The Arabidopsis Putative Selenium-Binding Protein Family: Expression Study and Characterization of SBP1 as a Potential New Player in Cadmium Detoxification Processes[1[W]]

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In Arabidopsis (*Arabidopsis thaliana*), the putative selenium-binding protein (SBP) gene family is composed of three members (*SBP1–SBP3*). Reverse transcription-polymerase chain reaction analyses showed that *SBP1* expression was ubiquitous. *SBP2* was expressed at a lower level in flowers and roots, whereas *SBP3* transcripts were only detected in young seedling tissues. In cadmium (Cd)-treated seedlings, *SBP1* level of expression was rapidly increased in roots. In shoots, *SBP1* transcripts accumulated later and for higher Cd doses. *SBP2* and *SBP3* expression showed delayed or no responsiveness to Cd. In addition, luciferase (LUC) activity recorded on Arabidopsis lines expressing the *LUC* gene under the control of the *SBP1* promoter further showed dynamic regulation of *SBP1* expression during development and in response to Cd stress. Western-blot analysis using polyclonal antibodies raised against SBP1 showed that SBP1 protein accumulated in Cd-exposed tissues in correlation with *SBP1* transcript amount. The *sbp1* null mutant displayed no visible phenotype under normal and stress conditions that was explained by the up-regulation of *SBP2* expression. SBP1 overexpression enhanced Cd accumulation in roots and reduced sensitivity to Cd in wild type and, more significantly, in Cd-hypersensitive *cad* mutants that lack phytochelatins. Similarly, in *Saccharomyces cerevisiae*, *SBP1* expression led to increased Cd tolerance of the Cd-hypersensitive *ycf1* mutant. In vitro experiments showed that SBP1 has the ability to bind Cd. These data highlight the importance of maintaining the adequate SBP protein level under healthy and stress conditions and suggest that, during Cd stress, SBP1 accumulation efficiently helps to detoxify Cd potentially through direct binding.

In mammals, selenium (Se) is an essential nutrient that is incorporated in the Se-amino acid Se-Cys required for the translation of numerous proteins having a critical role in cell defense and hormone regulation (Behne and Kyriakopoulos, 2001; Papp et al., 2007). Many reports also describe a relationship between insufficient Se intake and increased risk of cancer (Behne and Kyriakopoulos, 2001; Tapiero et al., 2003; Papp et al., 2007). In addition to its incorporation into selenoproteins, Se can be bound by other proteins. Mammalian selenium-binding protein 1 (SBP1) was first characterized in mouse liver in experiments designed to identify selenoproteins using ^{75}Se (Bansal et al., 1990). A protein with no Se-amino acid in its sequence was radiolabeled with ^{75}Se and was subsequently identified as a SBP. Today, homologs to SBP have been found in many organisms, such as nematodes, bacteria, scallops (Song et al., 2006; refs. therein), and diverse plant species (Flemetakis et al., 2002; Sawada et al., 2004; Agalou et al., 2005). No *SBP* homolog is present in yeast (*Saccharomyces cerevisiae*).

Although the function of SBP in mammals is still unclear, many data suggest its involvement in detoxification mechanisms. Many reports show that down-regulation of *SBP1* expression correlated with rapid tumor development in many organs (Kim et al., 2006; refs. therein) and, recently, *SBP1* was characterized as a biomarker for schizophrenia (Glatt et al., 2005). Its homolog, SBP2 (97% identity), was initially identified as the main hepatic target for the acetaminophen compound, a widely used analgesic, and was thus suggested to play a protective role as a scavenger of toxic electrophiles or oxidant species (Lanfear et al., 1993; Mattow et al., 2006). Other functions, such as intra-Golgi protein transport, have been assigned to mammalian SBP (Porat et al., 2000). Despite current multiple studies of mammalian SBP proteins, their physiological function is, however, still largely unclear.

Plant and mammalian SBPs share a high degree of similarity (68.5%–70.2% between Arabidopsis [*Arabidopsis*

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thaliana] SBP and their mammalian [mouse and human] counterparts), which suggests a shared biological role of these proteins among the different species (Agalou et al., 2005). To date, Se has not been demonstrated to be essential in land plants, but a Se-containing glutathione (GSH) peroxidase has been isolated from *Chlamydomonas reinhardtii* (Fu et al., 2002). Very few reports are available on plant SBPs. In *Lotus japonicus*, SBP1 was reported to be involved in nodule formation and function during the symbiosis of plants and rhizobia (Flemetakis et al., 2002). In rice (*Oryza sativa*), overexpression of *SBP1* led to enhanced tolerance to different pathogens (Sawada et al., 2004). More recently, a positive correlation between *SBP1* expression and selenite tolerance in Arabidopsis was reported, indicating a direct link between the protein and its probable ligand (Agalou et al., 2005).

In a previous article (Sarry et al., 2006), we showed from differential proteomics studies that SBP1 accumulated very early upon cadmium (Cd) application in Arabidopsis cells. Our global proteomic analysis aimed to explore the perturbations associated with Cd stress in plants to identify new molecular elements related to stress signaling and/or tolerance. Indeed, Cd is a widespread nonessential heavy metal, classified as a human carcinogen, and the uptake and accumulation of Cd in plants represent the main entry pathway into human and mammal food. Several molecular components involved in plant Cd uptake, accumulation, and tolerance have been identified (Sanita di Toppi and Gabrielli, 1999; Clemens, 2006a). Among them, the Fe^{2+}, Ca^{2+}, and Zn^{2+} transporters with low specificity have been suggested to enable Cd to enter into cells (Thomine et al., 2000; Connolly et al., 2002; Perfus-Barbeoch et al., 2002). Once inside the cell, Cd detoxification is mediated through Cd chelators, such as phytochelatins (PCs), metallothioneins, and organic acids (Cobbett and Goldsbrough, 2002; Roosens et al., 2005; Clemens, 2006a). In nonhyperaccumulator plants, such as Arabidopsis, PCs are one of the best-characterized Cd detoxification processes (Howden et al., 1995a, 1995b; Ebbs et al., 2002; Clemens, 2006a). PCs are thiol-rich peptides synthesized from GSH (Steffens et al., 1986; Grill, 1987; Clemens, 2006b). Cd is chelated by PCs and forms PC-Cd complexes, which are then sequestered in the vacuole (Vogeli-Lange and Wagner, 1990; Salt and Rauser, 1995).The accumulation of SBP1 in Arabidopsis cells in response to Cd stress provides a great opportunity to get new insight into the function of this protein and to discover new potential detoxification elements in response to this metal in plants. In the Arabidopsis genome, three *SBP* genes are present. *SBP1* and *SBP2* are located on chromosome IV in a head-to-tail arrangement, whereas *SBP3* is located on chromosome III (Agalou et al., 2005). The coding sequence of *SBP1* and *SBP2* share 85% identity, whereas they only show 69% identity with *SBP3*, the most divergent isoform. The aim of this article was to study the pattern of expression of the three *SBP* genes in planta under normal growth conditions and in response to Cd stress and to investigate whether this protein family could be involved in Cd detoxification mechanisms. This article provides interesting data concerning the expression and regulation of SBP proteins in plants and reveals SBP1 as a potential new player in Cd detoxification processes.

RESULTS

Tissue Expression Analyses of Members of the *SBP* Family in Arabidopsis

Using specific primers, semiquantitative reverse transcription (RT)-PCR was performed to analyze the expression of genes encoding the three SBP isoforms in Arabidopsis. Figure 1 shows the expression of *SBP1*, *SBP2*, and *SBP3* in roots and shoots of 7-d-old seedlings grown in vitro and in the leaves, stems, and flowers of 4-week-old plants grown on soil. *SBP1* transcripts were detected in all tissues analyzed and at the highest level in the shoots and roots of young seedlings and in the leaves of adult plants. A similar pattern of expression was observed for *SBP2* with a lower level of expression in roots and flowers (Fig. 1). Note that *SBP2* transcripts were detected in the roots and flowers using a higher number of PCR cycles (data not shown). In contrast to *SBP1* and *SBP2*, *SBP3* showed a more restricted pattern of expression. Its RNA was detected in the roots of young seedlings and at a much lower level compared to the two other isoforms because a higher number of PCR cycles (33 versus 26) was required to detect *SBP3* transcripts (Fig. 1). *SBP3* transcripts were detected in shoots when 35 cycles were performed (data not shown).

