

# Cu/Zn superoxide dismutases in developing cotton fibers: evidence for an extracellular form

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**Abstract** Hydrogen peroxide and other reactive oxygen species are important signaling molecules in diverse physiological processes. Previously, we discovered superoxide dismutase (SOD) activity in extracellular protein preparations from fiber-bearing cotton (*Gossypium hirsutum* L.) seeds. We show here, based on immunoreactivity, that the enzyme is a Cu/Zn-SOD (CSD). Immunogold localization shows that CSD localizes to secondary cell walls of developing cotton fibers. Five cotton CSD cDNAs were cloned from cotton fiber and classified into three subfamilies (Group 1: *GhCSD1*; Group 2: *GhCSD2a* and *GhCSD2b*; Group 3: *GhCSD3* and *GhCSD3s*). Members of Group 1 and 2 are expressed throughout fiber development, but predominant during the elongation stage. Group 3 CSDs are

also expressed throughout fiber development, but transiently increase in abundance at the transition period between cell elongation and secondary cell wall synthesis. Each of the three *GhCSDs* also has distinct patterns of expression in tissues other than fiber. Overexpression of cotton CSDs fused to green fluorescent protein in transgenic *Arabidopsis* demonstrated that *GhCSD1* localizes to the cytosol, *GhCSD2a* localizes to plastids, and *GhCSD3* is translocated to the cell wall. Subcellular fractionation of proteins from transgenic *Arabidopsis* seedlings confirmed that only c-myc epitope-tagged *GhCSD3* co-purifies with cell wall proteins. Extracellular CSDs have been suggested to be involved in lignin formation in secondary cell walls of other plants. Since cotton fibers are not lignified, we suggest that extracellular CSDs may be involved in other plant cell wall growth and development processes.

**Accession numbers:** The nucleotide sequence data reported here were deposited in the GenBank database under the following accession numbers: *GhCSD1*, AI727694; *GhCSD2a*, EU597268; *GhCSD2b*, EU597269; *GhCSD3*, EU597270; *GhCSD3s*, EU597271.

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## Abbreviations

CSD	Cu/Zn-type superoxide dismutase
DOA	Day of anthesis
DPA	Days post-anthesis
EC	Extracellular
FSD	Fe-type superoxide dismutase
GFP	Green fluorescent protein
MSD	Mn-type superoxide dismutase
PCW	Primary cell wall
Q-RT-PCR	Quantitative, reverse-transcription polymerase chain reaction
ROS	Reactive oxygen species
SCW	Secondary cell wall
SOD	Superoxide dismutases

## Introduction

Reactive oxygen species (ROS), including superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $\cdot OH$ ) are deleterious to living cells. To detoxify destructive ROS in the subcellular compartment, all aerobic cells have an antioxidant defense system (Bowler et al. 1992; Apel and Hirt 2004). ROS are involved in various physiological processes such as stomatal closure, root gravitropism, and allelopathy (Laloi et al. 2004) and function in the defense response to pathogen infection (Apel and Hirt 2004). In addition, ROS are involved in secondary cell wall (SCW) biosynthesis. Superoxide and  $H_2O_2$  play important roles in the covalent crosslinking between protein and carbohydrate cell wall components (Kieliszewski and Lamport 1994) and in lignin biosynthesis (Pomar et al. 2002), thereby increasing the tensile strength of the wall (Carpita and McCann 2000).  $H_2O_2$  has also been proposed to be involved in the induction of SCW cellulose biosynthesis (Potikha et al. 1999) and dimerization of cellulose synthase subunits (Kurek et al. 2002). In contrast, hydroxyl radical has been proposed to be a wall loosening agent working in concert with expansin, xyloglucan endotransglucosylase/hydrolase (XTH), and cellulase (Cosgrove 2005). Hydroxyl radicals converted from  $H_2O_2$  by the non-enzymatic Fenton reaction can cleave structural polysaccharides and, hence, loosen cell walls and activate cell elongation (Fry 1998; Chen and Schopfer 1999; Schweikert et al. 2000). The regulation of wall loosening and tightening will affect the final size and height of plants; therefore, the genes associated with cell wall loosening and tightening are expected to be highly regulated (Harmer et al. 2000). If  $H_2O_2$  is involved in wall tightening processes that lead to the cessation of cell enlargement, and  $\cdot OH$ , non-enzymatically generated from  $H_2O_2$ , is involved in wall loosening processes contributing to cell enlargement, then antioxidant enzymes regulating levels of extracellular (EC)  $H_2O_2$  must affect plant growth and development.

Superoxide dismutases (SODs) are important antioxidant enzymes catalyzing the conversion of  $O_2^-$  to  $H_2O_2$  and contain different metal cofactors (Fe, Mn, or Cu and Zn) at the active site (Bowler et al. 1992). Fe-type SODs (FSDs) are located in chloroplasts, Mn-type SODs (MSDs) in mitochondria and peroxisomes, and Cu/Zn-type SODs (CSDs) in chloroplasts, the cytosol, and the apoplast (Alscher et al. 2002). EC CSDs are well characterized in animal cells but comparatively little is known about these enzymes in plants. EC CSDs have been described previously from spinach (Ogawa et al. 1996), pine needles (Karpinska et al. 2001), and peas (Kasai et al. 2006).

Cotton (*Gossypium hirsutum*, L.) fibers are single-cell trichomes arising from the epidermis of developing cotton ovules. Cotton fiber development occurs in four overlap-

ping stages: initiation, elongation, SCW deposition, and maturation (Naithani et al. 1981). In addition to in planta development, cotton fibers develop in vitro, providing an exceptional experimental model for studying plant cell elongation and cellulose biosynthesis (Kim and Triplett 2001). Unlike multicellular organisms in which different cell types may be at different developmental or cell division stages, expression of genes and proteins in cotton fiber can be monitored independently during either the elongation or SCW deposition stage in a single cell type.

In an effort to characterize a cotton fiber cell-wall germin-like protein (*GhGLP1*) whose orthologs in some plants have weak Mn-SOD activity, we previously showed that EC SOD activity from developing cotton fibers could be separated from *GhGLP1* (Kim et al. 2004). In this paper, we have further characterized the cotton fiber EC SOD by immunoblotting, identified the putative metal cofactor, compared EC SOD cDNA with other cotton fiber SOD cDNAs, profiled transcript levels in fiber and other cotton tissues, and localized the enzymes by immunological and epitope tagging techniques.

## Materials and methods

### Plant materials and growth conditions

Cotton (*Gossypium hirsutum*, L., TM1) plants were grown under standard field conditions (naturally rain-fed with daytime high temperatures of  $\sim 32$ – $36^\circ C$  and nighttime low temperatures of  $20$ – $30^\circ C$ ) in New Orleans, LA and College Station, TX during the summers between 2002 and 2006. Immature seeds were harvested by 9 a.m. and frozen in liquid nitrogen. Fibers were carefully scraped from frozen seeds using a scalpel. Developing ovaries were collected at 4-day intervals from 8 to 24 DPA. Hypocotyls, roots and cotyledons were harvested from 10-day old seedlings. Fully expanded leaves (15 cm in diameter) and young leaves (5 cm in diameter), petals (DOA), bracts (DOA) were harvested from field-grown plants. All tissues were frozen in liquid nitrogen, and stored at  $-80^\circ C$ .

### Protein extraction and immunoblot analysis

EC proteins were eluted by bathing trichome-bearing seeds in 15 volumes of 1.0 M NaCl (Robertson et al. 1997; Kim et al. 2004). Tissues were vacuum-infiltrated by three 10 min exposures to 85 kPa, followed by gentle shaking for 16 h at  $4^\circ C$ . Plant material was recovered by filtration through two layers of cheesecloth followed by centrifugation at  $10,000\times g$  for 15 min. The supernatant liquid containing EC protein was concentrated with a Centriprep-10 centrifugal filter (Amicon, Beverly, MA).

Total proteins from transgenic *Arabidopsis* plants (3-weeks old) were extracted with 6 M urea, 100 mM Tris (pH 8.0), 0.1% SDS, and 10%  $\beta$ -mercaptoethanol. Soluble proteins were extracted from *Arabidopsis* plants (3-weeks old) with 100 mM Tris (pH 8.0) buffer containing Complete Tabs, a protease inhibitor (Roche Applied Science, Indianapolis, IN). Cell walls were prepared by washing insoluble fractions with 70% ethanol and 100% acetone. Cell wall proteins were extracted from the isolated cell walls with 6 M urea, 100 mM Tris (pH 8.0), 0.1% SDS, and 10%  $\beta$ -mercaptoethanol.

