

CHAPERONIN 20 mediates iron superoxide dismutase (FeSOD) activity independent of its co-chaperonin role in Arabidopsis chloroplasts

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Summary

- Iron superoxide dismutases (FeSODs; FSDs) are primary antioxidant enzymes in *Arabidopsis thaliana* chloroplasts. The stromal FSD1 conferred the only detectable FeSOD activity, whereas the thylakoid membrane- and nucleoid-co-localized FSD2 and FSD3 double mutant showed arrested chloroplast development.
- FeSOD requires cofactor Fe for its activity, but its mechanism of activation is unclear. We used reversed-phase high-performance liquid chromatography (HPLC), gel filtration chromatography, LC-MS/MS, protoplast transient expression and virus-induced gene silencing (VIGS) analyses to identify and characterize a factor involved in FeSOD activation.
- We identified the chloroplast-localized co-chaperonin CHAPERONIN 20 (CPN20) as a mediator of FeSOD activation by direct interaction. The relationship between CPN20 and FeSOD was confirmed by *in vitro* experiments showing that CPN20 alone could enhance FSD1, FSD2 and FSD3 activity. The *in vivo* results showed that CPN20-overexpressing mutants and mutants with defective co-chaperonin activity increased FSD1 activity, without changing the chaperonin CPN60 protein level, and VIGS-induced downregulation of CPN20 also led to decreased FeSOD activity.
- Our findings reveal that CPN20 can mediate FeSOD activation in chloroplasts, a role independent of its known function in the chaperonin system.

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Introduction

Reactive oxygen species (ROS) are harmful by-products of essential physiological reactions (Fridovich, 1978, 1983; Halliwell & Gutteridge, 2007). Superoxide dismutase (SOD) acts as a primary defense against ROS by converting O_2^- to O_2 and H_2O_2 , which requires a specific metal cofactor (Beyer *et al.*, 1991; Bowler *et al.*, 1992). Members of this enzyme family include the SODs copper–zinc (CuZnSOD), manganese (MnSOD), iron (FeSOD) and nickel (NiSOD), classified according to the metal cofactor required (McCord & Fridovich, 1969; Zelko *et al.*, 2002). Most eukaryotes harbor CuZnSOD and MnSOD (Weisiger & Fridovich, 1973; Marres *et al.*, 1985; Crapo *et al.*, 1992; Alscher *et al.*, 2002). FeSOD, found in plants and prokaryotes, is thought to originate in the plastid before moving to the nuclear genome (Alscher *et al.*, 2002; Fink & Scandalios, 2002). All three SOD groups can be found as dimers or tetramers, depending on the species (Alscher *et al.*, 2002). Because phospholipid membranes are impermeable to O_2^- , the existence of multiple SODs in different compartments ensures efficient protection in various organelles, particularly

chloroplasts, where molecular O_2 can be easily photoreduced from photosystem I (Mehler, 1951; Takahashi & Asada, 1983).

Arabidopsis has three CuZnSODs (*CSD1*, *CSD2* and *CSD3*), one MnSOD (*MSD1*) and three FeSODs (*FSD1*, *FSD2* and *FSD3*) genes. The use of KCN and H_2O_2 inhibitors revealed three bands of CuZnSOD and one band each of MnSOD and FeSOD as the major SOD activity signal (Kliebenstein *et al.*, 1998; Alscher *et al.*, 2002; Chu *et al.*, 2005). Among these SODs, FSD1, FSD2, FSD3 and CSD2 localize within chloroplasts at different sites. The localization of FSD1 was found to be stromal by the fractionation of chloroplasts and analysis of the proteome in the stroma (Kliebenstein *et al.*, 1998; Peltier *et al.*, 2006). FSD2, FSD3 and CSD2 were found to be attached to the thylakoid membranes: FSD2 was evenly distributed, whereas FSD3 co-localized with chloroplast nucleoids (Ogawa *et al.*, 1995; Myouga *et al.*, 2008). Hence, three of the four SODs in chloroplasts use Fe as a cofactor.

The *Arabidopsis* FSD T-DNA knockout mutants *fsd2* and *fsd3* showed slow growth, a pale-green color and abnormal chloroplasts, and the double mutant even showed a severe albino phenotype and arrested chloroplast development, possibly because of damage to the chloroplast nucleoids by superoxide anions (Myouga *et al.*, 2008). Therefore, *FSD2* and *FSD3* may be essential for the

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maintenance of early chloroplast development. FSD2 and FSD3, which formed a heterocomplex in chloroplasts, provided significant protection to chloroplast nucleoids and the chloroplast gene expression machinery. However, the *fsd1* mutant showed no phenotype change under normal growth conditions (Myounga *et al.*, 2008).

Although FeSODs are critical for protection against oxidative stress and chloroplast development, their activation mechanism has not yet been reported, and constitutes the topic of the present investigation. We isolated and characterized the co-chaperonin CHAPERONIN 20 (CPN20). We showed that CPN20 mediates FeSOD activity in chloroplasts, in addition to its well-known co-chaperonin function (Bertsch *et al.*, 1992; Hirohashi *et al.*, 1999; Weiss *et al.*, 2009).

Materials and Methods

Plants, yeast strains and growth conditions

The *Arabidopsis thaliana* (L.) Heynh *FSD1* (*At4g25100*) and *FSD2* (*At5g11100*) knockout lines, *fsd1-1* (SALK_029455), *fsd1-2* (SALK_036006) and *fsd2* (SALK_080457), were obtained from the Arabidopsis Biological Research Center (Ohio State University, Columbus, OH, USA). Seedlings were grown at 23°C with 16 h of light at 60–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Transgenic plants were created with the Arabidopsis Columbia ecotype by the floral dip method (Clough & Bent, 1998) and were selected by spraying seedlings with 0.4% BASTA herbicide (McDowell *et al.*, 1998).

The tomato (*Solanum lycopersicum* L.) cultivar L390 was grown at 25°C with 12 h of light at 60–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The fully expanded cotyledons of 10-d-old tomato underwent virus-induced gene silencing (VIGS) (Liu *et al.*, 2002). *Agrobacterium tumefaciens* GV3101 was used for infiltration. The infiltrated tomato plants were maintained at 20–22°C with 12 h of light for 2 wk; leaves were then obtained for further analysis.

The *Saccharomyces cerevisiae* strain BY4741 (*MATa*, *his3 Δ 1*, *leu2 Δ 0*, *met15 Δ 0*, *ura3 Δ 0*) was obtained from the *Saccharomyces* Genome Deletion Project (Stanford University, Stanford, CA, USA). Yeast was incubated at 30°C under aerobic conditions without shaking. Enriched yeast extract, peptone-based medium supplemented with 2% glucose (YPD) and synthetic dextrose (SD) medium were used to propagate yeast strains. Yeast transformation involved the lithium acetate procedure (Gietz & Schiestl, 1991). Yeast two-hybrid assay involved a Matchmaker Two Hybrid System 3 (Clontech, Palo Alto, CA, USA). Protein–protein interactions were examined on SD medium lacking leucine (Leu), tryptophan (Trp) and histidine (His) and supplemented with adenine (Ade) (Sigma).