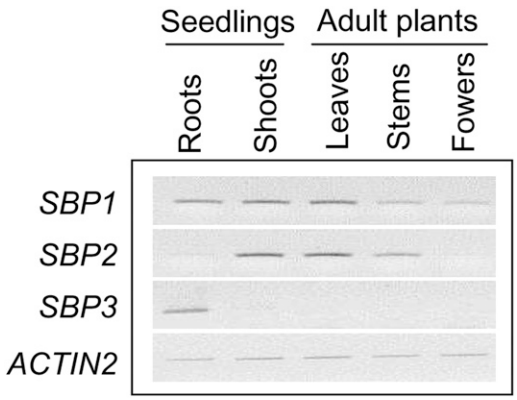

Figure 1. *SBP* gene expression in different tissues of Arabidopsis. Specific primers were designed for each isoform of SBP (*SBP1*, *SBP2*, and *SPB3*; Supplemental Table S1). PCR was performed with cDNA amplified from total RNA extracted from shoots and roots of 7-d-old seedlings grown on agar plates and leaves, stems, and flowers of 4-week-old adult plants grown on soil. Results show a representative experiment obtained from two independent sets of data. The number of PCR cycles performed was 26 for *SBP1* and *SBP2*, 33 for *SBP3*, and 25 for *ACTIN2* used as a control.

Expression Level of *SBP* Genes in Cd-Challenged Arabidopsis Seedlings

Because Cd enters the plant via the root system where it accumulates and is then translocated to the shoot to a lesser extent, the expression levels of *SBP* transcripts were examined separately in roots and shoots and for different times of Cd exposure (6 and 24 h for roots, 24 and 48 h for shoots). In addition, two Cd concentrations were used: 50 μM, which triggers significant Cd accumulation in roots, but not in shoots, and 500 μM Cd, which triggers significant accumulation in both tissues. In these conditions, at 24 h, Cd accumulation in roots was 250 and 5,900 ng/mg dry weight for 50 and 500 μM Cd treatment, respectively, whereas it only reached 71 and 960 ng/mg dry weight in shoots (data not shown). Expression levels of *SBP1*, *SBP2*, and *SBP3* were followed using semiquantitative RT-PCR analysis (Fig. 2). Accumulation of *SBP1* transcripts was observed in roots 6 h after Cd treatment and persisted up to 24 h at both concentrations (Fig. 2). In shoots, an increase in *SBP1* transcript levels was detected for the highest Cd concentration at 24 h and was maintained for 48 h (Fig. 2). No induction was observed at 6 h (data not shown). *SBP2* and *SBP3* expression in the roots was less responsive to Cd because there was no change in transcript level 6 h after treatment. However, an increase was observed at 24 h for *SBP2* transcripts at both concentrations and only at 50 μM Cd in the case of *SBP3* (Fig. 2). In shoots, no modification of *SBP2* and *SBP3* transcript levels was detected (Fig. 2). Therefore, *SBP1* is the most responsive SBP isoform upon Cd exposure.

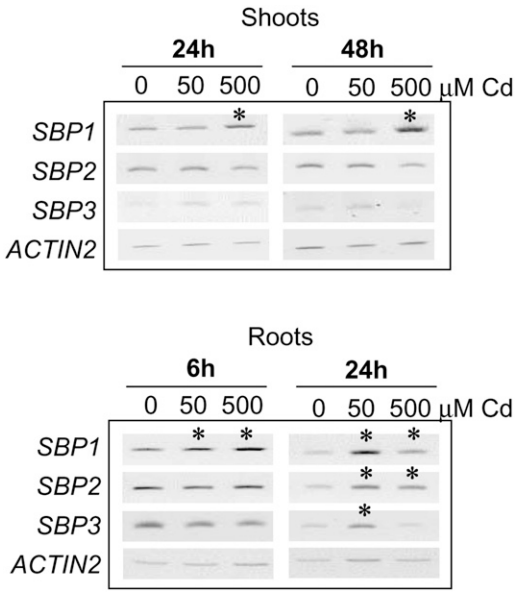

Figure 2. Expression analyses of the different *SBP* genes in Arabidopsis seedlings in response to Cd stress. *SBP* gene expression was analyzed by RT-PCR using total RNA extracted from roots and shoots of 7-d-old seedlings submitted to 0, 50, and 500 μM of $CdNO_3$ for 6, 24, and 48 h. Specific primers were used for each isoform of SBP (*SBP1*, *SBP2*, and *SPB3*; Supplemental Table S1). The number of PCR cycles performed for *SBP1* were 27 for roots (r) and shoots (s); for *SBP2*, 30 (r) and 28 (s); for *SBP3*, 32 (r) and 35 (s); and for *ACTIN2*, 24 (r) and 27 (s). Results show a representative experiment obtained from three independent sets of data. Asterisks show the increased accumulation of *SBP* transcripts in Cd-treated compared to control seedling tissues.

Luciferase Activity of Arabidopsis *SBP1*::*LUC* Lines in Healthy and Cd-Challenged Seedlings

Arabidopsis lines expressing the luciferase (*LUC*) reporter gene under the control of the *SBP1* promoter (*SBP1*::*LUC* lines) were constructed to test whether SBP1 transcription was increased in response to Cd stress and to further perform detailed metal dose responses. LUC activity was first analyzed on *SBP1*::*LUC* mature plants grown on soil, on seedlings grown on plates, and during the early stages of development (Fig. 3A). In mature plants, LUC activity was detected in stems, leaves, and flowers (Fig. 3A, I–IV) in accordance with transcript accumulation (Fig. 2) and in the peduncle and seeds (Fig. 3A, IV and V). Interestingly, LUC activity was high in young growing cauline (Fig. 3A, I) and rosette (Fig. 3A, VI and VII) leaves. In roots, LUC activity was very high at tips and this was clearly observed 12 d after germination when many secondary roots have developed (Fig. 3A, VI). During germination, LUC activity was detected as early as root tip emergence from the seeds (Fig. 3A, VIII). Figure 3B shows LUC bioluminescence recorded on *SBP1*::*LUC* whole seedlings challenged by Cd from 0 to 250 μM Cd for 72 h. In roots, bioluminescence was already increased at 5 μM treatment compared to control seedlings. Bioluminescence was first increased in the root tips, whereas at the highest concentrations it was observed in the whole roots (Fig. 3B). LUC activity was increased from 42 $\pm$ 6 photon/s in control roots, to 75 $\pm$ 2 and 430 $\pm$ 50 photon/s in roots treated with 5 and 50 μM Cd, respectively. In shoots, LUC bioluminescence was significantly increased from 2,106 $\pm$ 281 photon/s in control plants to 3,928 $\pm$ 581 and 6,030 $\pm$ 1,080 photon/s in 100 and 250 μM Cd-challenged seedlings, respectively. These data highlight the dynamic regulation of *SBP1* gene expression in healthy plants and during development and further show that *SBP1* transcription is induced in response to Cd stress in a dose-dependent manner.