The extracted proteins were separated on 15% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes in 25 mM Tris base–190 mM glycine–20% methanol at constant voltage (30 V) overnight at room temperature. The filters were blocked in 5% (w/v) skim milk–PBS-T [0.05% (v/v) Tween-20 in phosphate-buffered saline (PBS)] buffer for 2 h at room temperature, treated with primary antibodies [anti-plant CSD (1:6,000 dilution), anti-plant MSD (1:1,000 dilution), anti-polyclonal GFP (1:1,000 dilution) and anti-monoclonal c-myc (1:1,000 dilution)] in blocking buffer overnight at 4°C, washed three times with PBS-T, reacted with (1:1,000 dilution) horseradish peroxidase conjugated donkey anti-rabbit IgG or anti-mouse IgG (Pierce, Rockford, IL). The cross-reacting proteins were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

Peptide antibodies to conserved and distinct domains of plant CSDs and plant MSDs were prepared in rabbits (EnVirtue Biotechnologies, Inc., Winchester, VA). Polyclonal GFP antibody was purchased from Invitrogen (Carlsbad, CA) and monoclonal c-myc antibody was purchased from Sigma-Aldrich (St Louis, MO).

#### Cloning of *GhCSD1*, *GhCSD2*, and *GhCSD3*

The open reading frame sequence of *GhCSD1* was obtained by RT-PCR from cDNA template synthesized from *G. hirsutum* TM1 4 DPA ovules with forward primer (5'-CCCTCG AGAAATGGTGAAAGCCGTTGCCGTCC-3') and reverse primer (5'-TCGCTAGCGCCTTGCCAGACCAATAATACC GCA-3') designed from the sequence of AI727694 encoding a putative cytosolic CSD. Two full-length clones of *GhCSD2* were obtained using 3' RACE PCR following the manufacturer's protocol (Clontech, Palo Alto, CA). A specific primer (5'-AGCCATGGCTGCCCATATTTTCACGACAAC-3') for 3' RACE was designed from EST sequences (AI728663) encoding a putative leucoplast CSD. To obtain the sequences of the 5' UTR of *GhCSD3*, both 5' RACE PCR (Clontech) and GenomeWalker (BD Biosciences, Palo Alto, CA) were used. The gene-specific primers for 5' RACE and GenomeWalker were designed from EST sequences

(BM360311). The full-length cDNA of *GhCSD3* was obtained by RT-PCR. All PCR amplified products were cloned into pCR-XL-TOPO (Invitrogen, Carlsbad, CA) and sequenced by the DNA Sequencing Center, Auburn University.

#### Quantitative RT-PCR

Specific primers for *GhCSD1* (5'-GGGTGCATGTCAACT GGACC-3'/5'-ACCATGCTCTTTGCAGCA-3'), *GhCSD2* (5'-GGCTGCCCATATTTTCACGA-3'/5'-GGAAAAGG AAGGAGGTGG-3'), *GhCSD3* (5'-CCATGCTGGAG ATTTGGGTA-3'/5'-TCAGCAACCCATCAGGGC-3'), and *GhCSD3s* (5'-GATTTGGGAGTTGCTGAGGTCT-3'/5'-CTGTCCGCTAAGTGAATCTGC-3') were designed using Primer Express software (version 2.0, Applied Biosystems, Foster City, CA). The specificity of primer annealing was examined by monitoring product dissociation. Cotton 18S rRNA (5'-CGTCCCTGCCCTTTGTACA-3'/5'-AACCTTCACCGGACCATTCA-3') was used as a normalizer. All amplicon sizes were designed to be less than 150 bp to make amplification efficiencies equivalent.

Total RNAs from cotton tissues were isolated using a Spectrum Plant Total RNA kit (Sigma-Aldrich) and treated with DNase I (Sigma-Aldrich). First-strand complementary DNA was synthesized using 1  $\mu$ g of total RNA by priming with random hexamers at 48°C for 30 min followed by inactivation of MultiScribe™ Reverse Transcriptase (Applied Biosystems) at 95°C for 10 min. Q-RT-PCR was performed using the SYBR® Green PCR Master Mix in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Thermal cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s for denaturation and 60°C for 1 min for annealing and extension. The transcript levels of *GhCSDs* were normalized with respect to the transcript level of 18S ribosomal RNA. Reported values are the average of triplicate PCR reactions. The experiments were repeated twice independently beginning with RNA isolation.

Amplification efficiencies among samples varied by less than 0.01 for each cycle of amplification. Relative transcript levels were determined by a comparative  $C_T$  (threshold cycles) method according to Applied Biosystems' guidelines. Statistical analyses and construction of graphs were performed using Prism version 4.00 software (GraphPad Software, Inc., San Diego, CA).

#### Immunogold localization of CSD in developing cotton fibers

Cotton ovaries were harvested on the DOA, surface-sterilized in 95% ethanol, flamed briefly, and dissected under sterile conditions. Ovules were transferred to a liquid cul-

ture medium in the presence of 5.0  $\mu\text{M}$  indole-3-acetic acid and 0.5  $\mu\text{M}$  gibberellic acid (Beasley and Ting 1973). Cotton fibers cultured for 6, 14, and 23 days were fixed in 1.5% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 6.8) for 2 h at room temperature, washed in several exchanges of buffer, and then gradually dehydrated in ethanol (30, 50, 70, 100%). Fibers collected from each stage were gradually infiltrated with LR White resin (33, 66, 100%) (Electron Microscopy Sciences, Hatfield, PA) and polymerized in a gelatin capsule by UV light for 24 h at  $-18^\circ\text{C}$ , then at room temperature for an additional 24 h. Ultra-thin sections (75–80  $\mu\text{m}$ ) cut with an UltraCut E microtome (Leica, Germany) were mounted on nickel grids and washed with PBS buffer for 5 min. The grids were incubated in a series of solutions in the following order: blocking solution (1% Aurion BSA, Electron Microscopy Sciences, in PBS) for 2 h, plant CSD antibody (1:3,000 dilution in blocking solution) for 16 h at  $4^\circ\text{C}$ , PBS three times for 10 min each, donkey anti-rabbit IgG conjugated with 18 nm gold particles (1:100 dilution in blocking solution) for 16 h at  $4^\circ\text{C}$ . For the control, primary antibody was omitted and replaced with an equal dilution of pre-immune serum. After washing in PBS and Milli-Q water (Millipore, Billerica, MA) three times, 10 min each, the grids were stained with uranyl acetate and lead citrate and the specimens were observed with a transmission electron microscope (Philips CM 120, FEI Company, Hillsboro, OR) at 80 kV.

#### Transgenic lines of *GhCSDs*-GFP and *GhCSDs*-myc

Translational fusions of Green Fluorescent Protein (mGFP5) to the C-terminus of *GhCSD1*, *GhCSD2*, and *GhCSD3* were generated. The open reading frames of *GhCSD1*, *GhCSD2*, and *GhCSD3* were individually subcloned into pRT104-myc (Töpfer et al. 1987) and verified by sequencing. The CaMV 35S promoter and *GhCSDs* in pRT104-myc were digested with *HindIII* and *NheI* and ligated into the binary vector, pCAMBIA 1302 (CAMBIA, Canberra City, Australia), pre-digested with *HindIII* and *SpeI*. Translational fusions of c-myc to the C-terminus of *GhCSD1*, *GhCSD2*, and *GhCSD3* were also generated. The 35S promoter and *GhCSDs* in pRT104-myc were digested with *PstI* and *NheI* and ligated into the binary vector, pCAMBIA 1391z, pre-digested with *PstI* and *NheI*. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) and subsequently transformed into *Arabidopsis*, Columbia (Col-0), by the method of Clough and Bent (1998).

#### Subcellular localization of the *GhCSDs*-GFP protein

Transgenic *Arabidopsis* plants were selected on 0.5 X MS agar plates containing 50 mg/L hygromycin. For GFP

localization in cells, 2-week old transgenic seedlings or roots were transferred to Petri dishes and observed with a Leica DM RXA2 upright or a Nikon E800 microscope. To plasmolyze cells, the tissue samples were pretreated in 1 M NaCl or 600 mM mannitol for  $\sim 10$  min at room temperature. Images of differential interference contrast (DIC) and bright field were taken by HCX PL APO CS 40X N.A. 1.25 oil immersion objective lenses and SensiCam QE CCD camera (Cooke Corp., Auburn Hills, MI). The GFP filter set JP1 (exciter 470/20 nm and emitter 510/20 nm, Chroma, CT) was used to excite GFP. Exposure time was 1.0 s. The images were composed with ImageJ1.3.7 (<http://rsb.info.nih.gov/ij>) and PhotoShop (Adobe, San Jose, CA). Green color was added in the original black and white images through the Lookup Tables function in ImageJ (version 10.2) software.