Constructs

For green fluorescent protein (GFP) fusion protein, *FSD1* cDNA and a 33 N-terminal nucleotide-deleted *FSD1* cDNA (chloroplast transit peptide-deleted FSD1; Δ TP-FSD1) were cloned into the 326-GFP vector (Lee *et al.*, 2001). For glutathione-S-transferase (GST)-tagged protein, *FSD1*, *FSD2*, *FSD3* (*At5g23310*) and

CPN20 (*At5g20720*) cDNAs were cloned into pGEX-6P-1 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For yeast two-hybrid assay, genes were cloned into pGBKT7 or pACT2 (Clontech) in-frame with the GAL4 DNA binding domain (BD) or activation domain (AD), respectively. For fluorescence resonance energy transfer (FRET) assay, the 35S promoter of pPE1000 (Hancock *et al.*, 1997) was cloned into pECFP and pEYFP (Clontech) to create p35S-ECFP and p35S-EYFP. *FSD1* and *CPN20* cDNAs were subcloned into p35S-ECFP and p35S-EYFP, respectively. A transit peptide used to deliver a control cyan fluorescent protein (CFP) to chloroplasts was taken from the 71 N-terminal residues of the chloroplast-localized CSD2 (*At2g28190*). For transient expression of *CPN20* in protoplasts, *CPN20* cDNA (with stop codon) was cloned into p35S-EYFP. To generate *CPN20*-overexpressing lines, the genomic sequence of *CPN20* was cloned into pPZP200GB (Chu *et al.*, 2005). For the VIGS experiment, pTRV1 and pTRV2 were used (Liu *et al.*, 2002). A tomato *CPN20* (Hanania *et al.*, 2007; Solyc07g042250) fragment was cloned into pCR8/GW/TOPO (Invitrogen), and then recombined into a pTRV2, pYL279. pTRV2::GUS, pTRV2 containing a β -glucuronidase (*GUS*) gene, was used as a reference. All gene fragments were sequenced before making the constructs. The primers used in this study are given in Supporting Information Table S1. The construction and purification of the His-tagged CPN20 and mutants were performed as described by Bonshtien *et al.* (2007).

Recombinant protein purification and deactivation

The GST-fused proteins were affinity purified using a glutathione–agarose column (Sigma). For Holo-protein preparation, the GST tag was removed by PreScission protease (Sigma), passed through the glutathione–agarose column to remove the GST and then dialyzed against phosphate-buffered saline (PBS) before use. The absolute SOD activities of Holo-FSD1, Holo-FSD2 and Holo-FSD3 were 10–15, 1–2 and 10–13 units mg^{-1} protein min^{-1} , respectively. The inactive FSD1 (Apo-FSD1) was prepared according to the alkaline denaturation protocol (Yamakura, 1978). Briefly, the Holo-FSD1 was incubated with pH 11 alkaline buffer (0.2 M sodium carbonate and 1 mM sodium dithionite) at 30°C for 2 h, and dialyzed twice against pH 9.2 alkaline buffer (0.2 M sodium carbonate, 1 mM EDTA and 1 mM dithiothreitol (DTT)), and then against pH 7.8 buffer (5 mM potassium phosphate); thus, FSD1 lost cofactor Fe and became inactive.

RNA extraction, SOD activity assays and immunoblotting

RNA was isolated with Trizol reagent (Invitrogen). The in-gel SOD activity assay was performed as described by Chu *et al.* (2005). SOD identification involved the use of 8 mM KCN and H_2O_2 inhibitors (Pan *et al.*, 1999). KCN inhibits CuZnSOD activity, whereas H_2O_2 inhibits both CuZnSOD and FeSOD activities. For liquid SOD activity assay, 30 μl of protein mixture in a 96-well plate was mixed with 270 μl of reaction buffer (50 mM Hepes, 13 mM methionine, 0.025% Triton X-100, 0.1 mM EDTA, 2 μM riboflavin and 75 μM nitroblue tetrazolium (NBT),

pH 7.6) and illuminated on a light box for 15 min; the absorbance was measured at 560 nm. Immunoblotting was performed as described by Chu *et al.* (2005). The SOD activities in-gel and protein signals in-blot were quantified with the use of LAS-3000 (Fujifilm, Tokyo, Japan) and ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA).

Cellular extract preparation and FeSOD activity assay

Arabidopsis *fsd1* leaf cellular extracts were prepared using 150 mM Tris grinding buffer (Chu *et al.*, 2005). The cellular extract was heated at 100°C for 30 min and centrifuged at 13 000 *g* for 30 min; the supernatant was then collected. A G-25-microspin column (GE Healthcare, Salt Lake City, WI, USA) was used to desalt the cellular extract. An aliquot of 30 µg of cellular extract was treated with 2 units of proteinase K (Invitrogen) at 30°C for 16 h. The 16-h incubation ensures complete protease self-digestion. The liquid SOD activity assay involved the use of 0.5 µg of Holo-FSD1 (0.7 µM), 1 µg of Holo-FSD2 (1.1 µM) and 2 µg of Holo-FSD3 (2.8 µM). The in-gel SOD activity assay involved the use of 0.25 µg of Holo- and Apo-FSD1. In total, 15 and 4.5 µg of cellular extract were used for Holo- and Apo-FSD1 activation analyses, respectively. The reaction volume was set at 30 µl and was incubated at 30°C for 30 min.

Reversed-phase high-performance liquid chromatography (HPLC), gel filtration chromatography and LC-MS/MS

The lyophilized supernatant of the heated *fsd1* cellular extract was resuspended in H₂O with 0.1% trifluoroacetic acid (TFA; J.T. Baker, Phillipsburg, NJ, USA) and then separated by HPLC on a Nova-Pak C₁₈ column (3.9 × 150 mm; Waters, Milford, MA, USA). Solvent A contained 0.1% TFA in H₂O, and solvent B contained 80% acetonitrile (J.T. Baker) with 0.1% TFA. A linear gradient from 0% to 100% solvent B was run over 30 min at a flow rate of 1 ml min⁻¹. Samples were pooled every 1 min, and 30 fractions were collected separately. Each fraction was lyophilized and resuspended in 100 µl of H₂O; 5 µl was used for SOD activation *in vitro*.

For gel filtration chromatography, HPLC fractions at 19–27 min (from three independent experiments) were pooled, lyophilized and then resuspended in 35 µl of buffer containing 100 mM Tris and 150 mM NaCl, pH 7.5. The sample then underwent gel filtration on a BioSuite 125 SEC HPLC column (4 µm UHR SEC, 4.6 × 300 mm; Waters) pre-equilibrated with the same buffer, and eluted at a flow rate of 0.35 ml min⁻¹ for 80 min. Samples were pooled every 2 min and collected separately. In total, 10 µl of each fraction was used for SOD activation *in vitro*.

For LC-MS/MS, each fraction was lyophilized and dissolved in 25 mM ammonium bicarbonate (ABC) containing 0.1% RapiGest SF Surfactant (Waters); the fractions were digested with trypsin (Sigma) overnight at 37°C, and then analysed using an Agilent 1100 Series HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) and an LTQ-FT ICR hybrid mass spectrometer (Thermo Electron, Bremen, Germany). The MS/MS data were used for protein identification with the MASCOT search engine

(<http://www.matrixscience.com>) based on the International Protein Index databases (<http://www.ebi.ac.uk/IPI>).

Protoplast preparation and transfection

Protoplast preparation and transfection were performed as described by Yoo *et al.* (2007). An aliquot of 10 µg of DNA and 2 × 10⁴ protoplasts were used for subcellular localization, and 25–100 µg DNA and 2 × 10⁵ protoplasts were used for SOD activity assay. The fluorescence images were captured using a Leica TCS SP5 Confocal Spectral Microscope imaging system. Three channels were used for FRET measurements: the donor (CFP) channel, excitation at 458 nm and emission at 462–510 nm; the acceptor (yellow fluorescent protein, YFP) channel, excitation at 514 nm and emission at 518–580 nm; the FRET channel, excitation at 458 nm and emission at 518–580 nm. C-FRET was calculated using FRET SE Wizard (Leica Microsystems; <http://www.leica-microsystems.com>).

Statistical analysis

All experiments were independently repeated at least three times. Statistical analysis involved Student's *t*-test (two-tailed, unpaired). *P* < 0.05 was considered to be statistically significant.

Results

FSD1 confers the only detectable FeSOD activity in Arabidopsis chloroplasts

To identify the *FSD* genes responsible for in-gel detectable FeSOD activity, we analysed the SOD activity profile in Arabidopsis *fsd1* and *fsd2* mutants. FeSOD activity was absent in *fsd1-1* and *fsd1-2*, but present in *fsd2* (Fig. 1a). Subsequent immunoblotting revealed FSD1 protein at the position of the FeSOD activity band (data not shown). FSD2 and FSD3 activities were undetectable, and FSD1 conferred the only detectable FeSOD activity. The *fsd3* plant growth was arrested after germination. Therefore, we used *FSD1* in further analyses.