SBP1 Protein Accumulation in Cd-Challenged Seedlings

We were further interested to know whether SBP1 protein accumulates in response to Cd stress in planta. The recombinant SBP1 was overexpressed in *Escherichia coli* as a fusion protein, purified, and polyclonal antibodies were raised against the recombinant protein. SBP1 antibodies were used to perform western-blot analyses with total soluble proteins extracted from 7-d-old Arabidopsis seedlings and with the purified SBP1 protein (Fig. 4A). Surprisingly two bands were detected in Arabidopsis protein using the anti-SBP1 serum

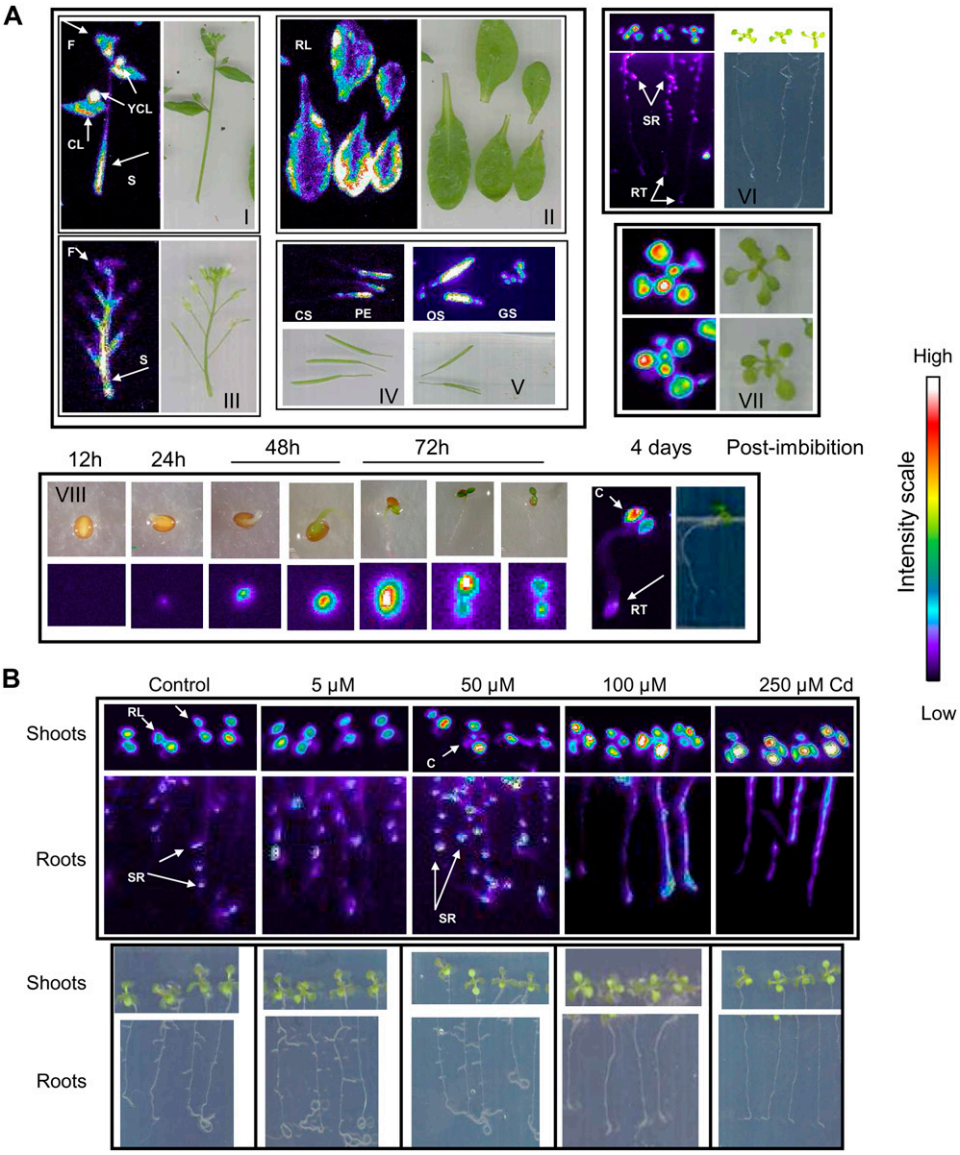

Figure 3. Analysis of *SBP1* promoter activity under healthy and Cd stress conditions through detection of LUC bioluminescence recorded on Arabidopsis *SBP1*::*LUC* transgenic lines. Bioluminescence was recorded using a CCD camera (see "Materials and Methods") and is presented as pseudocolored bioluminescence together with the corresponding white images. Bioluminescence intensity scale ranges from 0 to 300 arbitrary units in I to V, from 0 to 600 in roots and during germination, and from 0 to 6,000 in shoots (A, VI and VII, and B). A, LUC bioluminescence was recorded on 4-week-old mature *SBP1*::*LUC* transgenic lines (I–V) for 10 min on seedlings grown in vitro for 7 and 12 d (respectively, VI and VII) for 5 min, and during the early stages of development (VIII) for 10 min. B, LUC bioluminescence was recorded for 5 min on 10-d-old *SBP1*::*LUC* seedlings exposed to different concentrations of $CdNO_3$ for 72 h. S, Stem; F, flowers; CL, cauline leaf; YCL, young cauline leaf; RL, rosette leaf; PE, peduncle; CS, closed silique; OS, open silique; GS, green seeds; RT, root tips; SR, secondary root; C, cotyledon.

(Fig. 4A). One protein band migrated like the purified recombinant SBP1 protein (predicted molecular mass 59.5 kD) around 58 kD. The other had a lower molecular mass, approximately 52 kD. Incubation of the membrane with the preimmune serum revealed a nonspecific signal at approximately 35 kD (Fig. 4A). A T-DNA insertion line knockout for *SBP1* was used to identify the SBP1 protein (Fig. 4B). The T-DNA was inserted in the first exon and the corresponding transcript was not detectable compared to the wild-type plants in RT-PCR analysis (Fig. 4B). Whereas the 58-kD protein was still present in wild-type and *sbp1* protein extracts, the 52-kD protein was not detected in the *sbp1* null mutant in western-blot analysis using anti-SBP1 antibodies (Fig. 4B). The 52-kD protein was detected again in protein extracts of *sbp1* mutants that overexpressed the *SBP1* cDNA (Fig. 4C). These results show that the SBP1 protein in which expected molecular mass is 54 kD migrates around 52 kD in SDS-polyacrylamide gel. The SBP2 protein is recognized by the anti-SBP1 serum (Supplemental Fig. S1). In T-DNA insertion lines with reduced *SBP2* expression levels, these two bands were still detected (Supplemental Fig. S2B). The nature of the protein recognized by the antibodies at 58 kD thus remained unclear. This could be a nonspecific signal and was not further considered. In both roots and shoots of wild-type seedlings exposed to Cd, SBP1 protein abundance increased in a dose-dependent manner (Fig. 4D) and this was well correlated with *SBP1* expression levels observed in response to Cd (Figs. 2 and 3).

sbp Mutant Phenotype in Normal Conditions and upon Cd Stress

As described for *SBP1* (Fig. 4B), T-DNA insertion lines were also isolated for *SBP2* and *SBP3* (Supplemental Fig. S2A). The T-DNA, located either in the 5′