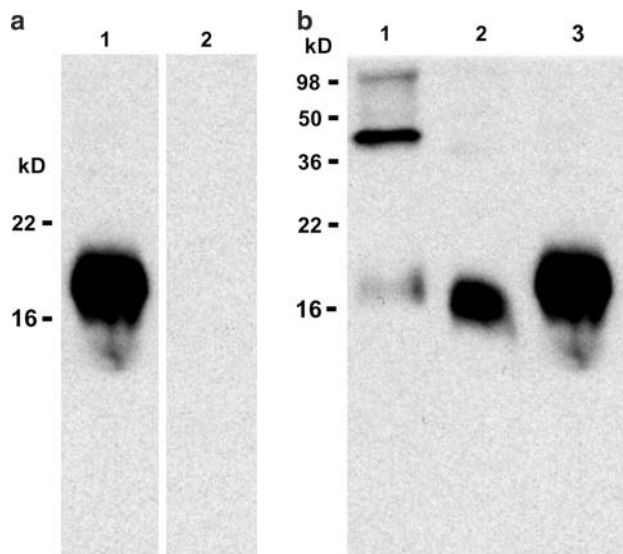
## Results and discussion

An extracellular SOD in developing cotton seeds is an oligomeric CSD

Previously, we purified a cotton-fiber cell-wall protein that was found to be a germin-like protein, *GhGLP1* (Kim et al. 2004). Germin-like proteins from other plants have been reported to have SOD and other enzyme activities; however, no activity was associated with purified *GhGLP1* (Kim et al. 2004). Developing cotton seeds<sup>1</sup> had strong EC SOD activity, and partially purified *GhGLP1* fractions from anion exchange and gel filtration chromatography also showed SOD activity; however, lectin-affinity chromatography separated *GhGLP1* from the SOD activity (Kim et al. 2004). As a result, we predicted that the EC protein fraction from immature cotton seeds contains an unglycosylated SOD having a similar molecular mass and *pI* to *GhGLP1* (native MW, 108 kDa; *pI* 5).

To determine which type of SOD is in the cell wall of cotton seeds, we tested the immunogenicity of immature cotton seed EC proteins with commercial peptide antibodies to plant CSD and MSD. Immunoblot analyses showed that immature cotton seed EC proteins contained a CSD, but not an MSD (Fig. 1a). MSD, an enzyme that localizes in mitochondria, should be present in soluble protein extracts. The lack of detectable MSD in the EC protein extract (Fig. 1a, lane 2) suggests that the EC protein extract contains little or low levels of contaminating intracellular proteins.

<sup>1</sup> Cotton fibers (trichomes) cover the cotton seed throughout development. Approximately 25–30% of the epidermal cells of the seed become fibers.



**Fig. 1** Identification of EC *GhCSD* protein from fiber-bearing cotton seeds. **a** Immunoblot analysis of EC protein separated on a 15% SDS-polyacrylamide gel and blotted to nitrocellulose. Primary antibodies were: *lane 1* anti-plant CSD (1:6,000 dilution), *lane 2* anti-plant MSD (1:1,000 dilution). Secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2,000 dilution) with detection by SuperSignal West Pico Chemiluminescent Substrate. **b** Immunoblot of EC proteins subjected to different denaturing conditions. EC protein (8  $\mu$ g) was denatured, loaded onto a 15% SDS-polyacrylamide gel, blotted to nitrocellulose, and treated with anti-plant CSD (1:6,000 dilution). Denaturation conditions were: *lane 1* SDS, *lane 2* SDS and heat (100°C) for 20 min, *lane 3* SDS and heat (100°C) for 20 min in the presence of 100 mM dithiothreitol

Under conditions where the reducing agent was omitted and the sample was not heated prior to electrophoresis, protein bands of three different sizes (17, 44, and 110 kDa) were detected by the plant CSD antibodies (Fig. 1b, lane 1). Denatured, extracellular *GhCSD* migrated with an apparent molecular mass of 17 kDa on 15% SDS-PAGE (Fig. 1b, lanes 2 and 3). Despite the identical amounts of EC protein extracts loaded onto lanes 2 and 3 of Fig. 1b, the EC protein extract denatured by heat alone (Fig. 1b, lane 2) has a less prominent band than that denatured by heat and in the presence of dithiothreitol (Fig. 1b, lane 3). The CSD antibodies used for the blot were produced against a conserved metal-binding domain of plant CSDs. Crystallographic studies on CSDs reveal that the metal-binding domain is located in densely packed interiors of the folded CSD (Tainer et al. 1982; Hough and Hasnain 1999). *GhCSD* fully denatured by heat and dithiothreitol (Fig. 1b, lane 3) exposed more epitopes than the partially denatured *GhCSD* (Fig. 1b, lane 2). Oligomeric forms found in the absence of dithiothreitol and heat indicate the formation of disulfide linkages between the SDS-dissociated subunits. Both HipI-SOD, an EC CSD from pine needles (Streller and Wingsle 1994; Karpinska et al. 2001) and *SoCSD1*, a spinach CSD that localizes to cell walls (Kanematsu and Asada 1990; Ogawa

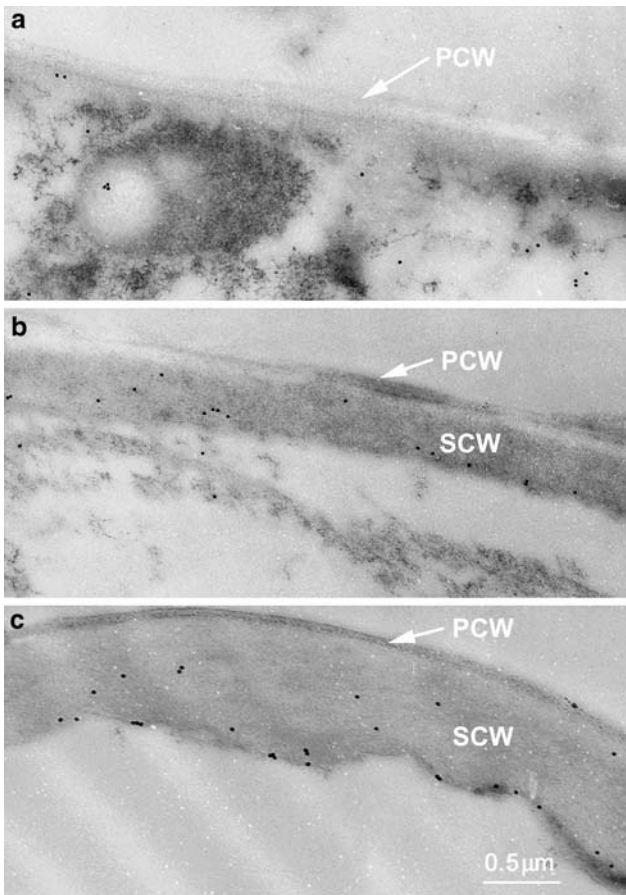
et al. 1996, 1997), were reported to be dimers; however, mammalian EC CSDs are tetrameric enzymes (Cannio et al. 2000). Further studies using gel filtration and/or X-ray diffraction are necessary to determine exactly the oligomeric structure of EC *GhCSD*.

#### *GhCSD* is detected in SCWs of cotton fibers by immunogold localization

To determine if EC *GhCSD* is present in cell walls of developing cotton fibers, we fixed fibers (*G. hirsutum*) at 6, 14, and 23 DPA in preparation for immunogold localization. The 6 DPA fibers are in the elongation stage and have only a thin primary cell wall (PCW); the 14 DPA fibers are in the transition from elongation to SCW deposition and have a thin PCW and some SCW; and the 23 DPA fibers are in the SCW deposition stage and have a thin PCW and a thicker SCW (Fig. 2). Overall, immunogold-labeled *GhCSD* was found only in the SCW of 14 and 23 DPA cotton fibers and was rarely detected in PCWs at any stage of development. A low level of labeling was found in the cytosol, and a pre-immune serum control showed little to no labeling of cell walls (data not shown). The localization patterns of two proposed EC SODs, *SoCSD1* from spinach and HipI-SOD from pine were similarly found to be only in the SCW, suggesting a possible involvement in lignin formation (Ogawa et al. 1996, 1997; Karpinska et al. 2001; Schinkel et al. 2001; Karlsson et al. 2005). Since cotton fibers are not lignified, there must be other potential functions for this EC enzyme. The localization of EC *GhCSD* in SCWs of developing cotton fibers (Fig. 2) suggests that hydrogen peroxide produced by EC *GhCSD*s may be involved in cotton fiber SCW development. In cotton fiber cells, hydrogen peroxide was proposed to be involved in the induction of SCW cellulose biosynthesis (Potikha et al. 1999) and dimerization of cellulose synthase subunits (Kurek et al. 2002).  $H_2O_2$  produced by EC *GhCSD* could also be used by EC peroxidases to crosslink the wall matrix (Kieliszewski and Lamport 1994).