Although FSD1 has been reported to be located in different cellular compartments of chloroplasts and cytosol (Kliebenstein *et al.*, 1998; Peltier *et al.*, 2002, 2006; Ferro *et al.*, 2003; Brugière *et al.*, 2004; Kleffmann *et al.*, 2004; Marmagne *et al.*, 2004; Myouga *et al.*, 2008), we showed its localization in chloroplasts using a GFP fusion product (Fig. 1b). When the 11 residues at the N-terminus of FSD1 were deleted (ΔTP-FSD1), based on a sequence alignment from different species (Fig. S1), the resulting ΔTP-FSD1 was present in cytoplasm and was still active (Fig. 1b,c).

Arabidopsis *fsd1* cellular extract facilitates FSD1 activation

Through the analysis of the activation of affinity-purified FSD1 proteins (Fig. S2a) *in vitro*, we screened the factor(s) involved in FeSOD activation. GST-FSD1 and Holo-FSD1 (with the GST tag removed) were active, whereas the apo form of FSD1 (Apo-FSD1) was inactive because of the absence of the cofactor Fe (Figs 2a, S3).

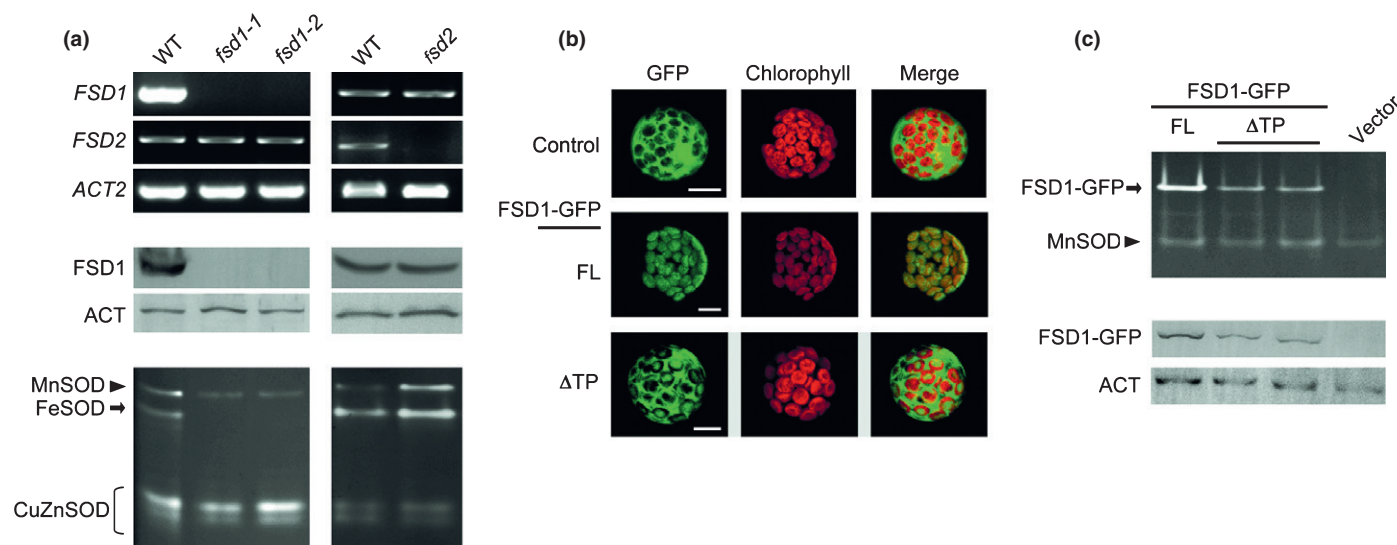


Fig. 1 FSD1 activity and localization. (a) *FSD1* and *FSD2* mRNA (top), FSD1 protein (middle) and superoxide dismutase (SOD) activity (bottom) in Arabidopsis wild-type (WT), *fsd1-1*, *fsd1-2* and *fsd2* lines. Subcellular localization (b) and activity (c) of green fluorescent protein (GFP) fusion of full-length FSD1 (FL-FSD1) and FSD1 with 11 N-terminal amino acids deleted (Δ ATP-FSD1) in Arabidopsis WT protoplasts. Immunoblotting involved FSD1, actin (ACT) and GFP antibodies. *Actin2* (ACT2) was used as an internal control. Bars, 10 μ m.

On treatment with Fe (0.5 or 1 mM) without *fsd1* cellular extract (Fig. 2b, lanes 3 and 5), Holo-FSD1 activity increased by 1.2-fold; with the cellular extract without Fe (lane 2), the activity was enhanced by 1.6-fold. With both cellular extract and Fe present, the activity was enhanced 2.2-fold (lanes 4 and 6). Apo-FSD1 activation showed a similar profile (Fig. 2c); however, activity was recovered with only Fe and the cellular extract combined.

Apo-FSD1 was unable to use Fe for activation at concentrations of up to 12 mM (Fig. 2c). Holo-FSD1 activity was increased by 1.2-fold with 0.5 mM Fe, but the activity was not proportional to the increase in Fe concentration (Fig. 2b). Therefore, the 1.2-fold activity enhancement may reach the limit for FSD1 activation through spontaneous, unaided acquisition of the cofactor Fe. Moreover, desalting of the cellular extract before the activation assay had no effect on the ability to enhance FSD1 activity (Fig. S4), which ruled out the effects of ions in the extract.

The total Fe in Arabidopsis leaf is about 1.83 μ mol g⁻¹ dry weight (Abdel-Ghany *et al.*, 2005b). However, the labile Fe pool of embryonic axes of sorghum (*Sorghum bicolor*) is c. 8 nmol g⁻¹ fresh weight (Jasid *et al.*, 2008), which is equivalent to 1–2 μ M, a concentration much lower than that of the Fe supplied on a millimolar scale in our *in vitro* experiments. Therefore, the efficiency of Fe incorporation by FSD1 itself may be negligible *in vivo*. Hence, we suggest the requirement for a factor(s) assisting FSD1 activation.

CPN20 as an assisting factor in the activation of FSD1

Arabidopsis *fsd1* cellular extracts from supernatant heated at 100°C for 30 min to remove most of the cellular proteins (Fig. 3a) still retained the ability to recover and enhance the activity of Holo-FSD1 and Apo-FSD1 (Fig. 3b). Hence, to identify the factor(s) assisting FSD1 activation, we used the heated supernatant

with reversed-phase HPLC, gel filtration chromatography and LC-MS/MS analyses.

Fractions eluted at 19–27 min of HPLC could restore Apo-FSD1 activity (Fig. 4a,b), but the ability was lost when the fractions were treated with proteinase K (Fig. 4c); this suggests that the assisting factor is heat stable and proteinaceous. These fractions were combined and subjected to gel filtration chromatography (Fig. 4d). Fractions eluting at 25–42 min of gel filtration retain the ability to assist in FSD1 activation (Fig. 4e). We then subjected the reversed-phase HPLC fractions at 19–26 min and the gel filtration fractions at 25–36 min to LC-MS/MS analysis (Notes S1, S2, respectively). The MS data revealed 14 proteins which are consistently present in these fractions (Table 1). We found four chloroplast proteins: two photosynthetic oxygen-evolving complex subunits, one chloroplast ribosomal protein and CPN20 (also called CHAPERONIN 21; *At5g20720*); we then focused on CPN20 for further analysis because of its heat stability and consistently high score on MS data.

CPN20 is a well-known chloroplast-localized co-chaperonin (a GroES homolog; Bertsch *et al.*, 1992; Hirohashi *et al.*, 1999; Koumoto *et al.*, 1999; Weiss *et al.*, 2009; Fig. S5) and assists chaperonin CPN60s (a GroEL homolog; Horwich *et al.*, 2007) in protein folding. An additional role for CPN20 unrelated to its co-chaperonin function has not been reported.