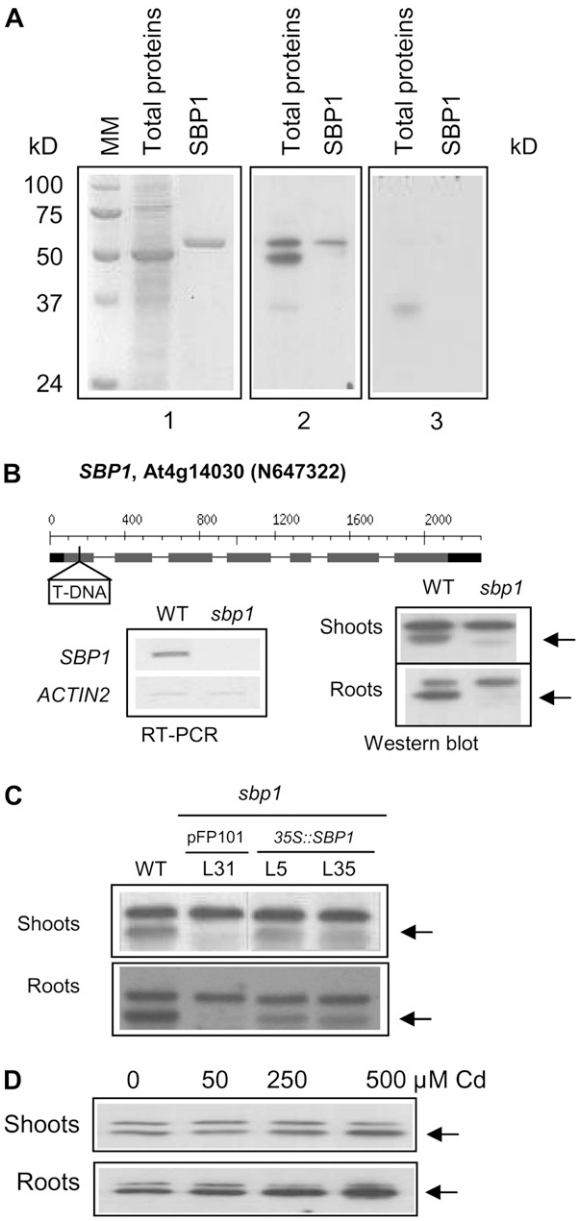

Figure 4. Characterization of SBP1 protein using *sbp1* knockout mutant and Cd-challenged seedlings. A, Total proteins extracted from 7-d-old seedlings (15 μg) and purified recombinant His-SBP1 protein (2 μg) were separated by SDS-PAGE and stained (1) or blotted onto membrane and analyzed by immunodetection with anti-SBP1 (2) and preimmune serums (3); 25 ng of purified SBP1 were used for the immunodetection analyses. MM, Molecular markers. B, T-DNA insertion line for *SBP1* (N647322) has been obtained from the NASC. The map shows the localization of the T-DNA. RT-PCR was made on total RNA extracted from 7-d-old seedlings to test the level of expression of *SBP1* in the T-DNA insertion line. Primers are described in Supplemental Table S1. Number of PCR cycles was 25 for *SBP1* and 23 for *ACTIN2*. No *SBP1* transcript was detected even for a higher number of PCR cycles (data not shown). In parallel, western blot was performed on proteins extracted from wild type and *sbp1* using anti-SBP1 antibodies. Five micrograms of total proteins were loaded per lane. C, Western blots were performed on wild-type and *sbp1* lines expressing the *35S::SBP1* construct (lines L5 and L35) or the empty vector pFP101 (line L31), with anti-SBP1 antibodies. Twenty micrograms of total proteins were loaded per lane. *sbp1 35S::SBP1* lines L5 and L35 showed about 50% of *SBP1* transcript accumulation compared to the wild-type control lines based on RT-PCR analyses (data not shown). D, Analysis of SBP1 level in wild-type seedlings exposed to Cd. Total proteins were extracted from roots and shoots of 7-d-old seedlings after 72 h of exposure to 0, 50, 250, or 500 μM $CdNO_3$ blotted with the anti-SBP1 antibodies. Five micrograms of total proteins were loaded per lane. Arrows point to SBP1 protein.

or 3′ noncoding region for *SBP2*, reduced gene expression (Supplemental Fig. S2A). The T-DNA was located in the last exon for *SBP3* and completely abolished gene expression (Supplemental Fig. S2A). The double mutant *sbp1sbp3* was generated by crossing. None of these mutants showed altered developmental phenotypes when grown under no-stress conditions (Supplemental Fig. S3A) and sensitivity to Cd was similar to wild-type plants (Supplemental Fig. S3B). However, when we looked at the molecular level, *SBP2* transcript amount was increased in both *sbp1* and *sbp1sbp3* mutants compared to the wild type (Fig. 5A). In the *sbp2* and *sbp3* mutants, no noticeable changes in other *SBP* transcript levels were observed (Fig. 5A). *SBP2* gene expression was also enhanced in the roots of *sbp1* and *sbp1sbp3* mutants exposed to Cd compared to wild-type plants (Fig. 5B). In the shoots, however, no induction of *SBP2* was observed in the mutants or the wild type. To check that the loss of *SBP1* was responsible for *SBP2* overexpression, *SBP2* expression level was analyzed in the *sbp1* mutant complemented with the *SBP1* cDNA (Fig. 5C). As expected, *SBP2* transcript levels in the two *sbp1 35S::SBP1* lines (L5 and L35) were restored back to the wild-type level (Fig. 5C). These data clearly indicate that the absence of *SBP1* resulted in the up-regulation of *SBP2* gene expression. This compensatory phenomenon suggests functional redundancy in this gene family and may explain why the *sbp1* mutant does not show any visible phenotype under both normal and stress conditions.

Phenotype of Wild-Type Arabidopsis Lines Overexpressing *SBP1* in Response to Cd

We further investigate the role of SBP1 in response to Cd by overexpressing *SBP1* in Arabidopsis wild-type plants. Western-blot experiments were performed to select the best SBP1-accumulating lines. Among 14 independent lines carrying the *35S::SBP1* construct, four lines (L3, L4, L6, and L35) showed the highest level of accumulation of SBP1 in roots (2.3-, 1.8-, 2.0-, and 2.2-fold, respectively; Fig. 6A) compared to control lines. The increase in SBP1 accumulation in shoots was less pronounced than that observed in roots (Fig. 6A). When wild-type control lines were challenged with Cd, no toxic effect of Cd was observed up to 10 μM and root growth started to be significantly inhibited at 25 μM (15%–20% inhibition; $P < 0.002$; Fig. 6B). In *35S::SBP1* lines L4, L6, and L35, 25 μM Cd did not affect root growth ($P > 0.5$ for the three lines) and root growth inhibition was reduced for line L3. At higher Cd concentration (50 μM), they were still less

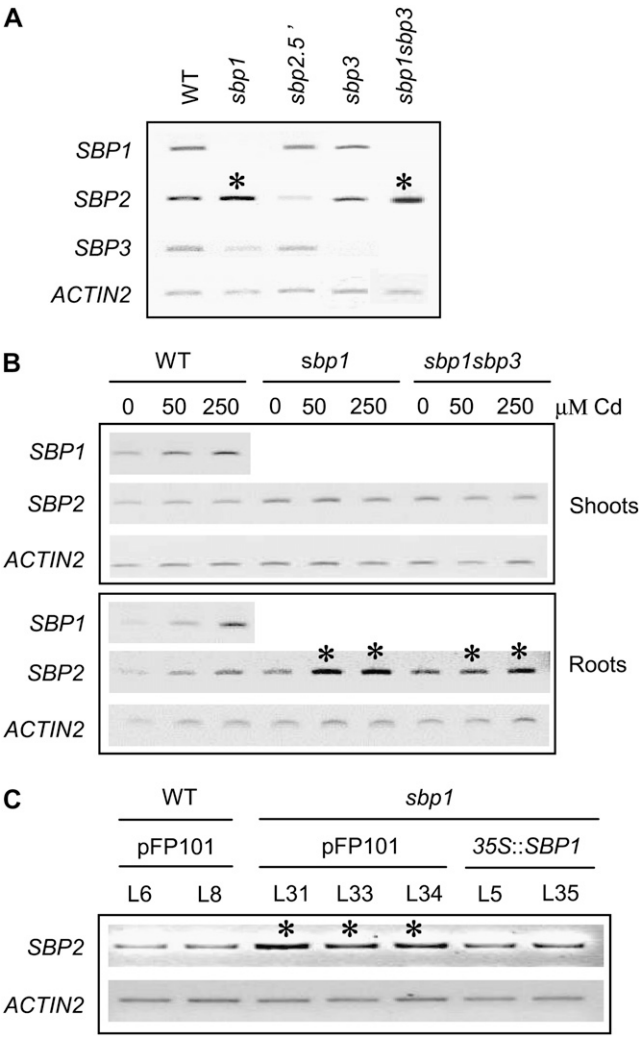

Figure 5. Analysis of *SBP* expression in *sbp* Arabidopsis mutants in healthy or Cd-stressed conditions. A, *SBP* transcript level in healthy *sbp* mutants. RT-PCR was performed on total RNA extracted from 7-d-old wild type and *sbp* mutants (*sbp1*, *sbp2.5'*, *sbp3*, and the double mutant *sbp1sbp3*). Results show a representative experiment obtained from two independent sets of data. The number of PCR cycles performed was 27, 27, 35, and 23 for *SBP1*, *SBP2*, *SBP3*, and *ACTIN2*, respectively. B, Expression analysis of *SBP2* in response to Cd in the *sbp1* and *sbp1sbp3* mutants. RT-PCR was made on total RNA extracted from roots and shoots of 7-d-old seedlings submitted to 0, 50, and 250 μM $CdNO_3$ for 24 h. The number of PCR cycles performed for roots and shoots was 29 and 27, respectively, for *SBP1*, 31 and 27 for *SBP2*, and 27 and 23 for *ACTIN2*. Asterisks show the increased accumulation of *SBP2* transcripts in the *sbp1* and *sbp1sbp3* mutants compared to wild-type seedlings. C, Expression analysis of *SBP2* in *sbp1 35S::SBP1* lines L5 and L35 in comparison with wild-type and *sbp1* lines transformed with the empty vector pFP101 (lines L6, L8, and lines L31, L33, L34, respectively). RT-PCR analyses were made on total RNA extracted from roots of 7-d-old seedlings. The number of PCR cycles performed was 29 for *SBP2* and 25 for *ACTIN2*. Primers are described in Supplemental Table S1. Asterisks show the increased accumulation of *SBP2* transcripts in the *sbp1* control lines compared to wild-type control lines.