#### There are three groups of *GhCSD*s in developing cotton fibers

*Arabidopsis* contains three different types of CSDs: cytosolic *AtCSD1* (AT1G08830), chloroplastic *AtCSD2* (AT2G28190), and a putative peroxisomal form *AtCSD3* (AT5G18100). Putative orthologs of all three *AtCSD* isoforms were found in public databases of cotton DNA sequences. Four cotton Expressed Sequence Tags (ESTs; AI731179, AI727694, AI727387, and AI730588) isolated from 6 DPA cotton (*G. hirsutum*) fibers were most similar to cytosolic *AtCSD1* (AT1G08830). From the open reading frame of AI727694, primers were designed and a putative



**Fig. 2** Immunogold TEM localization of EC *GhCSD* in developing cotton fibers at 6 (a), 14 (b), and 23 DPA (c). *PCW* primary cell wall, *SCW* secondary cell wall

ortholog of *AtCSD1* was amplified by RT-PCR and named *GhCSD1* (Fig. 3). Comparison of deduced amino acids between *GhCSD1* and *AtCSD1* shows 85% sequence similarity. *GhCSD1* encodes a deduced protein of 152

amino acids with a *pI* of 5.47 and a molecular mass of 15,208 Da.

Two 3'-truncated cotton ESTs (AI726214 and AI728663) isolated from fibers were 72% similar to chloroplastic *AtCSD2* (AT2G28190). From AI728663, primers were designed and two different full-length cDNAs were obtained and named *GhCSD2a* and *GhCSD2b* (Fig. 3). Both cDNAs encode deduced proteins of 214 amino acids with *pIs* of 6.02 and molecular masses of 22,097 Da. The deduced protein sequences of *GhCSD2a* and *GhCSD2b* are identical except for two amino acids and the 3'-untranslated region. Since allotetraploid cotton, *G. hirsutum* contains two different subgenomes, it is likely that *GhCSD2a* and *GhCSD2b* are homeologous genes. *GhCSD2a* and *GhCSD2b* are expected to translocate into plastids since both contain a transit peptide of 59 amino acids, predicted by Chlorop 1.1 (Emanuelsson et al. 1999). After cleavage of the transit peptide, the estimated *pI* and molecular mass are 5.04 and 15,820 Da, respectively.

The sequences of two 5'-truncated ESTs (BG446025 and BM360311) from *G. arboreum* were 72% similar to the putative peroxisomal *AtCSD3* (AT5G18100), and we named the full-length cDNA *GhCSD3*. We were unable to obtain 5'-terminal sequences of *GhCSD3* using 5' RACE PCR, a technique that had successfully amplified *GhCSD1* and *GhCSD2* with the same cDNA template. We suspect that lower endogenous transcript levels of *GhCSD3* contributed to the failure of its amplification by RACE-PCR. Much lower levels of *AtCSD3* than *AtCSD1* and *AtCSD2* were also reported in *Arabidopsis* (Kliebenstein et al. 1998). Using a GenomeWalker kit, we were able to obtain the 5'-terminal sequences of *GhCSD3*. From this sequence information, we designed primers and amplified the coding sequences of *GhCSD3* with RT-PCR. Interestingly, the product amplified by RT-PCR contained two bands. The

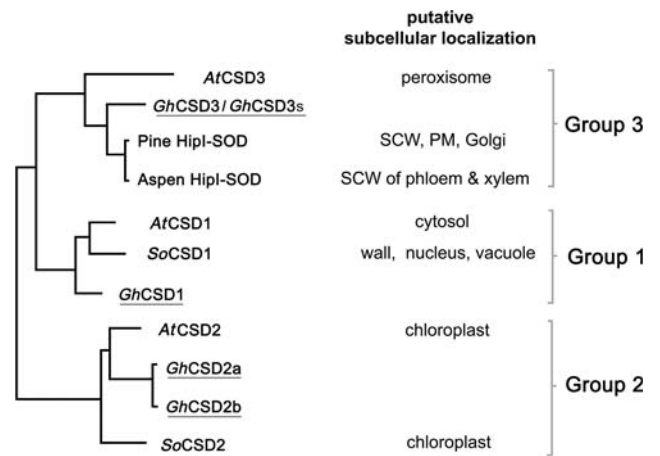
**Fig. 3** Multiple alignment of *GhCSD* proteins. Deduced protein sequences from cotton *GhCSD1*, *GhCSD2a*, *GhCSD2b*, *GhCSD3*, and *GhCSD3s*. Two transit peptides of 59 amino acids, predicted by Chlorop 1.1 (Emanuelsson et al. 1999), are shown in *bold* and *italicized* font. Conserved sequences are highlighted. The two amino acids that differ between *GhCSD2a* and *GhCSD2b* are *underlined*

<i>GhCSD1</i>	-----	55
<i>GhCSD2a</i>	<b><i>MAAHIFTTTPSHLALSFPSSTNPSNPPVLLSSFRGVSLKLPKQSLSLAATIPKKP</i></b>	55
<i>GhCSD2b</i>	<b><i>MAAHIFTTTPSHLALSFPSSTNPSNPPVLLSSFRGVSLKLPKQSLSLAATIPKKP</i></b>	55
<i>GhCSD3</i>	-----M	1
<i>GhCSD3s</i>	-----M	1
<i>GhCSD1</i>	-----MVKAVAVLSSNEGVSGTVFFSQEGDGPPTVTGNLSGLKPLGHGFVHALGDTTN	54
<i>GhCSD2a</i>	<b><i>FSVF</i></b> AVTKKAVAVLKGNSEVEGVVTLTQENDGPTTVNVRITGLTPGPHGFHLHEYGDTTN	115
<i>GhCSD2b</i>	<b><i>FSVF</i></b> AVTKKAVAVLKGNSEVEGVVTLTQETDGPPTTVNVRITGLTPGPHGFHLHEYGDTTN	115
<i>GhCSD3</i>	ECGSKATLKAVALITGDTNVRGFIHFTQIPNGITHVQKQITGLSPGLHGFHIALGDTTN	61
<i>GhCSD3s</i>	ECGSKATLKAVALITGDTNVRGFIHFTQIPNGITHVQKQITGLSPGLHGFHIALGDTTN	61
<i>GhCSD1</i>	GCMSTGPHFNPAGKEHGAPEDENRHAGDLGNVTVGDDGCASFSTITDKQIPLTGPNSIIGR	114
<i>GhCSD2a</i>	GCMSTGAHFNPNNMTHGAPEDVVRHAGDLGNIIANADGVAEATIVDNQIPLSGPNAVVG	175
<i>GhCSD2b</i>	GCMSTGAHFNPNNMTHGAPEDVVRHAGDLGNIIANADGVAEATIVDNQIPLSGPNAVVG	175
<i>GhCSD3</i>	GCMSTGPHFNPPLKDHGAPSDGERHAGDLGNIIANADGVAEATIKDWQIPLSGQHSILGR	121
<i>GhCSD3s</i>	GCMSTGPHFNPPLKDHGAPSDGERHAGDLG-----VAEVSIKDWQIPLSGQHSILGR	121
<i>GhCSD1</i>	AVVVHADPDDLKGGHELKSKSTGNAGGRVACGIIGLQG-----	152
<i>GhCSD2a</i>	AFVVHELEDDLKGGHELKSLTTGNAGGRVACGVVGLTPV-----	214
<i>GhCSD2b</i>	ASVVHELEDDLKGGHELKSLTTGNAGGRVACGVVGLTPV-----	214
<i>GhCSD3</i>	AVVAHADPDDLKGGHELKSKTTGNAGARVCGCIIGLQSSV----	161
<i>GhCSD3s</i>	AVVAHADPDDLKGGHELKSKTTGNAGARVCGCIIGLQSSV----	153

larger product was named *GhCSD3*, and the shorter product was named *GhCSD3s* (Fig. 3). *GhCSD3* encodes a deduced protein of 161 amino acids with a *pI* of 6.22 and a molecular mass of 16,461 Da. *GhCSD3s* was identical to *GhCSD3* except for eight amino acids in the middle of the open reading frame that were missing in *GhCSD3s*.