CPN20 interacts with FSD1

The use of GFP fusion proteins and immunogold labelling has shown that CPN20 and FSD1 are localized to chloroplasts (Kliebenstein *et al.*, 1998; Koumoto *et al.*, 1999, 2001). To investigate the interaction between CPN20 and FSD1, we performed yeast two-hybrid assay with FSD1 fused to the DNA BD and CPN20 fused to the AD of GAL4 (Fig. 5a). CPN20

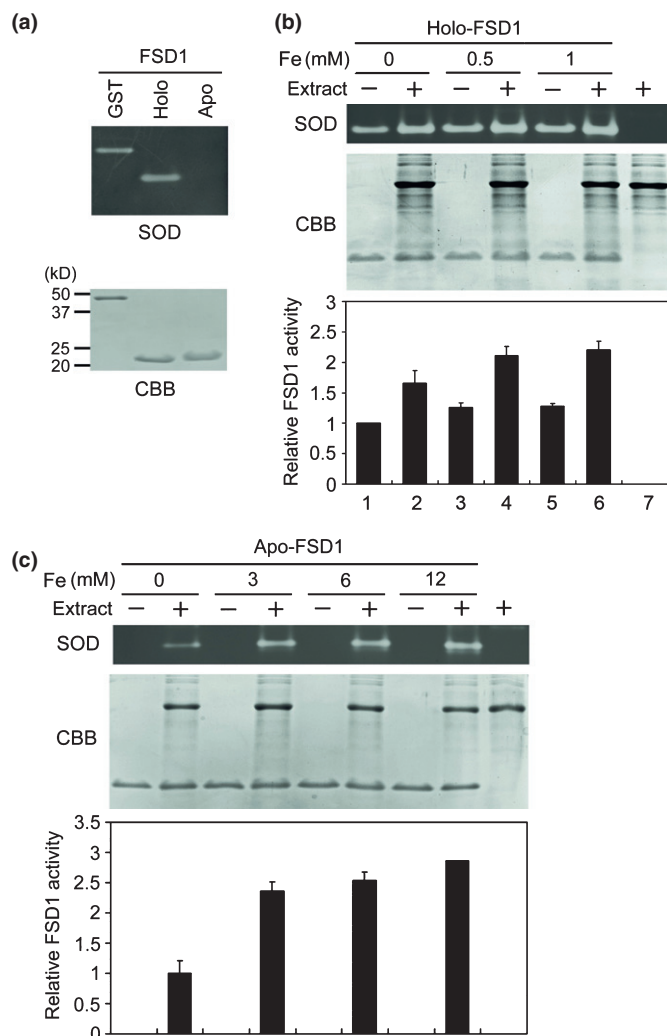


Fig. 2 FSD1 activation by incubation with Fe and *fsd1* cellular extract. (a) GST-FSD1, Holo-FSD1 and Apo-FSD1 activity assay (SOD) and protein profile by Coomassie Brilliant Blue staining (CBB). Activity (SOD) and protein profile (CBB) of Holo-FSD1 (b) and Apo-FSD1 (c) incubated with different amounts of Fe ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$) without or with *fsd1* cellular extract. Data are relative to that of untreated Holo-FSD1 or Apo-FSD1 treated with extract only (mean $\pm$ SD, $n = 5$).

showed specific interaction with FSD1, and none of the protein interacted with CSD1, a control for cytoplasmic CuZnSOD. In addition, we performed FRET assay to confirm the interaction of CPN20-YFP and FSD1-CFP fusion proteins in Arabidopsis protoplasts. We also constructed a control for chloroplast-directed CFP, chl-CFP (Fig. 5b). The FRET result revealed an interaction between CPN20-YFP and FSD1-CFP, but not chl-CFP (Fig. 5c,d), which indicates a specific interaction between CPN20 and FSD1 in chloroplasts.

CPN20 facilitates FSD1 activation *in vitro*

To determine the effect of CPN20 on FSD1 activity, we measured the activity of Holo-FSD1 incubated with GST, GST-CPN20 or Holo-CPN20 (with the GST tag removed; Fig. 6a). Holo-FSD1 activity was increased significantly by incubation with increasing

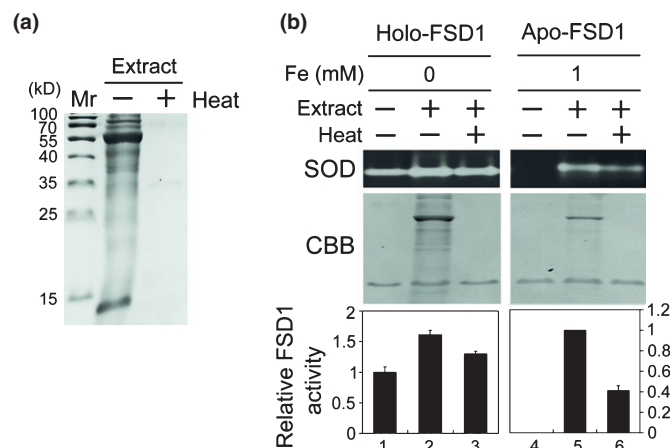


Fig. 3 FSD1 activation by incubation with Fe and *fsd1* heated cellular extract. (a) Coomassie Brilliant Blue (CBB) staining of supernatant of *fsd1* cellular extract without or with 30 min of 100°C treatment. (b) Activity of Holo-FSD1 and Apo-FSD1 without or with *fsd1* cellular extract and heat. Data are relative to those of untreated Holo-FSD1 or Apo-FSD1 treated with 1 mM Fe and nonheated cellular extract (mean $\pm$ SD, $n = 5$).

amounts of GST-CPN20 and Holo-CPN20, but not GST (Fig. 6b), without Fe. The enhanced Holo-FSD1 activity reached 1.45- and 1.78-fold on incubation with 8 μg GST-CPN20 and Holo-CPN20, respectively. Tris grinding buffer, various pH buffers and ions commonly found in cells did not enhance significantly FSD1 activity in this system (Fig. S6), which rules out the artifactual effects in these buffer components. The Apo-FSD1 activity was also enhanced by incubation with Holo-CPN20 (Fig. S7), suggesting that Holo-FSD and Apo-FSD share a similar trend in the activity assay *in vitro*.

We also tested the cellular extract prepared from CPN20-YFP-overexpressing protoplasts (Fig. 6c), and found that it induced significantly (1.4-fold) Holo-FSD1 activity (Fig. 6c, top). When the extract was heated to 100°C for 30 min, CPN20-YFP protein was no longer present in the supernatant (Fig. 6c, bottom), possibly because of the heat-labile GFP tag, and the FSD1 enhancement effect was lost. Of note, the endogenous CPN20 in the supernatant remained soluble after heating, with a slight decrease in level (Fig. 6c, bottom).

CPN20 facilitates FSD1 activation *in vivo*

In Arabidopsis *CPN20*-overexpressing lines generated under the control of the 35S promoter (Fig. 7), *CPN20* transcript and protein levels were both increased, and the FSD1 protein and activity levels were enhanced by 2.5- and 3.7-fold, respectively. However, the *FSD1* transcript levels did not increase accordingly. Of note, the increase in CPN20 expression did not affect CPN60s protein levels, as determined by the use of a CPN60 antibody that cross-reacted with all the chloroplast CPN60 subtypes (Fig. S8a).