affected than wild-type control plants (Fig. 6B). Similar results were obtained based on fresh-weight measurement (Fig. 6C). In addition, overaccumulation of SBP1 in roots of *35S::SBP1* lines led to an increase in Cd content in this tissue of about 40% compared to control lines (Fig. 6D). No difference was observed in Cd accumulation in shoots (Fig. 6D). Together, these data indicate that overexpression of *SBP1* in the wild-type background slightly, but significantly, reduced wild-type sensitivity to Cd. The phenotypes of wild-type and *35S::SBP1* line L4 are shown in Supplemental Figure S4.

Phenotype of Arabidopsis *cad* Mutant Overexpressing *SBP1* in Response to Cd

The ability of the SBP1 protein to enhance Cd tolerance was further analyzed in two Cd-hypersensitive mutants, *cad2-1* and *cad1-3*. These two mutants are affected in γ-glutamyl-Cys synthetase (GSH1) and phytochelatin synthase (PCS1) activity, respectively (Howden et al., 1995a, 1995b), and thus lack GSH and PC for *cad2-1*, and PC only for *cad1-3*. In the *cad2-1* background, among 10 independent lines carrying the *35S::SBP1* construct, four lines (L7, L17, L28, and L29) accumulated the highest amount of SBP1 in roots (2.9-, 3.0-, 2.9-, and 3.7-fold, respectively; Fig. 7A) compared to the control lines. In these lines, little or no overaccumulation of SBP1 in the shoots was observed (Fig. 7A). In the *cad2-1* control lines, root growth started to be significantly inhibited by Cd at 5 and 10 μM (30%–40% inhibition; $P < 0.0001$) compared to wild-type control lines (Fig. 6B). In the four *35S::SBP1* lines, no reduction of root growth was observed at these two concentrations ($P \geq 0.5$), and sensitivity to Cd started to be significant at 25 μM Cd, although it was still reduced when compared to control lines (Fig. 7B). Based on fresh-weight measurement, similar results were observed (Fig. 7C). Together, these results show that overaccumulation of SBP1 in *cad2-1* mutants partially restores wild-type growth sensitivity to Cd. The phenotypes of *cad2-1* control and *35S::SBP1* lines are shown in Figure 7D and Supplemental Figure S5. Similar results were obtained in the *cad1-3* background (Supplemental Fig. S6). As in the wild-type background, accumulation of SBP1 led to an increase in Cd content of about 20% in the roots of *cad2-1 35S::SBP1* lines (Fig. 7E).

Effect of Cd on *SBP1* Overexpressing Yeast Cell Growth

No homolog of the *SBP* genes has been found in yeast. To test whether SBP can confer Cd tolerance to yeast, we introduced *SBP1* cDNA into a wild-type strain and the yeast Cd factor (*ycf1*) mutant, which is Cd hypersensitive (Li et al., 1996). As controls, yeast cells expressing the GUS protein were used. As shown in Figure 8A, in both wild type and *ycf1* controls, the addition of Cd greatly slowed down cell growth and *ycf1* cells showed higher Cd sensitivity than the wild

type, as expected (Fig. 8A, white symbols). In the wild-type background, SBP1 expression had no marked effect on Cd sensitivity (Fig. 8A, black symbols versus white symbols). Higher Cd concentrations gave similar results (data not shown). In the *ycf1* background, the expression of SBP1 led to better growth of the cells in the presence of Cd compared to the control *ycf1*-GUS cells (Fig. 8A). When 15 μM Cd was used, the higher tolerance observed in *ycf1*-SBP1 cells reached a maximum at 32 h, whereas at 25 μM, better growth was observed later, with a peak at 48 h (Fig. 8A). Although tolerance was increased in *ycf1*-SBP1 cells, wild-type growth was not fully restored (Fig. 8A). Similar results were obtained with *ycf1*-SBP2-expressing cells (data not shown). Cd measurements showed that SBP1 and control cells accumulated similar amounts of Cd, indicating that the observed increased tolerance was not linked to a reduced Cd uptake in *ycf1*-SBP1 cells (Fig. 8B). Together, these data show that SBPs decrease Cd sensitivity in the *ycf1* background.

Binding Ability of Recombinant SBP1 Protein toward Cd

The first hypothesis to explain why SBP1 would help to protect against Cd toxicity is that SBP1 is able to bind the metal. Recombinant GST-SBP1 protein was thus overexpressed in *E. coli* and purified as described in ''Materials and Methods''. Binding experiments were performed with the recombinant SBP1 protein and in parallel with GST alone as a control. One nanomole of each protein was incubated with increasing amounts of Cd. Bound and free Cd were separated by chromatography through a Sephadex G-25 column. As shown in Figure 9, Cd coeluted with SBP1-containing fractions. When 1 nmol of Cd was used, all the Cd was eluted with SBP1 protein. Increasing the amount of Cd from 1 to 20 nmol increases the amount of Cd retained by SBP1 with approximately a maximum of 3 nmol of Cd/nmol of SBP1 protein. When 1 nmol of GST alone was incubated with Cd (1–20 nmol), no Cd was retrieved in the protein fractions. These data clearly indicate that SBP1 protein has the ability to bind Cd.

DISCUSSION

From differential proteomic studies (Sarry et al., 2006), we selected a Cd-accumulating protein identified as Arabidopsis putative SBP1 as a good candidate for deeper exploration of its role in the Cd response. In