Although it might be questioned whether *GhCSD3s* is functional, two cotton fiber ESTs found in DFCI Cotton Gene Index Release 8.0 (<http://compbio.dfci.harvard.edu/tgi/plant.html>) also are lacking the same eight amino acids as *GhCSD3s*, suggesting that *GhCSD3s* is likely to be expressed in fibers. Co-production of two closely related Group 3 CSDs has also been reported in aspen xylem cells (Srivastava et al. 2007), and a recent proteomic investigation showed that EC CSDs of two different sizes are ionically bound to cell walls in the elongation zone of maize roots (Zhu et al. 2006). Both *GhCSD3* and *GhCSD3s* show 67% amino acid sequence similarity with *GhCSD1*, have seven additional amino acids in the N-terminus compared to *GhCSD1*, but do not contain signal peptides as shown by SignalP 3.0 analysis (Emanuelsson et al. 2007). *GhCSD3* is expected to localize in the cytoplasm according to algorithms for predicting protein subcellular localization such as PSORT (Nakai and Kanehisa 1991), WoLF PSORT (Horton et al. 2007), Plant-PLoc (Chou and Shen 2007), and ESLpred (Bhasin and Raghava 2004). Interestingly, *GhCSD3* and *GhCSD3s* lack a peroxisomal targeting sequence (Ala-Lys-Leu; Keller et al. 1991) that was found at the carboxyl terminus of *AtCSD3* (Kliebenstein et al. 1998). Thus, unlike *AtCSD3*, *GhCSD3* does not appear to target to the peroxisome.

Our phylogenetic comparison of cotton *GhCSDs* with other CSDs from *Arabidopsis* (Kliebenstein et al. 1998), spinach (Ogawa et al. 1995, 1996), pine (Karpinska et al. 2001) and aspen (Schinkel et al. 2001) was performed using MAFFT (Katoh et al. 2002) and led to the classification of three subfamilies (Fig. 4). *GhCSD1* grouped with *AtCSD1* and *SoCSD1*. *AtCSD1* is considered a cytosolic CSD since the product was found in the soluble fraction of a total protein extract by immunoblot analysis (Kliebenstein et al. 1998), whereas *SoCSD1* was reported to localize in the apoplast (44%), nucleus (24%) and tonoplast (23%) of spinach mesophyll cells (Ogawa et al. 1996). *GhCSD2a* and *GhCSD2b* were classified with *AtCSD2* and *SoCSD2* that contain transit peptides and localize in chloroplasts of *Arabidopsis* (Kliebenstein et al. 1998) and spinach (Ogawa et al. 1995). *GhCSD3* grouped with *AtCSD3* (Kliebenstein et al. 1998), pine HipI-SOD (Karpinska et al. 2001), and aspen HipI-SOD (Schinkel et al. 2001). Although *AtCSD3* was assumed to be a peroxisomal CSD due to a peroxisomal targeting sequence (Keller et al. 1991), the subcellular localization of *AtCSD3* has not been tested directly because even affinity-purified *AtCSD3* antibody reacts with



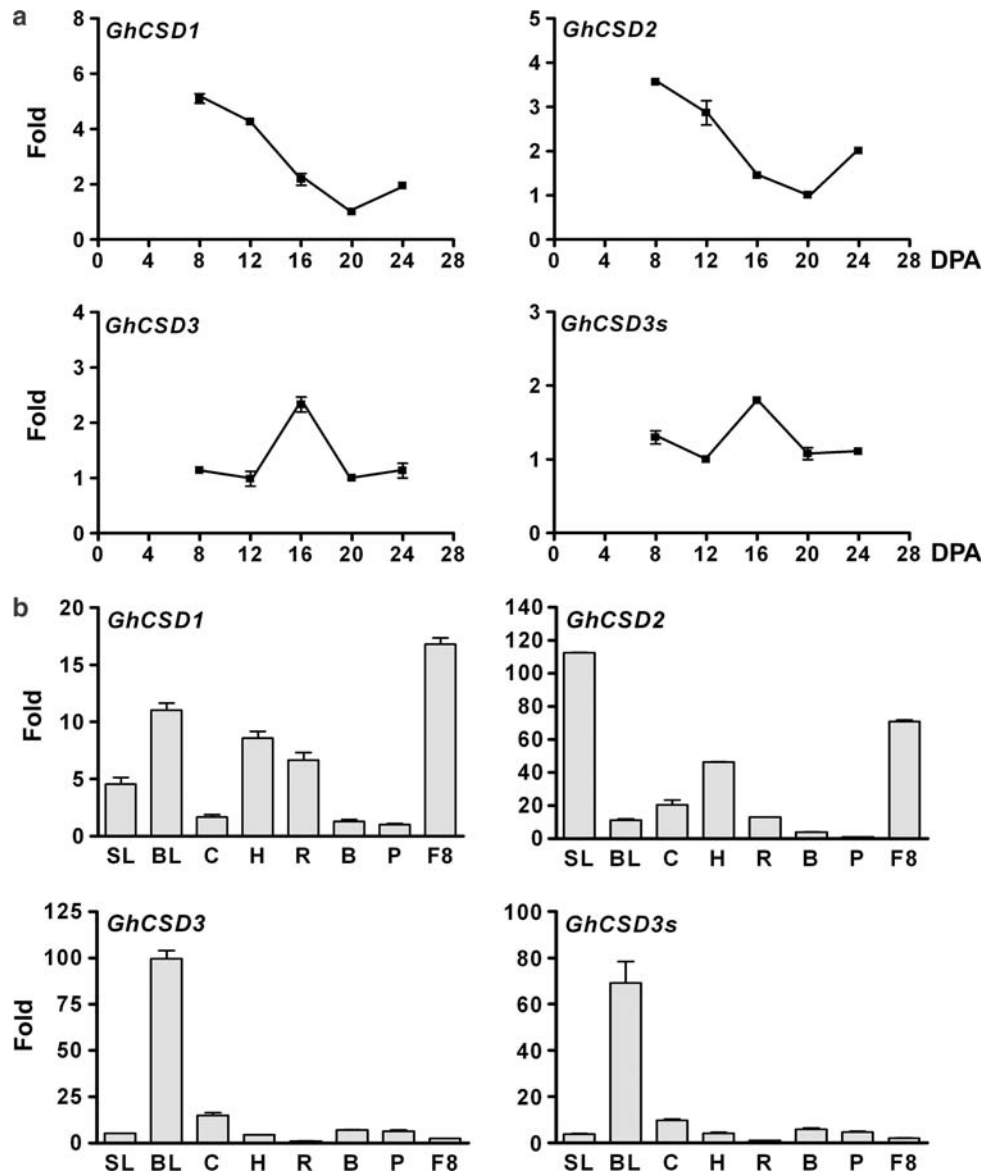
**Fig. 4** Phylogenetic tree and putative subcellular localization of CSDs. Deduced protein sequences of *GhCSD1*, *GhCSD2*, and *GhCSD3* were analyzed by MAFFT in comparison with the deduced proteins of *AtCSD1* (*Arabidopsis thaliana*, AT1G08830), *AtCSD2* (AT2G28190), *AtCSD3* (AT5G18100), pine HipI-SOD (*Pinus sylvestris*, CAA05633), aspen HipI-SOD (*Populus tremula* × *Populus tremuloides*, CAC33847), *SoCSD1* (*Spinacia oleracea*, CAA37866), *SoCSD2* (BAA01088). Citations identifying the putative subcellular localization of CSDs: *AtCSDs* (Kliebenstein et al. 1998), *SoCSDs* (Ogawa et al. 1995, 1996), pine HipI-SOD (Karpinska et al. 2001), and aspen HipI-SOD (Srivastava et al. 2007). SCW secondary cell wall, PM plasma membrane

other CSDs (Kliebenstein et al. 1998). A contributing factor to this lack of specificity may be the low endogenous levels of *AtCSD3* compared with the other forms of CSD (Kliebenstein et al. 1998). Immunogold labeling showed that HipI-SOD is localized in the plasma membrane, Golgi apparatus, SCW, and intercellular spaces of pine (Karpinska et al. 2001). Immunolocalization of HipI-SOD in cross-sections of stems of hybrid aspen plants showed that this CSD localized in the lignified SCWs of phloem fibers and xylem vessels (Srivastava et al. 2007).