CPN20 silencing decreases FeSOD activity

Screening for a *CPN20*-knockout or *CPN20*-knockdown mutant in Arabidopsis failed (data not shown). Interference with *CPN20* expression has been successful only with the use of VIGS in tobacco

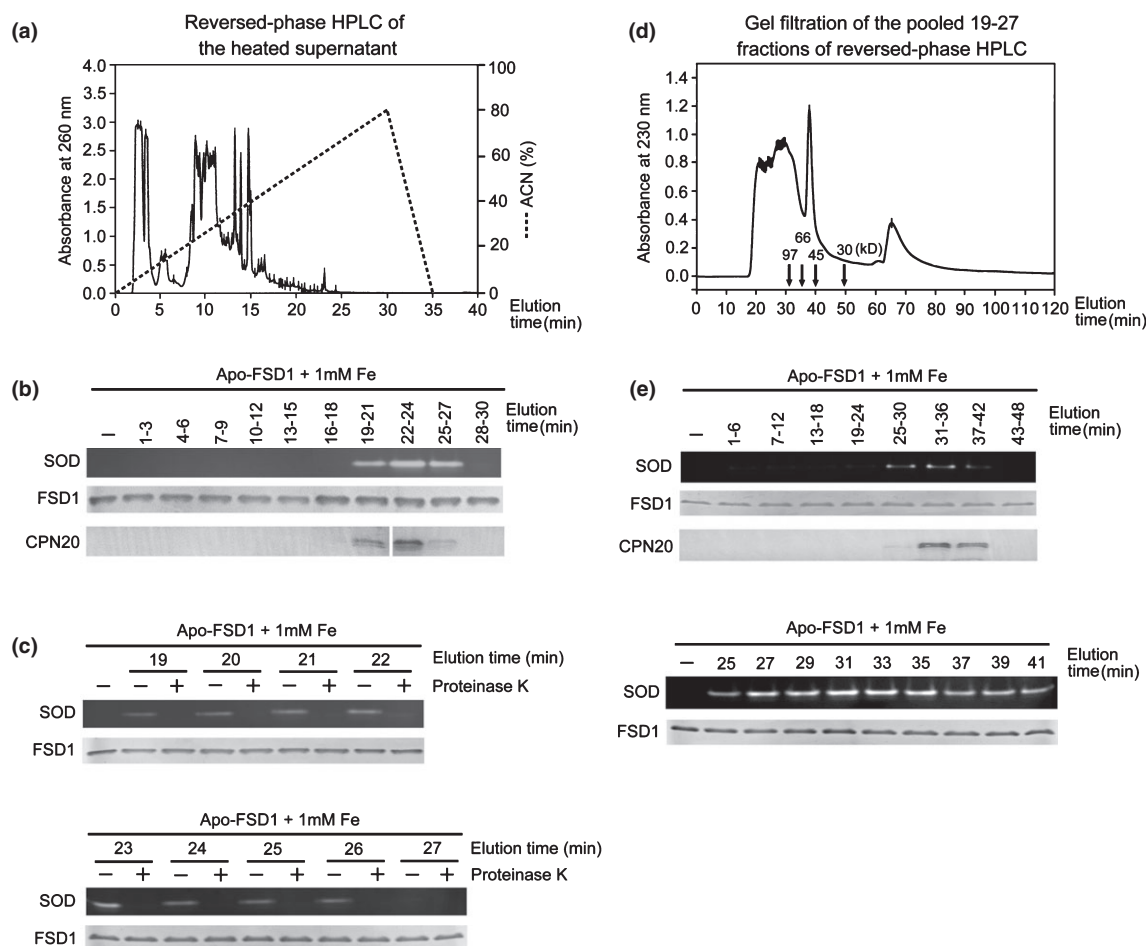


Fig. 4 Apo-FSD1 activation in the presence of fractions from reversed-phase high-performance liquid chromatography (HPLC) and gel filtration chromatography. (a) HPLC elution profile (A_{260}) of the supernatant of *Arabidopsis fsd1* heated cellular extracts. ACN, acetonitrile. (b) The three HPLC fractions were combined and tested for their effects on Apo-FSD1 activity (SOD). Coomassie Brilliant Blue (CBB) staining of Apo-FSD1 (FSD1) and immunoblotting of CHAPERONIN 20 (CPN20) with CPN20 antibody (CPN20). (c) Each of the fractions at 19–27 min in (b) was treated without or with proteinase K before the tests. (d) Gel filtration chromatography elution profile (A_{230}) of the HPLC fractions at 19–27 min combined. Arrows indicate the molecular weight markers (kDa) separated on the same column (top). (e) Effect of gel filtration fractions on Apo-FSD1 activity. Overall screening of fractions at 6-min intervals (top) and details of fractions at 25–42 min at 2-min intervals (bottom).

Table 1 Protein candidates identified by LC-MS/MS analysis

Accession no.	Description (TAIR protein)	Scores	Mass	Matches	pl
Chloroplast proteins					
IPI00537354	20-kDa chaperonin (AT5G20720)	475	26 785	19	8.86
IPI00532582	Isoform 2 of oxygen-evolving enhancer protein 3-1 (AT4G21280)	162	23 781	7	9.64
IPI00548616	Oxygen-evolving enhancer protein 3-2 (AT4G05180)	141	24 628	5	9.72
IPI00540051	50S ribosomal protein L12-3 (AT3G27850)	35	71 607	1	6.41
Other localized proteins					
IPI00520773	Peptidyl-prolyl <i>cis-trans</i> isomerase CYP19-1 (AT2G16600)	117	18 480	4	8.65
IPI00545561	Acyl CoA binding protein (AT1G31812)	168	10 466	5	5.19
IPI00536021	FAM10 family protein (AT4G22670)	53	46 591	1	4.83
IPI00532377	DREPP plasma membrane polypeptide family protein (AT4G20260)	551	24 569	22	4.99
IPI00524841	Nodulin-related protein (AT1G13930)	124	16 154	5	4.82
IPI00541725	RNA binding/translation elongation factor (AT4G29060)	255	103 718	6	4.95
IPI00521110	Isoform 1 of cysteine proteinase inhibitor 6 (AT3G12490)	80	26 278	2	5.86
IPI00525190	Cysteine proteinase inhibitor 1 (AT5G12140)	166	11 249	8	5.07
IPI00523226	Putative uncharacterized protein F4F15.260 (AT3G52150)	85	59 745	3	6.77
IPI00542179	Eukaryotic translation initiation factor 5A-2 (AT1G26630)	341	17 129	13	5.55

The accession numbers and descriptions of the proteins were obtained from the International Protein Index databases (<http://www.ebi.ac.uk/IPI>).

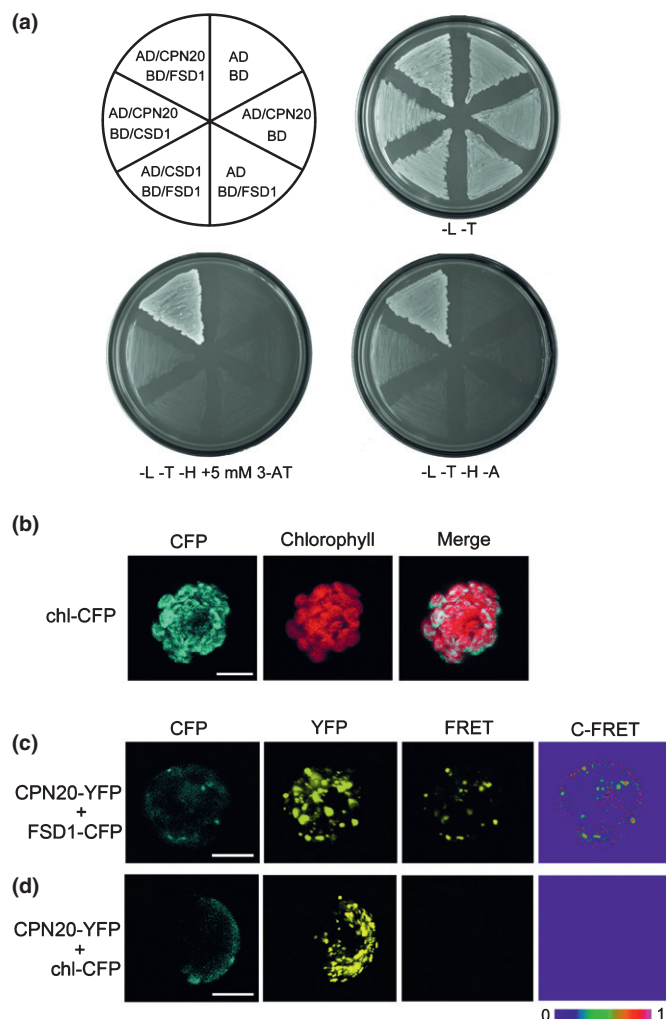


Fig. 5 CHAPERONIN 20 (CPN20) and FSD1 interaction. (a) Yeast two-hybrid analysis. (b) Localization of a chloroplast-directed CFP (chl-CFP) control in Arabidopsis protoplasts. Fluorescence resonance energy transfer (FRET) analysis of Arabidopsis wild-type (WT) protoplasts co-transfected with CPN20-YFP and FSD1-CFP (c) or chl-CFP (d). Fluorescence images were captured with the cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) and FRET channels. Autofluorescence from chlorophyll indicates the location of chloroplasts. The corrected FRET (C-FRET) is in a pseudocolour mode. Bars, 10 μ m.