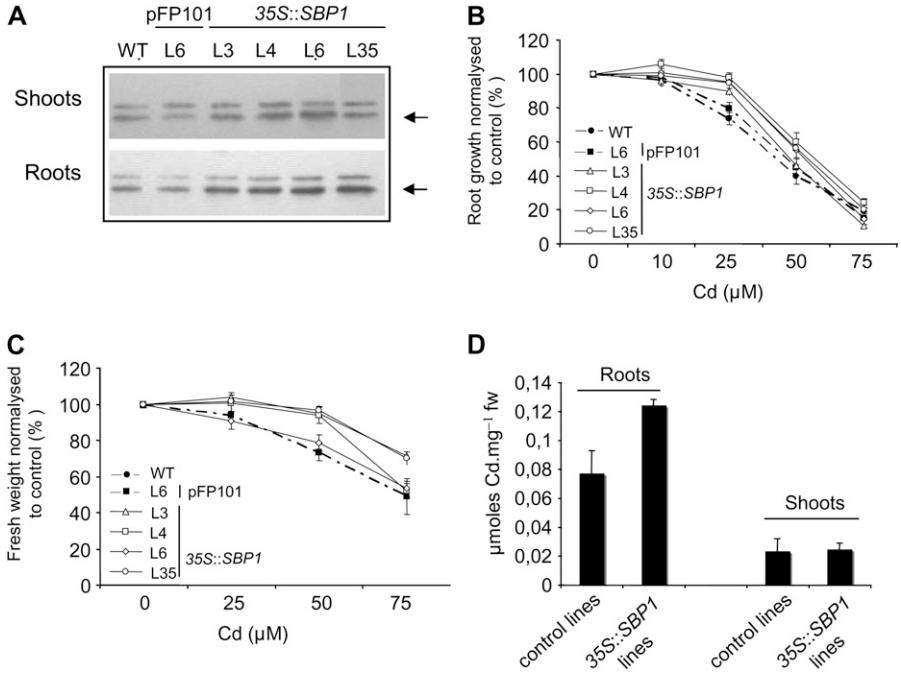

Figure 6. SBP1 protein level in wild-type Arabidopsis seedlings overexpressing *SBP1* and effect on Cd tolerance and accumulation. A, Western blot was performed on proteins extracted from roots and shoots of untransformed wild-type Arabidopsis or from transgenic lines expressing the empty vector pFP101 (lines L6) or the *35S::SBP1* construct (lines L3, L4, L6, and L35) with anti-SBP1 antibodies. Five micrograms of total proteins were loaded per lane. Arrows point to SBP1 protein. B and C, Root growth (B) and fresh weight (C) of Arabidopsis lines tested by western blot in A exposed to Cd stress. Four-day-old seedlings were exposed to different $CdNO_3$ concentrations (0–75 μM Cd) and root lengths and fresh weights were measured after 6 d of contact with the toxic metal. Data are expressed as a percent of the growth and fresh weight measured for each line in control conditions (0 μM Cd). A representative experiment performed on 12 seedlings per line is shown. Error bars show the mean $\pm$ SE. Where not apparent, error bars are hidden by the symbol. D, Cd content in roots and shoots of wild type tested by western blot in A. Cd content was determined by ICP-MS in roots and shoots of 7-d-old seedlings treated for 3 d with 25 μM Cd. Results include data obtained on three control lines and four *35S::SBP1* lines.

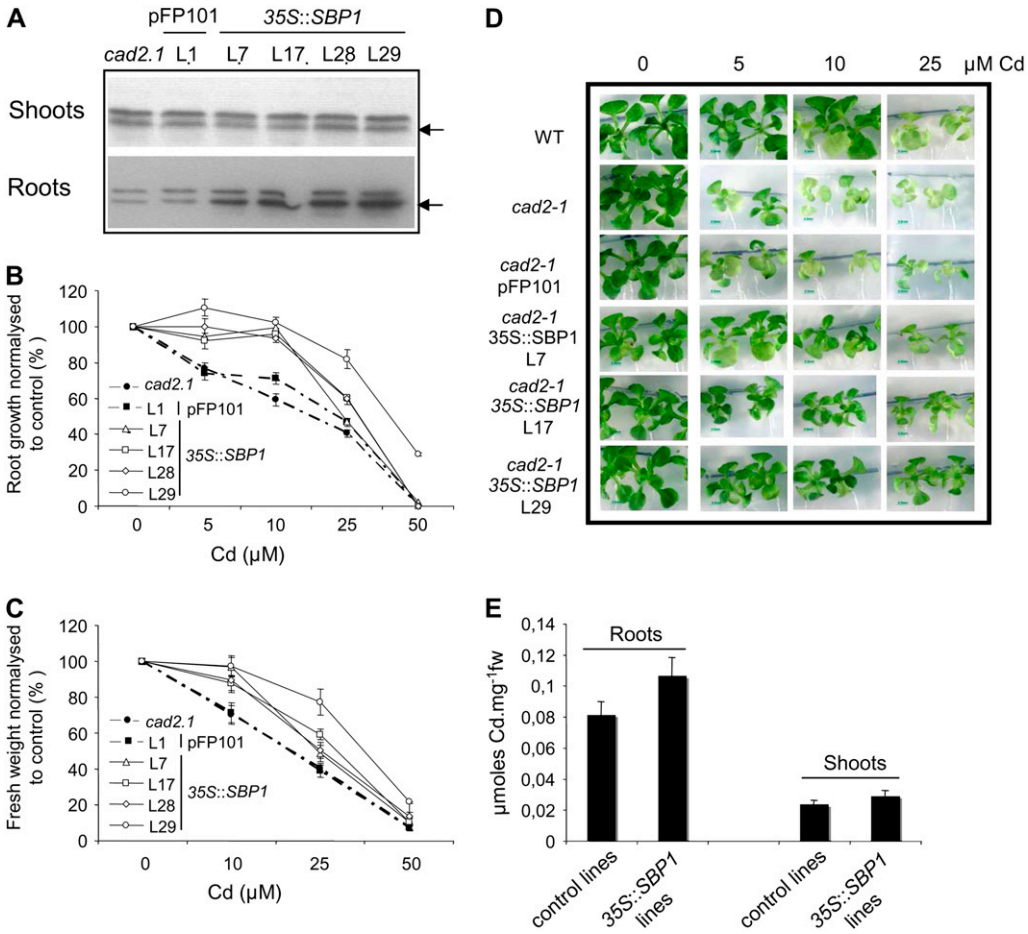

Figure 7. SBP1 protein level in *cad2-1* Arabidopsis seedlings overexpressing *SBP1* and effect on Cd tolerance and accumulation. A, Western blot was performed on proteins extracted from roots and shoots of untransformed Arabidopsis *cad2-1* mutant and from transgenic lines expressing the empty vector pFP101 (line L1) or the *35S*::*SBP1* construct (lines L7, L17, L28, and L29) with anti-SBP1 antibodies. Five micrograms of total proteins were loaded per lane. Arrows point to SBP1 protein. B and C, Root growth (B) and fresh weight (C) of Arabidopsis lines tested by western blot in A exposed to Cd stress. Four-day-old seedlings were exposed to different $CdNO_3$ concentrations (0–50 μM Cd) and root lengths and fresh weights were measured after 6 d of contact with the toxic metal. Data are expressed as a percent of the growth and fresh weight measured for each line in control conditions (0 μM Cd). A representative experiment performed on 12 seedlings per line is shown. Error bars show the mean ± SE. Where not apparent, error bars are hidden by the symbol. D, Phenotype of the plants after 10 d of contact with the toxic in comparison with wild-type plants. E, Cd content in roots and shoots of *cad2*-1 lines tested by western blot in A. Cd content was determined by ICP-MS in roots and shoots of 7-d-old seedlings treated for 3 d with 25 μM Cd. Results include data obtained on three control lines and four *35S*::*SBP1* lines.

Arabidopsis, the SBP protein family has been poorly studied until now. In this article, we established the pattern of expression of the three *SBP* genes in healthy plants as well as in response to Cd. We also analyzed the phenotype of Cd-challenged *sbp* mutants and *SBP* overexpressors, with the aim of getting new insights into detoxification mechanisms that plants could use to face this heavy metal.

We showed that, among the three *SBP* homologs, *SBP1* was ubiquitously expressed in young as well as in mature plants, whereas *SBP2* was notably less expressed in roots and flowers. The expression of *SBP3*, the most divergent isoform, was restricted to very faint levels, mainly in roots. Similarly, a different pattern of expression was observed for the two *SBP* isoforms expressed in mice regarding the organ analyzed (Lanfear et al., 1993; Mattow et al., 2006). *SBP1* ubiquitous expression was also reported in human (Kim et al., 2006) and in *L. japonicus* (Sawada et al., 2004). The Arabidopsis *SBP1* promoter was found active in many organs in *SBP1*::*LUC* transgenic lines and it showed high activity in young active growing tissues, peduncles, and stems during the early stage of development, indicating that *SBP1* gene expression is regulated during plant growth. Another interesting feature about *SBP* gene expression was revealed in the *sbp1* null mutant in which up-regulation of *SBP2* expression was observed more particularly in roots, where *SBP2*