All *GhCSDs* are developmentally regulated in cotton fibers and preferentially expressed in different tissues

The expression patterns of *GhCSDs* were determined by Q-RT-PCR (Fig. 5). Specific primer pairs for *GhCSD1*, *GhCSD3* and *GhCSD3s* were designed to amplify unique cDNAs; however, the primers for *GhCSD2* detected both *GhCSD2a* and *GhCSD2b* because their sequences are almost identical. *GhCSD1*, 2, 3, and 3s are expressed in fibers and exhibit developmentally regulated expression patterns (Fig. 5a). The expression patterns of *GhCSD1* and *GhCSD2* are almost identical to each other, but are very different from those of *GhCSD3* and *GhCSD3s*. *GhCSD1* and *GhCSD2* levels peaked during the elongation stage (8–12 DPA), and declined coincident with the initiation of SCW synthesis (16 DPA). Many genes involved in cell elongation or SCW synthesis in cotton fibers are transcrip-

**Fig. 5** Relative transcript abundance of *GhCSD* genes in fiber and other tissues. **a** Relative transcript abundance of *GhCSD* genes in 8–24 DPA fibers measured by Q-RT-PCR. The fold difference is relative to the lowest transcript level present in 20 DPA fibers for *GhCSD1* and *GhCSD2* and 12 DPA fibers for *GhCSD3s*. **b** Relative transcript abundance of *GhCSDs* in different cotton tissues measured by Q-RT-PCR. RNA was isolated from young, small leaves (*SL*), fully expanded big leaves (*BL*), 1-week old cotyledons (*C*), 1-week old hypocotyls (*H*), 1-week old roots (*R*), DOA bracts (*B*), DOA petals (*P*), and 8 DPA fibers (*F8*). The fold difference is relative to the lowest transcript level present in petals for *GhCSD1* and *GhCSD2* and roots for *GhCSD3* and *GhCSD3s*



tionally regulated, and there are multiple developmental programs controlling gene expression throughout cotton fiber development (Smart et al. 1998; Haigler et al. 2005). The expression patterns of *GhCSD1* and *GhCSD2* are similar to the patterns of genes expressed primarily during the cell elongation stage of fiber development (Whittaker and Triplett 1999; Kim and Triplett 2004). Interestingly, the patterns and scale of down-regulation of *GhCSD1* and *GhCSD2* are almost identical to those of *G. hirsutum* cellulose synthase catalytic subunits 3 and 5 (*GhCesA3* and *GhCesA5*) that are involved in cellulose biosynthesis for the PCW during fiber elongation (Kim and Triplett 2007). In contrast, *GhCSD3* and *GhCSD3s* transcript abundance increased at the transition to SCW biosynthesis.

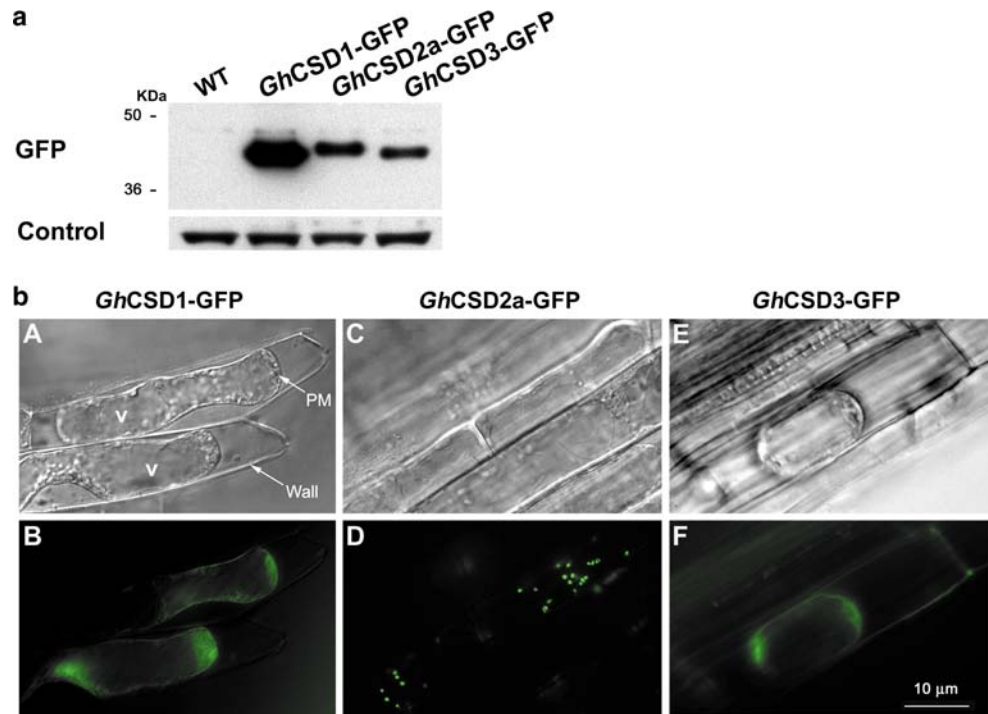
Although it is inappropriate to compare directly the expression of one gene to another by relative Q-RT-PCR since genes with different sequences may not be reverse

transcribed with the same efficiency, the number of threshold cycles ( $C_T$ ) can provide an indirect estimation of transcript accumulation levels of individual members of a highly similar gene family. The  $C_T$  numbers for *GhCSD1*, 2, and 3 suggested that *GhCSD3* was less abundantly expressed than *GhCSD1* or *GhCSD2*. In 8 DPA fibers, the transcript level of *GhCSD3* was ~73 times lower than *GhCSD1* and 17 times lower than *GhCSD2*. In 16 DPA fibers, the transcript level of *GhCSD3* was ~15 times lower than *GhCSD1* and 3 times lower than *GhCSD2*.

To compare the expression patterns of *GhCSD1*, 2, 3, and 3s in specific tissues, Q-RT-PCR was also performed using RNA isolated from young leaves, fully expanded leaves, 1-week old cotyledons and hypocotyls, roots, bracts, and petals, in addition to 8 DPA elongating fibers (Fig. 5b). All four *GhCSDs* were expressed in the tested tissues with tissue-preferential expression patterns. Both



**Fig. 6** **a** Immunoblot analysis of *GhCSDs*-GFP with total proteins of transgenic *Arabidopsis*. A non-specific band recognized by antipolyclonal GFP was used as a loading control. **b** Subcellular localization of *GhCSD1*-GFP, *GhCSD2a*-GFP, and *GhCSD3*-GFP. To view GFP fluorescence in the cell wall, roots of *GhCSD1*-GFP and *GhCSD3*-GFP were plasmolyzed. *GhCSD1*-GFP (panels A and B), *GhCSD2a*-GFP (panels C and D), *GhCSD3*-GFP (panels E and F), live images (panels A, C, and E), fluorescent images (panels B, D, and F). V vacuole, PM plasma membrane



*GhCSD1* and *GhCSD2* showed a high level of expression in elongating tissues such as 1-week old hypocotyls and roots, 8 DPA elongating fiber, and young leaves. Unlike *GhCSD2* that is preferentially expressed in expanding young leaves, *GhCSD1* was expressed in both young and fully expanded leaves. In contrast, *GhCSD3* and *GhCSD3s* are more abundantly expressed in fully expanded leaves than any other tested tissue. Preferential expression (70–100 times more) in fully expanded leaves suggests that *GhCSD3* and *GhCSD3s* may be involved in processes unrelated to cell enlargement in these tissues.

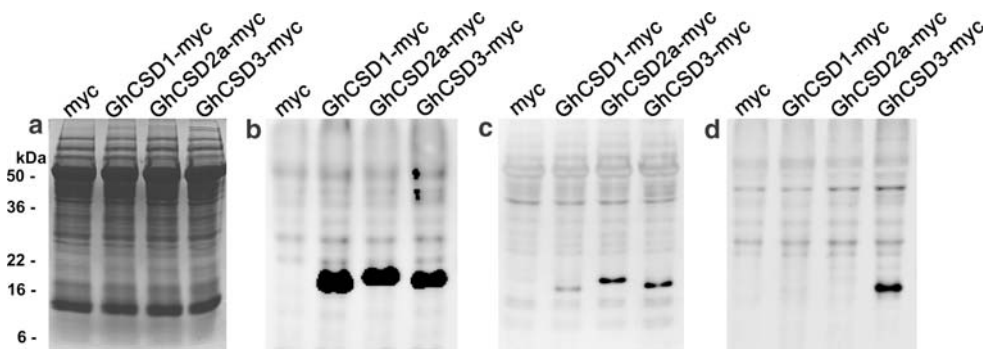
**Localization of *GhCSD1*-GFP, *GhCSD2a*-GFP, and *GhCSD3*-GFP**

To avoid some of the potential artifacts associated with immunolocalization of proteins that have great sequence similarity and very different endogenous levels, we prepared GFP constructs linked to each type of *GhCSD* and used them to transform *Arabidopsis*. Immunoblot analysis of total proteins from these transgenic plants using a polyclonal GFP antibody shows that the fusion proteins of *GhCSD1*-GFP, *GhCSD2a*-GFP, and *GhCSD3*-GFP are abundant, but not all at the same level (Fig. 6a).