(*Nicotiana benthamiana*) and tomato (Hanania *et al.*, 2007). The silencing of *CPN20* resulted in a pale-green phenotype and poorly developed mesophylls, with a reduced number of chloroplasts, which suggests a role for *CPN20* in chloroplast development. Moreover, specific silencing of *CPN20* in tomato fruit resulted in seedless tomatoes (Hanania *et al.*, 2007). *CPN20* may play a role in seed abortion, which might explain the failure to obtain a stable knockout mutant in Arabidopsis.

To confirm the function of CPN20 in FeSOD activation, tomato *CPN20* silencing by VIGS was conducted. Both *CPN20* transcript and protein levels were reduced in VIGS plants (Fig. 8a,b), and FeSOD activity was also decreased accordingly (Fig. 8c). Therefore, the decreased CPN20 level was accompanied by reduced FeSOD activity *in vivo*. The VIGS plants also showed similar results (Hanania *et al.*, 2007), with a pale-green phenotype (Fig. S9).

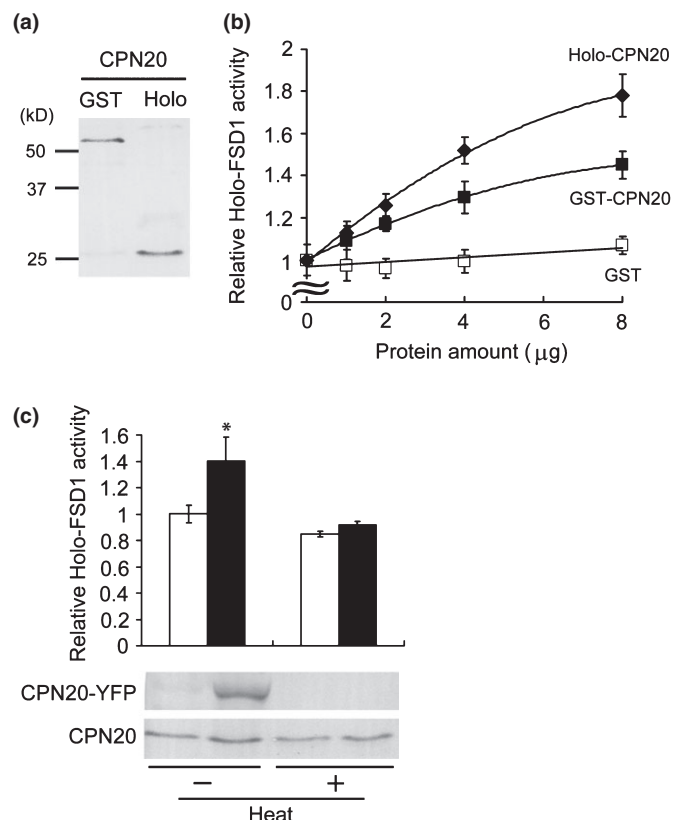


Fig. 6 FSD1 activation by CHAPERONIN 20 (CPN20) *in vitro*. (a) Immunoblotting of GST-CPN20 and Holo-CPN20 with CPN20 antibody. (b) Activity of Holo-FSD1 incubated with the indicated amounts of glutathione-S-transferase (GST), GST-CPN20 or Holo-CPN20. Data are relative to those of an untreated control (mean $\pm$ SD, $n = 5$). (c) Activity of Holo-FSD1 in supernatant of 100°C/30 min heat- or non-heat-treated cellular extract of Arabidopsis wild-type (WT) (white bars) and CPN20-YFP-overexpressing (black bars) protoplasts. Data are relative to those of the WT and non-heated supernatant (mean $\pm$ SD, $n = 3$). *, $P < 0.05$. Immunoblotting of CPN20-YFP and endogenous CPN20 (CPN20) with CPN20 antibody. YFP, yellow fluorescent protein.

CPN20 enhances FSD2 and FSD3 activities *in vitro*

CPN20 may have similar effects on the activation of FSD2 and FSD3; however, the *CPN20*-overexpressing lines were not suitable for the evaluation of this possibility because FSD2 and FSD3 activities were still undetectable in these plants (data not shown). We used the affinity-purified FSD2 and FSD3 (Fig. S2c,d) as shown in Fig. 6.

The activities of GST-removed Holo-FSD2 and Holo-FSD3 (Fig. 9a,b) on incubation with GST or GST-CPN20 were analysed, and were found to be increased significantly with GST-CPN20, but not GST (Fig. 9c,d). Thus, the activating role of CPN20 for FSD2 and FSD3 was similar to that for FSD1.

CPN20 functions independently of its co-chaperonin activity

CPN20 consists of two homologous CPN10-like domains joined head-to-tail by a short chain of amino acids (Hirohashi *et al.*, 1999; Weiss *et al.*, 2009; Fig. S5). Each domain contains a 'mobile loop'

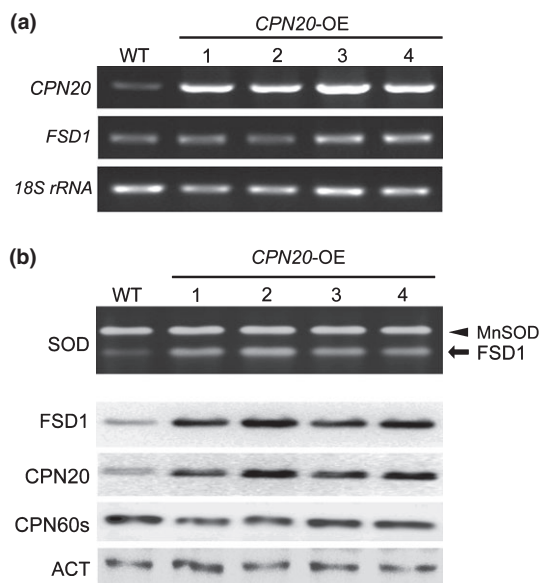


Fig. 7 FSD1 transcript and activity levels in *CHAPERONIN 20* (CPN20)-overexpressing plants. RNA and protein were prepared from T₃-transgenic plants. (a) Transcript level of CPN20 and FSD1 in Arabidopsis wild-type (WT) and four CPN20-overexpressing lines (CPN20-OE1–4). (b) Activity of FSD1 (top) and FSD1, CPN20 and CPN60s protein levels (bottom). 18S rRNA and actin (ACT) were used as internal controls.

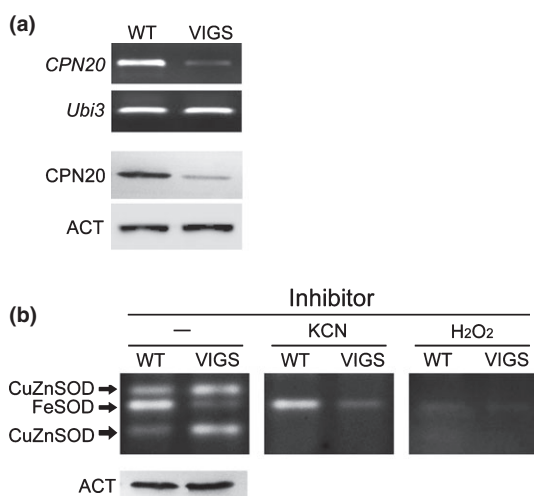


Fig. 8 Iron superoxide dismutase (FeSOD) activity in tomato *CHAPERONIN 20* (CPN20)-silenced plants. (a) CPN20 transcript (top) and protein (bottom) levels in tomato wild-type (WT) and CPN20 virus-induced gene-silenced (VIGS) plants. *Ubiquitin 3* (Ubi3) and actin (ACT) were used as internal controls. (b) FeSOD activity assay.

