentrations of H_2O_2 could exquisitely regulate the expression of an *Arabidopsis* flavin-amine oxidase (*ATFAO1*) involved in PA catabolism of *Arabidopsis* (Lim and others 2006). On the other hand, FAOs in turn can affect the endogenous H_2O_2 levels because H_2O_2 is also an oxidative product of FAOs. Thus, the SOD activity and the

Fig. 5 *In situ* detection of H_2O_2 production in wild-type, AS-*MSOD1*, and S-*MSOD4* plants. DAB staining was performed as described in Materials and Methods
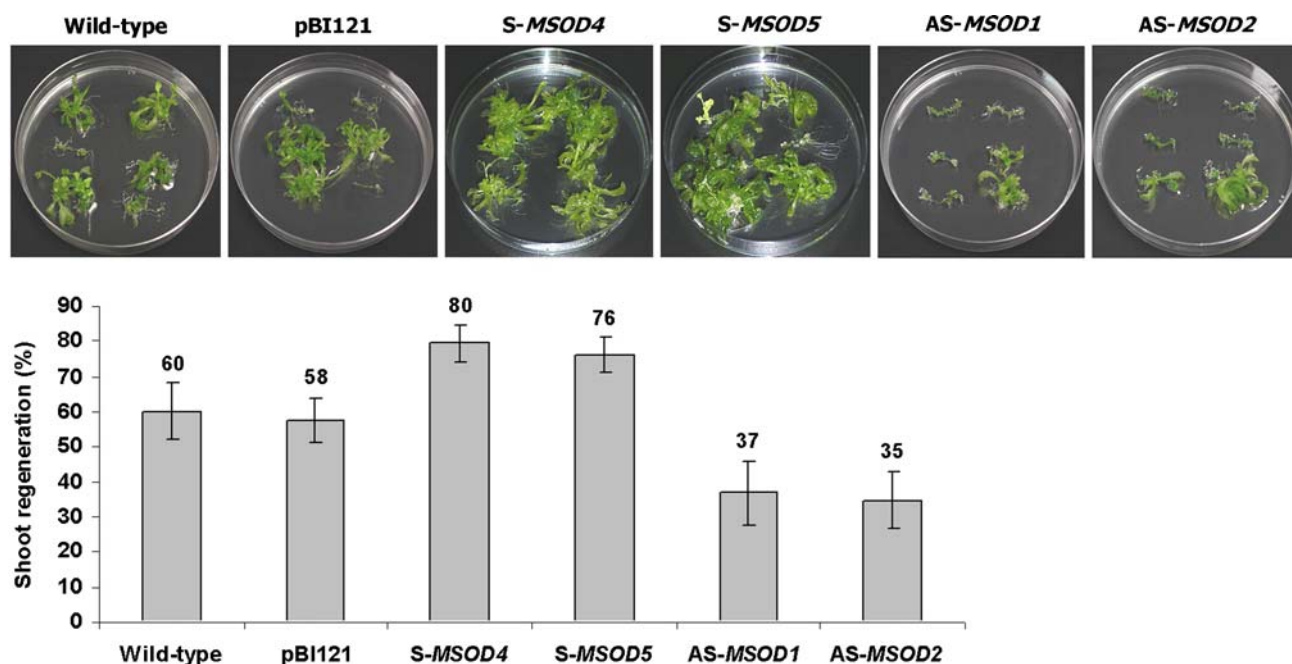
Fig. 6 Effects of SOD activity on shoot regeneration. Shoot regeneration from root segments of untransformed wild-type and transgenic plants (pBI121, S-*MSOD4*, S-*MSOD5*, AS-*MSOD1*, AS-*MSOD2*). Root segments were preincubated on CIM for 4 days and then transferred to SIM. Wild-type and pBI121 transgenic plants served as controls. Representative plates after 20 days in culture are shown on the top, whereas statistical analysis of shoot regeneration is shown below. Explants were evaluated in terms of percent shoot regeneration, which was calculated based on the number of explants forming shoots as a percentage of the total number of explants (18). Data were converted to Arcsin-transformed values before being analyzed statistically. Vertical bars represent mean ± SE of three replicates

corresponding H_2O_2 levels may affect shoot regeneration partly via the effect of PAs. However, in this study we did not find that altered levels of PAs in the SOD transgenic lines were relevant to the regenerative capacity of these plants. Therefore, the molecular mechanism of regulation of shoot regeneration by the SOD activity needs to be investigated further.

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