Since EC *GhCSD* was found in SCWs of developing cotton fibers (Fig. 2), we first tested if GFP signals from the fusion proteins can be detected in xylem SCW in transgenic *Arabidopsis* stems. The high level of lignin autofluorescence in xylem, however, precluded these localization studies (data not shown). Root cells of untransformed wild-type

showed little autofluorescence, so we monitored GFP signals in transgenic plant roots. Without plasmolysis, the GFP signals from *GhCSD1*-GFP and *GhCSD3*-GFP were almost indistinguishable. After gentle plasmolysis, the GFP signal of *GhCSD1*-GFP was found in the cytosol and nuclei of root cells (Fig. 6b, panels A and B). The signal of *GhCSD2a*-GFP containing a transit peptide was specifically found in organelles that appear to be unpigmented plastids of root cells (Fig. 6b, panels C and D). The signal of *GhCSD2a*-GFP was specifically detected in guard cell chloroplasts (data not shown). The GFP signal of *GhCSD3*-GFP in plasmolyzed root cells was found in cell walls in addition to the cytosol and nuclei (Fig. 6b, panels E and F). As a result, we conclude that *GhCSD3*-GFP was translocated to root cell walls in transgenic *Arabidopsis*, but *GhCSD1*-GFP and *GhCSD2a*-GFP were not.

Although GFPs are routinely used to determine the subcellular localization of proteins, there is the possibility that erroneous GFP signals can result from overexpressing high levels GFP or using a pH-sensitive GFP. Other highly overexpressed GFP constructs that localize to the cytosol have also resulted in nuclear GFP fluorescence (Lertpiriyapong and Sung 2003; Zhou and Li 2005). Fluorescence of some GFPs is often quenched at acidic pH, such as that found in plant cell walls (Ashby et al. 2004). Expression of all three *GhCSD*-GFPs was under the control of the *CaMV35S* promoter, and all three fusion proteins were found abundantly in transgenic plants (Fig. 6a). Notably, the abundance of *GhCSD1*-GFP fusion protein was much higher than those of *GhCSD2*-GFP or *GhCSD3*-GFP (Fig. 6a). Since the



**Fig. 7** Subcellular distribution of *GhCSD1-myc*, *GhCSD2a-myc*, and *GhCSD3-myc* in transgenic *Arabidopsis*. **a** Coomassie blue stained *Arabidopsis* soluble proteins (60 µg) in 12% SDS-PAGE gel for a loading control. **b** Immunoblot analyses of total proteins (10 µg). **c** Immunoblot analyses of soluble proteins (10 µg). **d** Immunoblot

analyses of cell wall proteins (10 µg). Monoclonal c-myc antibody (1:1,000 dilution) was the primary antibody for all immunoblots. Secondary antibody was horseradish peroxidaseconjugated donkey anti-mouse IgG (1:1,000 dilution) with detection by SuperSignal West Pico Chemiluminescent Substrate

more abundant fusion protein, *GhCSD1-GFP* failed to translocate to cell walls (Fig. 6b, panel B) whereas the less abundant *GhCSD3-GFP* can translocate to cell walls (Fig. 6b, panel F), we conclude that abundance of *GhCSD3-GFP* did not affect its localization pattern. To avoid the quenching of GFP signals in acidic cell walls, we used mGFP-5 that was engineered for removal of a cryptic intron and increased thermostability (Siemering et al. 1996) and was detected in the cell wall when fused with ELD1 (Lertpiriyapong and Sung 2003). The GFP signal of mGFP-5 fused with *GhCSD3* (this paper) was strong enough to be detected in the cell wall.

#### *GhCSD3-myc* co-purifies with a cell wall fraction

*GhCSD1* of Group 1 and *GhCSD3* of Group 3 are similar to each other; there is a high sequence similarity, both deduced proteins lack signal peptides and are predicted to localize in the cytoplasm by PSORT (Nakai and Kanehisa 1991). Cytosolic *SoCSD1*, also of Group 1, was reported to localize to spinach cell walls (Ogawa et al. 1996), although there is a possibility that *SoCSD1* antibody may crossreact with other CSDs. Nevertheless, we tested if low levels of *GhCSD1*, also belonging to Group 1, can be translocated to cell walls. Since weak GFP signals of *GhCSD1-GFP* in the acidic wall can be quenched and may not be detected, we decided to discern the subcellular localizations of *GhCSD1* and *GhCSD3* using a c-myc epitope tag (Jarvik and Telmer 1998).

The short c-myc sequence (ten amino acids) was fused with the C-terminus of three forms of *GhCSD* and transformed into *Arabidopsis* plants. A construct containing myc alone was used as a negative control. The levels of myc alone, *GhCSD1-myc*, *GhCSD2a-myc*, and *GhCSD3-myc* controlled by a *CaMV35S* promoter in each transgenic line were estimated by immunoblot analyses of multiple trans-

formants. Subsequently, transgenic lines expressing approximately equivalent levels of *GhCSD1*, *GhCSD2a*, and *GhCSD3* were selected and used for further analyses (Fig. 7a, b). Monoclonal c-myc antibody detected *GhCSD1-myc* (17 kDa), *GhCSD2-myc* (20 kDa), and *GhCSD3-myc* (18 kDa) in total protein preparations from *Arabidopsis* plants (Fig. 7b). All three *GhCSDs* were detected in soluble proteins extracts (Fig. 7c) and *GhCSD3-myc* was specifically detected in cell wall extracts (Fig. 7d). The results of these immunoblot analyses confirm that *GhCSD3* translocates to the cell wall, but *GhCSD1* and *GhCSD2* do not.

#### Conclusions

Using several complementary approaches, we show here that a CSD is associated with cell walls. First, a peptide antibody designed to a conserved domain of plant CSDs recognized an EC protein extracted from immature cotton seeds. Secondly, the same antibody labeled fiber SCWs as shown by immuno-gold labeling. By overexpressing six different constructs in *Arabidopsis*, i.e., three *GhCSDs* fused to GFP and three *GhCSDs* fused to c-myc, we show that only *GhCSD3* can translocate to the cell wall. Furthermore, since only *GhCSD3* localized to *Arabidopsis* cell walls, we propose that *GhCSD3* is the wall protein detected by the peptide CSD antibody.

Cell wall lignification has been suggested as the principle function of CSDs that localize to plant SCWs (Ogawa et al. 1996, 1997; Karpinska et al. 2001; Schinkel et al. 2001; Karlsson et al. 2005); however, cotton fiber SCWs are unique in that they are not lignified. Additional evidence for the function of Group 3 CSDs comes from a recent report of antisense lines of transgenic hybrid aspen (Srivastava et al. 2007). Reduced expression of HipI-SOD

resulted in increased levels of ROS with major effects on plant development. Antisense-HipI-SOD plants had more lateral branches and were severely dwarfed due to reductions in both cell division and expansion; one line was impaired in apical dominance. The transgenic lines had only a slight decrease in lignin content relative to wild-type, but contained elevated levels of phenolic acids. Microarray analysis indicated that expression of many genes involved in cell signaling, stress responses, and lignification changed in the transgenic lines. Similarly, the activity of an EC CSD was found to be responsive to biotic and abiotic stresses (Kasai et al. 2006; Zhang et al. 2008). Together, these recent investigations provide additional support that Group 3 CSDs play important roles in plant development by regulating the ROS content of cell walls.

The dwarfed phenotype of transgenic aspen in which EC CSD levels were reduced (Srivastava et al. 2007) raises the question of whether EC CSDs may be required for wall elongation and expansion of PCWs. Recent advances in characterizing the cell wall proteome also suggest potential involvement of EC CSDs in PCWs of elongating roots (Zhu et al. 2006, 2007). Our observation that *GhCSD3*-GFP translocated to PCW of *Arabidopsis* root cells also suggest the potential involvement of EC CSDs in PCW biosynthesis in some plant tissues; however, the precise physiological roles of EC CSD in PCW biosynthesis remain to be discovered.

In summary, we propose that EC H<sub>2</sub>O<sub>2</sub> levels are regulated by redox status-related enzymes including EC *GhCSD3* and that developmentally regulated changes in the EC levels of *GhCSD3* can influence plant cell wall growth.