known to be important for co-chaperonin binding to chaperonins (Landry *et al.*, 1993, 1996; Baneyx *et al.*, 1995; Richardson *et al.*, 2001). The 'IVL' motif within the mobile loop is primarily responsible for the binding affinity, whereas the preceding amino acids of GG allow for the formation of the hairpin structure that is adopted on binding to chaperonins (Landry *et al.*, 1993, 1996; Bertsch & Soll, 1995; Richardson & Georgopoulos, 1999; Richardson *et al.*, 2001). The role of the mobile loops of CPN20 binding to chaperonins was tested previously with proteins

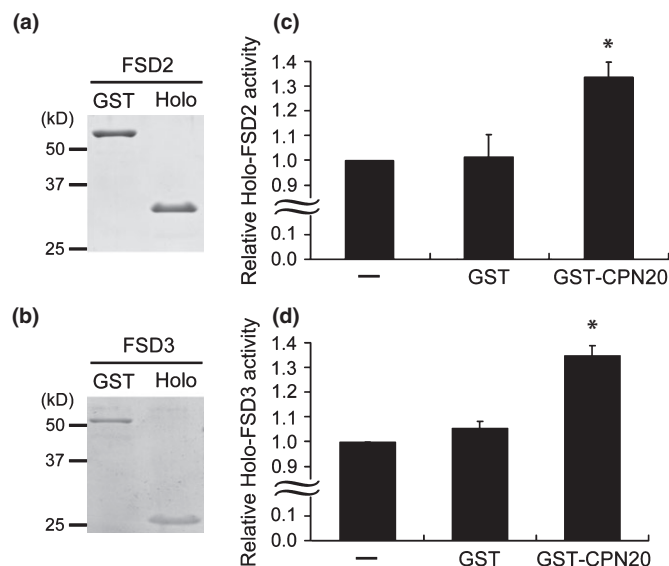


Fig. 9 FSD2 and FSD3 activation by *CHAPERONIN 20* (CPN20) *in vitro*. Coomassie Brilliant Blue (CBB) staining of the affinity-purified GST- and Holo-proteins of FSD2 (a) and FSD3 (b). Activity of Holo-FSD2 (c) and Holo-FSD3 (d) incubated with glutathione-S-transferase (GST) or GST-CPN20. Data are relative to those of an untreated control (mean $\pm$ SD, $n = 3$). *, $P < 0.05$.

mutated in one of the highly conserved Leu residues in the IVL motif (i.e. L35A, L133A and LDM for Leu double mutation) or at the conserved Gly position (i.e. G32A, G130A and GDM for Gly double mutation). Among these mutations, L35A, LDM and GDM completely lost their co-chaperonin activity (Bonshtien *et al.*, 2007).

We used the same affinity-purified CPN20 (Fig. S8b; free of bacterial GroEL contamination) and its mutants provided by one of the authors (A. Azem; Bonshtien *et al.*, 2007) in FSD1 activation analysis *in vitro* (Fig. 10a). Although all of the mutants exhibited diminished assisting ability (c. 18–50%) when compared with CPN20, the mutants completely lacking co-chaperonin activity (L35A, LDM and GDM) could still enhance significantly FSD1 activity.

To determine whether the mobile loop was involved in FSD1 activity *in vivo*, we co-expressed FSD1-YFP with CPN20 or its mutants G32A (slightly decreased co-chaperonin activity) and L35A (no co-chaperonin activity) in Arabidopsis protoplasts; the FSD1-YFP activity and protein levels with CPN20 mutants were increased to a similar extent as CPN20 (Fig. 10b–e). Such enhancement was not observed on co-expression of CPN20 with CSD1, a control for CuZnSOD (Fig. S10). Thus, CPN20 had a specific effect on FSD1 activity, independent of its co-chaperonin activity.

Discussion

Chloroplast localization of FSD1

Proteomic studies of different plant cell organelles have been reported (see reviews by Peck, 2005; Baginsky & Gruissem, 2006),

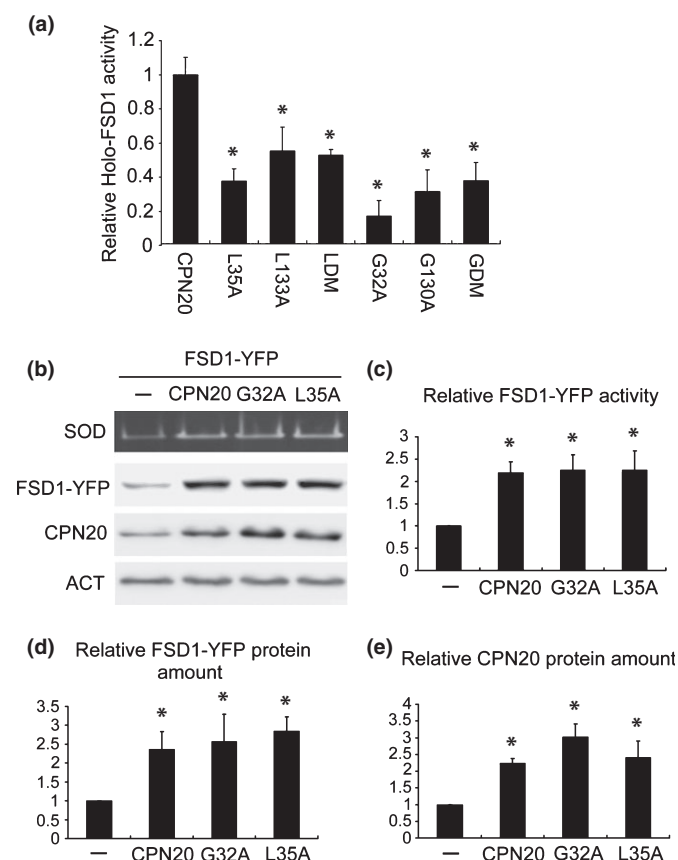


Fig. 10 Effect of CHAPERONIN 20 (CPN20) and mutants with defective co-chaperone activity on FSD1 activity. (a) Activity of Holo-FSD1 incubated with the histidine (His)-tagged CPN20 (CPN20) and mutants as indicated *in vitro*. Data are relative to those with CPN20 treatment (mean $\pm$ SD, $n = 5$). *, $P < 0.05$. (b) FSD1-YFP co-expression with CPN20 or mutants in Arabidopsis wild-type (WT) protoplasts. Activity of FSD1-YFP (top) and FSD1-YFP and CPN20 protein levels (bottom) *in vivo*. CPN20 and mutants lacking the His tag were used. (c–e) Data are relative to FSD1-YFP without co-expression (mean $\pm$ SD, $n = 3$). *, $P < 0.05$. YFP, yellow fluorescent protein.

and have shown Arabidopsis FSD1 in the soluble fraction of chloroplasts (Kleffmann *et al.*, 2004; Peltier *et al.*, 2006), peripheral thylakoid (Peltier *et al.*, 2002) and envelope of purified chloroplasts (Ferro *et al.*, 2003). Different localization results have been shown in different plant materials; for example, the Arabidopsis PEND (plastid envelope DNA binding), a well-characterized DNA binding protein (Sato *et al.*, 1993), was located in the inner envelope of chloroplasts (Terasawa & Sato, 2005), but PEND-CFP was observed in the cell nucleus and chloroplast nucleoids in tobacco protoplasts (Myouga *et al.*, 2008). Myouga *et al.* (2008) expressed FSD1-GFP in tobacco and found that it was localized in the cytoplasm, whereas our results showed that it was localized to chloroplasts; this may be because the tobacco chloroplast protein transport machinery was unable to discriminate this noncanonical N-terminal chloroplast transit of FSD1. The 11 N-terminal residue-deleted FSD1, Δ ATP-FSD1, was present in the cytoplasm and functional (Fig. 1b,c); this supports the chloroplast localization of FSD1.