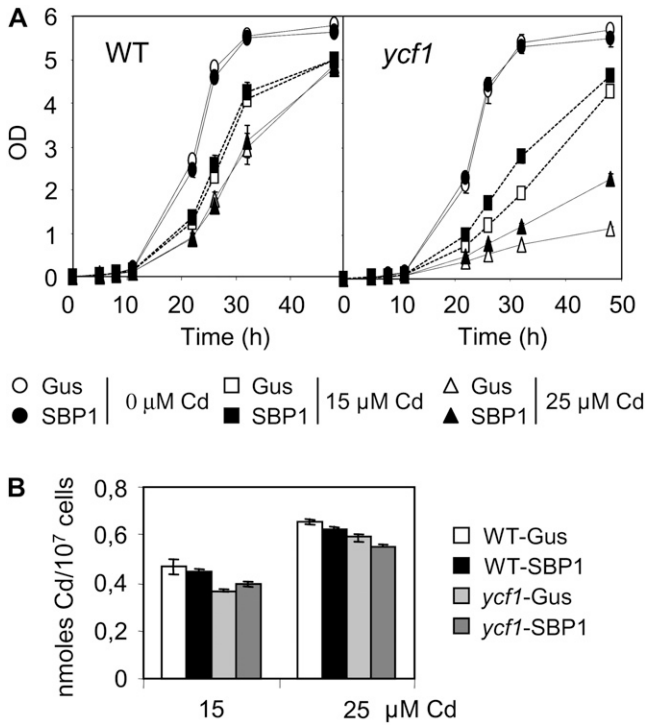

Figure 8. Effect of SBP1 expression on yeast cell sensitivity to Cd. A, Time-course study of the growth of SBP1 and GUS yeast transformants in liquid culture supplemented with or without $CdNO_3$. The graph represents yeast growth expressed as OD as a function of time. Error bars show the mean ± SE of three independent clones. Where not apparent, error bars are hidden by the symbol. These graphs show a typical experiment performed on three independent clones for each construct and was reproducible on at least three independent experiments. B, Cd measurements in the transformants. Yeast cells were sedimented by centrifugation at the end point of the experiment shown in A and Cd content was determined by ICP-MS. Error bars show the mean ± SE of data collected from three independent clones. This graph shows a representative experiment.

was less expressed. These data suggest the existence of a regulatory network to maintain adequate SBP protein levels, as well as redundancy in this gene family. In addition, accumulation of SBP1 in Arabidopsis *35S::SBP1* lines was observed in roots, but not in shoots. This phenomenon was particularly obvious in the *cad2-1* mutant background that lacks *GSH1* (involved in GSH synthesis), where for the highest SBP1 accumulation in roots (3- to 4-fold higher) the lowest accumulation in shoots was observed (0- to 1.5-fold). SBP1 protein level in both tissues was correlated to *SBP1* transcript level (data not shown). These data highlight the fact that SBP expression level is tightly controlled and different between shoot and root tissues.

In response to Cd stress, *SBP1* expression was enhanced and the protein accumulated first in roots, where the level of accumulation was approximately 2 to 4 times higher than in control plants. In the same conditions, *SBP2* and *SBP3* transcript accumulation was delayed. Accumulation of SBP1 protein was less important in shoots where Cd accumulation was less pronounced, suggesting a direct correlation between Cd content and SBP1 accumulation. Data obtained on SBP1-overexpressing Arabidopsis plants strongly argue in favor of a role for SBP1 in Cd detoxification. In all three backgrounds analyzed (i.e. wild type and both Cd-hypersensitive *cad* mutants), SBP1 overaccumulation led to an enhanced tolerance to Cd. The *cad* mutants are devoid of PCs, one of the main Cd detoxification mechanisms in Arabidopsis, because of impairment in PC synthase activity or absence of their precursor, GSH (Howden et al., 1995a, 1995b). In *SBP1*-overexpressing *cad* lines, Cd sensitivity based on root growth assay was abolished and restored back to wild-type sensitivity up to 25 μM Cd. SBP1-enhanced tolerance to Cd was more significant in the *cad* than in the wild-type background, suggesting that SBP1 action may be relevant when the Cd-dedicated detoxification mechanism is overcome. Correlated to these results, the expression of SBP1 in Cd-hypersensitive *ycf1* cells led to an increased tolerance to Cd, whereas no effect was observed in the wild-type background. In yeast, the Cd detoxification process relies on the direct complexation of Cd to GSH and on the import of Cd-GSH complexes to the vacuole via YCF1, an ATP-binding cassette transporter (Li et al., 1996). Because SBP homologs are absent in yeast, we might suggest that the slighter effect of SBP1 in Cd tolerance observed in yeast versus plants could be that the protein is not fully functional in a yeast background (missing partners, different subcellular localization, etc.) or that the N-terminal hemagglutinin extension interferes with SBP1 activity.

The fact that the Cd hypersensitivity of the PC-deficient *cad* mutants could be rescued by *SBP1* overexpression and that *SBP1*-overexpressing lines showed increased Cd content in roots suggested that this protein could take part in Cd detoxification processes through direct binding of the metal. This hypothesis was confirmed by in vitro Cd-binding experiments that showed that the purified recombinant SBP1 protein was able to bind Cd^{2+} ions with a stoichiometry of about 3 nmol Cd^{2+}/nmol protein, which correlates to the number of putative metal-binding sites within the protein. Indeed, the ability of SBP1 to bind Cd might be explained by the presence of several putative metal-binding domains (three His-rich domains and a CXXC domain) within the protein that belong to the diverse highly conserved motifs of SBP sequences from different organisms (Supplemental Fig. S7; Flemetakis et al., 2002). In Arabidopsis, the Cdl19 protein, a putative metal-binding protein in which overexpression enhanced tolerance to Cd stress, was shown to bind the metal via the CXXC motif (Suzuki et al., 2002). The SBP-conserved CXXC motif is also characteristic of proteins involved in the redox control of target proteins (Meyer et al., 1999; Flemetakis et al., 2002). Cd is known to induce oxidative stress and *SBP* expression is induced under both Cd and oxidative stress (Desikan et al., 2001; this article). Under Cd stress, SBP structure and function (i.e. metal-binding capacity) might be

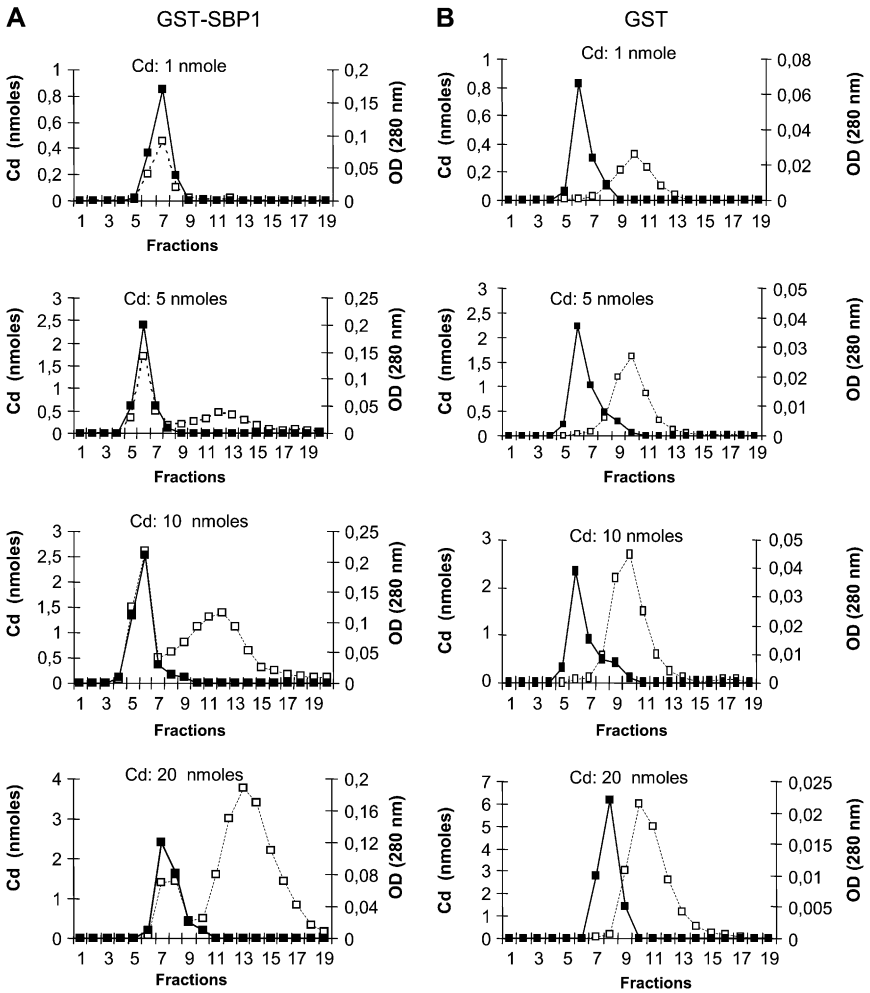

Figure 9. In vitro binding ability of Arabidopsis SBP1 toward Cd. One nanomole of recombinant GST-SBP1 (A) and GST alone (B) was incubated with 1 to 20 nmol of $CdNO_3$ and separated from free Cd by chromatography on a Sephadex G-25 column. Elution of proteins was followed by measuring the A_{280} (black square) and Cd (white square) content was assayed by ICP-MS.