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## References

- Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot* 53:1331–1341
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399
- Ashby MC, Ibaraki K, Henley JM (2004) It's green outside: tracking cell surface proteins with pH sensitive GFP. *Trends Neurosci* 27:257–261
- Beasley CA, Ting IP (1973) The effects of plant growth substances on in vitro fiber development from fertilized cotton ovules. *Am J Bot* 60:130–139
- Bhasin M, Raghava GPS (2004) ESLpred: SVM based method for subcellular localization of eukaryotic proteins using dipeptide composition and PSI-BLAST. *Nucleic Acids Res* 32:414–419
- Bowler C, Montagu MV, Inze D (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 43:83–116
- Cannio R, Fiorentino G, Morana A, Rossi M, Bartolucci S (2000) Oxygen: friend or foe? Archaeal superoxide dismutases in the protection of intra- and extracellular oxidative stress. *Front Biosci* 5:D768–D779
- Carpita N, McCann M (2000) The cell wall. In: Buchanan BB, Gruissem W, Jones RL (eds) *Biochemistry & molecular biology of plants*. American Society of Plant Physiologists, Rockville, pp 52–109
- Chen SX, Schopfer P (1999) Hydroxyl-radical production in physiological reactions. A novel function of peroxidase. *Eur J Biochem* 260:726–735
- Chou KC, Shen HB (2007) Large-scale plant protein subcellular location prediction. *J Cell Biochem* 100:665–678
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Cosgrove DJ (2005) Growth of the plant cell wall. *Nat Rev Mol Cell Biol* 6:850–861
- Emanuelsson O, Nielsen H, Heijne GV (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides. *Protein Sci* 8:978–984
- Emanuelsson O, Brunak S, Heijne GV, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP, and related tools. *Nat Protoc* 2:953–971
- Fry SC (1998) Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochem J* 332:507–515
- Haigler CH, Zhang D, Wilkerson CG (2005) Biotechnological improvement of cotton fibre maturity. *Physiol Plant* 124:285–294
- Harmer SL, Hogenesch JB, Straume M, Chang H, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290:2110–2113
- Horton P, Park K, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007) WoLF PSORT: protein localization predictor. *Nucleic Acid Res* 35:W585–W587
- Hough MA, Hasnain S (1999) Crystallographic structures of bovine copper-zinc superoxide dismutase reveal asymmetry in two subunits: functionally important three and five coordinate copper sites captured in the same crystal. *J Mol Biol* 287:579–592
- Jarvik JW, Telmer CA (1998) Epitope tagging. *Annu Rev Genet* 32:601–618
- Kanematsu S, Asada K (1990) Characteristic amino acid sequences of chloroplast and cytosol isozymes of CuZn-superoxide dismutase in spinach, rice, and horsetail. *Plant Cell Physiol* 31:99–112
- Karlsson M, Melzer M, Prokhorenko I, Johansson T, Wingsle G (2005) Hydrogen peroxide and expression of hipI-superoxide dismutase are associated with the development of secondary cell walls in *Zinnia elegans*. *J Exp Bot* 56:2085–2093
- Karpinska B, Karlsson M, Schinkel H, Steller S, Süß KH, Melzer M, Wingsle G (2001) A novel superoxide dismutase with a high isoelectric point in higher plants. Expression, regulation and protein localisation. *Plant Physiol* 126:1668–1677
- Kasai T, Suzuki T, Ono K, Ogawa K, Inagaki Y, Ichinose Y, Toyoda K, Shiraishi T (2006) Pea extracellular Cu/Zn-superoxide dismu-

- tase responsive to signal molecules from a fungal pathogen. *J Gen Plant Pathol* 72:265–272
- Katoh K, Misawa K, Kuma KI, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 30:3059–3066
- Keller GA, Krisans S, Gould SJ, Sommer JM, Wang CC, Schliebs W, Kunau W, Brody S, Subramani S (1991) Evolutionary conservation of a microbody targeting signal that targets proteins to peroxisomes, glyoxysomes, and glycosomes. *J Cell Biol* 114:893–904
- Kieliszewski JJ, Lamport DTA (1994) Extensin: repetitive motifs, functional sites, post-translational codes and phylogeny. *Plant J* 5:157–172
- Kim HJ, Triplett BA (2001) Cotton fiber growth in planta and in vitro. Models for plant cell elongation and cell wall biogenesis. *Plant Physiol* 127:1361–1366
- Kim HJ, Triplett BA (2004) Cotton fiber germin-like protein. I. Molecular cloning and gene expression. *Planta* 218:516–524
- Kim HJ, Triplett BA (2007) Cellulose synthase catalytic subunit (*Cesa*) genes associated with primary or secondary wall biosynthesis in developing cotton fibers (*Gossypium hirsutum*). In: Proceedings of the World Cotton Res Conference 4, Lubbock, TX, <http://wrc.confex.com/wrc/2007/techprogram/P1764.HTM>
- Kim HJ, Pesacreta TC, Triplett BA (2004) Cotton-fiber germin-like protein. II: Immunolocalization, purification, and functional analysis. *Planta* 218:525–535
- Kliebenstein DJ, Monde R, Last RL (1998) Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol* 118:637–650
- Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M, Delmer D (2002) Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. *Proc Natl Acad Sci USA* 99:11109–11114
- Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. *Curr Opin Plant Biol* 7:323–328
- Lertpiriyapong K, Sung ZR (2003) The *elongation defective 1* mutant of *Arabidopsis* is impaired in the gene encoding a serine-rich secreted protein. *Plant Mol Biol* 53:581–595
- Naithani SC, Rama-Rao N, Krishnan PN, Singh YD (1981) Changes in *o*-diphenol oxidase during fibre development in cotton. *Ann Bot* 48:379–385
- Nakai K, Kanehisa M (1991) Expert system for predicting protein localization sites in gram-negative bacteria. *Protein* 11:95–110
- Ogawa K, Kanematsu S, Takabe K, Asada K (1995) Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immuno-gold labeling after rapid freezing and substitution method. *Plant Cell Physiol* 35:565–573
- Ogawa K, Kanematsu S, Asada K (1996) Intra- and extra-cellular localization of cytosolic CuZn-superoxide dismutase in spinach leaf and hypocotyls. *Plant Cell Physiol* 37:790–799
- Ogawa K, Kanematsu S, Asada K (1997) Generation of superoxide anion and localization of CuZn-superoxide dismutase in the vascular tissues of spinach hypocotyls: their association with lignification. *Plant Cell Physiol* 38:1118–1126
- Pomar F, Caballero N, Pedreno M, Ros Barcelo A (2002) H<sub>2</sub>O<sub>2</sub> generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. *FEBS Lett* 529:198–202
- Potikha TS, Collins CC, Johnson DI, Delmer DP, Levine A (1999) The involvement of hydrogen peroxide in the differentiation of secondary walls in cotton fibers. *Plant Physiol* 119:849–858
- Robertson D, Mitchell GP, Gilroy JS, Gerrish C, Bolwell GP, Slabas AR (1997) Differential extraction and protein sequencing reveals major differences in patterns of primary cell wall proteins from plants. *J Biol Chem* 272:15841–15848
- Schinkel H, Hertzberg M, Wingsle G (2001) A small family of novel CuZn-superoxide dismutases with high isoelectric points in hybrid aspen. *Planta* 213:272–279
- Schweikert C, Liszakay A, Schopfer P (2000) Scission of polysaccharides by peroxidase-generated hydroxyl radicals. *Phytochemistry* 53:565–570
- Siemerling KR, Golbik R, Sever R, Haseloff J (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr Biol* 6:1653–1663
- Smart LB, Vojdani F, Maeshima M, Wilkins TA (1998) Genes involved in osmoregulation during turgor-driven cell expansion of developing cotton fibers are differentially regulated. *Plant Physiol* 116:1539–1549
- Srivastava V, Schinkel H, Witzell J, Hertzberg M, Torp M, Srivastava MK, Karpinska B, Melzer M, Wingsle G (2007) Downregulation of high-isoelectric-point extracellular superoxide dismutase mediates alterations in the metabolism of reactive oxygen species and developmental disturbances in hybrid aspen. *Plant J* 49:135–148
- Streller S, Wingsle G (1994) *Pinus sylvestris* L. needles contain extracellular CuZn superoxide dismutase. *Planta* 192:195–201
- Tainer JA, Getzoff ED, Beem KM, Richardson JS, Richardson DC (1982) Determination and analysis of the 2 Å structure of copper, zinc superoxide dismutase. *J Mol Biol* 160:181–217
- Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss H-H (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res* 15:5890
- Whittaker DJ, Triplett BA (1999) Gene-specific changes in  $\alpha$ -tubulin transcript accumulation in developing cotton fibers. *Plant Physiol* 121:181–188
- Zhang H, Xia Y, Wang G, Zhenguang S (2008) Excess copper induces accumulation of hydrogen peroxide and increases lipid peroxidation and total activity of copper–zinc superoxide dismutase in roots of *Elsholtzia haichowensis*. *Planta* 227:465–475
- Zhou A, Li J (2005) *Arabidopsis* BRS1 is a secreted and active serine carboxypeptidase. *J Biol Chem* 280:35554–35561
- Zhu J, Chen S, Alvarez S, Asirvatham VS, Schachtman DP, Wu Y, Sharp RE (2006) Cell wall proteome in the maize primary root elongation zone. I. Extraction and identification of water-soluble and lightly ionically bound proteins. *Plant Physiol* 140:311–325
- Zhu J, Alvarez S, Marsh EI, LeNoble ME, Cho I, Sivaguru M, Chen S, Nguyen HT, Wu Y, Schachtman P, Sharp RE (2007) Cell wall proteome in the maize primary root elongation zone. II. Region-specific changes in water soluble and lightly ionically bound proteins under water deficit. *Plant Physiol* 145:1533–1548