CPN20 has a dual function

We investigated the factor(s) mediating FeSOD activation, and found CPN20, a plastid-specific co-chaperonin, present in all steps of isolation (Fig. 4). Chaperonin systems play a vital role in protein folding in both eukaryotic and prokaryotic cells (Horwich *et al.*, 2007; Hartl & Hayer-Hartl, 2009). The well-studied type I chaperonin system involves the bacterial chaperonin (GroEL) and co-chaperonin (GroES) proteins. Co-chaperonins help chaperonins in their protein-folding function in an ATP-dependent manner and facilitate the folding of the bound polypeptide substrate to its native form (Horwich *et al.*, 2007; Hartl & Hayer-Hartl, 2009).

Plant chloroplasts harbor the 10-kDa GroES homologs, the CPN10s and a doubled CPN10 known as CPN20 (Bertsch *et al.*, 1992; Hirohashi *et al.*, 1999; Weiss *et al.*, 2009). Similar to GroES, CPN20 was found to be active as a chaperonin helper protein *in vitro* (Bertsch *et al.*, 1992). In this study, CPN20 mediated significantly FSD1 activity *in vitro* in the absence of the chaperonin homolog CPN60 (Fig. 6); therefore, this function is distinct from its co-chaperonin activity. We also confirmed that CPN20 mutants defective in co-chaperone activity could still mediate FSD1 activity (Fig. 10), which supports the dual functions of CPN20.

CPN20 post-transcriptional regulation of FeSOD activity

CPN20 interacts with FSD1 *in vivo* (Figs 5, 7, 8) and facilitates the activation of three FSDs *in vitro* (Figs 2–4, 6, 9). The FSD1 protein and activity levels were enhanced in CPN20-overexpressing lines, with CPN60 chaperonin levels remaining unaltered (Fig. 7); this implies that CPN20 functions independently of the chaperonin system. Indeed, CPN20 has been suggested previously to play a role other than as a co-chaperonin (Weiss *et al.*, 2009). CPN20 itself may assist in FSD1 activation in chloroplasts.

Meanwhile, CPN20 overexpression increased FSD1 protein and activity levels, but not the mRNA level (Fig. 7). From this result, we propose a post-transcriptional regulation of FSD1 by CPN20. Moreover, CPN20 enhanced Holo-FSD activities *in vitro* without Fe (Figs 6c, 9, 10a); thus, the enhanced activity levels might result from the role of CPN20 as an Fe chaperone, in protein folding and/or in stabilization. As CPN20 shows Fe binding ability on the basis of our preliminary results (data not shown), we propose that CPN20 is an Fe chaperone for FeSOD; however, the precise mechanism awaits further investigation.

Factors in addition to CPN20 for FeSOD activation in the cytosol

Examples of SOD activation in different compartments have been demonstrated for CuZnSOD and MnSOD. The activation of Arabidopsis CSD1, the cytoplasmic CuZnSOD, occurs via Cu chaperone for SOD (CCS)-dependent and CCS-independent pathways; of CSD2, the chloroplast-localized CuZnSOD, via a CCS-dependent pathway; and of CSD3, peroxisome-localized CuZnSOD, mainly by a CCS-independent pathway (Abdel-Ghany *et al.*, 2005a; Chu *et al.*, 2005; Huang *et al.*,

2012a,b). Yeast mitochondrial MnSOD and the cytoplasmic-localized MnSOD (Δ TP-MnSOD) are active, but only the mitochondrial MnSOD is inactivated with deletion of a mitochondrial carrier protein (MTM1). These findings suggest different activation processes for MnSOD in mitochondria and cytoplasm (Luk *et al.*, 2003).

Our results for FeSOD activation are similar to those for MnSOD, with both FSD1 and Δ TP-FSD1 being active (Fig. 1c). A cytosolic FeSOD has been identified in nodules of cowpea (*Vigna unguiculata*; Moran *et al.*, 2003) and the CPN20 homologs are present only in chloroplasts (Koumoto *et al.*, 1996, 1999, 2001; Schlicher & Soll, 1996); this suggests that other factors might be able to activate Δ TP-FSD1 in cytoplasm. Indeed, the lower assisting ability of the heated cellular extract could be caused by the loss of other heat-labile factors that may contribute to FSD1 activation (Figs 3b, 6c).

Despite the possible existence of different activating factors in other localities, physiological effects can only be achieved with SODs activated in their native compartments. Hence, although Δ TP-FSD1 was found to be active when localized in the cytoplasm, its actual physiological activity would be in the chloroplast, where CPN20 is located and is involved in its activation process. Further studies are required to elucidate the complete activation mechanism of FeSOD by CPN20 in plants.

SOD functions other than ROS disproportion

Although only FSD1 showed detectable activity (Fig. 1a), knock-out of this gene produced no change in phenotype under normal growth conditions (Myouga *et al.*, 2008). In contrast, although FSD2 and FSD3 showed undetectable activities, knockout of *FSD2* and *FSD3* caused a severe developmental defect in chloroplasts. The weak activities resulting in such a severe phenotype may be explained, first, by the location, which allows for immediate and efficient dismutation of the superoxide anions in the chloroplast nucleoid *in situ* and explains the failure of the stromal FSD1 to compensate for the requirement of thylakoid membrane-attached FSD2 and FSD3 activities. Notably, CSD2 is another thylakoid membrane-attached CuZnSOD with detectable activity much stronger than that of FSD2 and FSD3 (Fig. 1a; Kliebenstein *et al.*, 1998; Chu *et al.*, 2005). In terms of SOD activity, this CSD2 could compensate for the effects of FSD2 and FSD3 because of a similar localization and relatively strong activity. However, the knockout phenotypes suggest that CSD2 cannot replace the roles of FSD2 and FSD3.

Second, FeSOD may play a role in Fe buffering, similar to the physiological role in Cu buffering suggested for CuZnSOD (Culotta *et al.*, 1995; Cohu *et al.*, 2009). This situation may explain the failure of CSD2 to compensate for the effect of FSD2 and FSD3; however, FSD1 should be able to buffer Fe in the chloroplast. Thus, the reason for the severe phenotypes in *fsd2* and *fsd3* still cannot be explained by the location and/or function in Fe buffering. A function other than the disproportionate of ROS for FeSOD might be implicated. CPN20, which connects the ROS scavenging function and chaperonin system, might offer an important clue.

In conclusion, we have described the existence of an assisting factor, CPN20, in the activation of FeSOD, in addition to its co-chaperonin activity in plants. The effect of CPN20 on FSD2 and FSD3 activities was similar to its effect on FSD1 activity, which supports a common role of CPN20 in the activation of FeSOD for oxidative stress protection and chloroplast development.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sequence alignment of the N-termini of iron superoxide dismutases (FeSODs) from different species.

Fig. S2 Affinity purification of the glutathione-S-transferase (GST)-tagged FSD1, FSD2, FSD3 and CHAPERONIN 20 (CPN20).

Fig. S3 Active and inactive FSD1 in affinity-purified Holo-FSD1 and Apo-FSD1.

Fig. S4 Holo- and Apo-FSD1 activation by incubation with *fsd1*-desalted cellular extract.

Fig. S5 Sequence alignment of the CHAPERONIN 20 (CPN20) N- and C-domains and CPN10 among different species.

Fig. S6 Effect of Tris buffer, various pH buffers and metal ions on Holo-FSD1 activity.

Fig. S7 CHAPERONIN 20 (CPN20) facilitated Apo-FSD1 activation *in vitro*.

Fig. S8 The CHAPERONIN 60 (CPN60) antibody cross-reacted with all the chloroplast CPN60 subtypes and the affinity purified CPN20 without bacterial GroEL contamination.

Fig. S9 Phenotype of tomato wild-type (WT) and *CHAPERONIN 20* (CPN20) virus-induced gene silenced plants.

Fig. S10 CSD1 activity and protein levels were not affected by the co-expression of CHAPERONIN 20 (CPN20) and mutants with defective co-chaperone activity.

Table S1 Primers used in this study

Notes S1 LC-MS/MS analysis of high-performance liquid chromatography (HPLC) fractions.

Notes S2 LC-MS/MS analysis of gel filtration fractions.

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