affected because it is known for other CXXC motif-containing enzymes (Meyer et al., 1999; Rollin-Genetet et al., 2004). In addition, it is interesting to note that, in mammals, SBP2 was found to bind to the toxic compound, acetaminophen (see introduction). In mice, GSH is the major acetaminophen-detoxification process, but SBP2 is able to replace it by binding this toxic compound when the GSH level becomes too low (Mattow et al., 2006). Therefore, we cannot exclude that additional functions of SBP1 may help to reduce Cd toxicity. An increase in *SBP1* expression was also observed in the condition of sulfur starvation (Nikiforova et al., 2003). In response to Cd, sulfur metabolite fluxes are redirected to the GSH/PC synthesis pathways and ultimately lead to GSH drop when PC synthesis is too high (Ducruix et al., 2006; Herbette et al., 2006; Sarry et al., 2006). *SBP1* expression under Cd stress could therefore be linked to perturbation of the sulfur metabolism.

Microarray analyses tend to suggest that Arabidopsis *SBP1* is a general stress-responsive gene. Indeed, increased expression of *SBP1* was observed in response to hydrogen peroxide, auxin, aphids, sulfur starvation, or drought (Desikan et al., 2001; Zhao et al., 2002; Liu and Baird, 2003; Nikiforova et al., 2003; Zhu-Salzman et al., 2004). *SBP1* overexpression has been linked to enhanced tolerance against different pathogens, selenite, or Cd (Sawada et al., 2004; Agalou et al., 2005; this article), suggesting a general role of this protein in the detoxification process and cell defense, as it is observed in mammals. However, *SBP1* expression is ubiquitous and not restricted to stress conditions. By now, the physiological role of SBP in plants is far from being understood and we cannot exclude the possibility that plant SBP protein may have several functions as it was proposed in mammals. Increased tolerance to selenite has been observed in SBP-overexpressing plants (Agalou et al., 2005). Up to now, Se has not been demonstrated to be essential in land plants (Sors et al., 2005) and the physiological role of SBP1 toward this metalloid in nonstressed conditions is thus quite unclear. However, we might suggest that SBP is able to bind diverse metal(loid) ions and thus participate in metal(loid) homeostasis, as has been proposed for metallothioneins and PC (Cobbett and Goldsbrough, 2002; Roosens et al., 2005; Zimeri et al., 2005). The pattern of expression of *SBP1* in sites of active transportation, such root tips, peduncles, and

stems, are in accordance with such a potential role. *SBP1* is overexpressed in response to oxidative stress and the protein contains a CXXC motif characteristic of proteins involved in the redox control of target proteins (Flemetakis et al., 2002), also suggesting that SBP1 might be involved in redox processes.

CONCLUSION

To engineer plants capable of cleaning Cd-polluted soils using phytoremediation techniques or to limit nutritional disease by preventing the introduction of heavy metals into the food chain, it is necessary to better understand the mechanism by which plants recognize and uptake Cd, the signaling pathways triggering tolerance mechanisms, and the different detoxification processes. This article provides good evidence that the Arabidopsis SBP1 protein can function as a potential new player in Cd detoxification, acting in parallel with GSH and PC. Further studies are now under investigation to test the ability of SBP1 to bind other metal ions, to understand the function of SBP1 under other stress, and to identify how SBP1 expression is regulated. This will definitely help to get new insights into the function of the SBP protein family in plants.

MATERIALS AND METHODS

Plant Material

All experiments were performed using Arabidopsis (*Arabidopsis thaliana*) wild-type in the Col-0 background. T-DNA insertion lines in *SBP1* (N647322), *SBP2* (N515271 and N558073), and *SBP3* (N569596) were obtained at the Salk Institute from the Nottingham Arabidopsis Stock Center (NASC). Homozygous lines carrying the T-DNA insertion were isolated by PCR using gene-specific primers (Supplemental Table S1) and each mutant was back-crossed once in the wild-type background. Double mutant *sbp1sbp3* was generated by crossing *sbp1* with *sbp3*. Arabidopsis *cad1.3* and *cad2.1* mutants, respectively affected in PC and GSH synthesis (Howden et al., 1995a, 1995b), were kindly provided by C. Cobbett (University of Melbourne). Transgenic lines overexpressing SBP1 (*35S::SBP1*) or the *LUC* gene under the control of the SBP1 promoter (*SBP1::LUC*) gene were generated as described later in the "Materials and Methods" section.

Plant Growth Condition and Cd Application

Arabidopsis seeds were sterilized, stratified for 4 d at 4°C, and sown on basic 0.5× Murashige and Skoog medium (M0404; Sigma-Aldrich) supplemented with 5 g/L Suc, 0.5 g/L MES (pH 5.7), and 8 g/L agar type A). Plates were then placed in a controlled-environment growth chamber, in a long-daylength condition, at 56% humidity and 21°C (day) or 20°C (night). Irradiance was set at 120 μE m^{-2} s^{-1}. Plates were grown vertically to allow root and shoot collection. Depending the experiment performed, 4- or 7-d-old seedlings were transferred on 0.5× Murashige and Skoog medium, containing or not $CdNO_3$, for 6 h to 6 d. Seedling root length and fresh weight were measured as an indicator of Cd sensitivity. For seed collection and tissue expression analysis, plants were grown in soil under the same growth conditions as described above.

SBP cDNAs and Promoter Cloning and Plasmid Constructions for Expression in Plants

The sequence of the different primers used in this section is presented in Supplemental Table S1. *SBP* cDNA was amplified using gene-specific primers with restriction sites at both ends (*Xba*I [5′] and *Bam*HI [3′] for *SBP1* and *SBP3*, and *Xba*I [5′] and *Sal*I [3′] for *SBP2*), from cDNA synthesized from Arabidopsis cells treated with Cd (Sarry et al., 2006). PCR was performed using Pfu polymerase for 28 cycles and TA cloning was performed using the pGEM-T Easy vector (Promega). All cDNA sequences were checked (Genome Express). To generate plasmid for overexpression of SBP proteins in plants, each cDNA was cloned into the pFP101 vector under the control of the *35S* promoter (Bensmihen et al., 2004) after release of the cDNA from pGEM-T Easy vector using the specific restriction enzymes added at the 5′ and 3′ ends described above. The *SBP1* promoter region was amplified using primers with *Bam*HI restriction sites at both ends (see Supplemental Table S1) from genomic DNA isolated from Arabidopsis Col-0. PCR was performed using Pfu polymerase for 28 cycles and PCR products were cloned into the pGEM-T Easy vector. Promoter sequences were checked. The *Bam*HI DNA fragments containing the *SBP1* promoter were introduced into the pATM-Domega plasmid, kindly provided by Andrew Millar (University of Warwick), which contains the *LUC* reporter gene (Welsh et al., 2005). The resulting *Sma*I cassettes containing *SBP1* promoter, *LUC* gene, and terminator sequences were further cloned into pFP100 vector (Bensmihen et al., 2004).

Vectors for the production of GFP-SBP1 and GFP-SBP2 fusion proteins were generated using Gateway technology (Invitrogen). cDNA encoding *SBP1* (At4g14030) and *SBP2* (At4g14040) were provided in entry clones (pENTR/SD/D-TOPO, respectively, U15803 and U15274), by the Arabidopsis Biological Resource Center. LR reactions were performed following the manufacturer's instructions, using the destination vector pK7WGF2, containing the enhanced GFP gene (Karimi et al., 2005) kindly provided by the Flanders Interuniversity Institute for Biotechnology. For the production of the GFP-SBP3 fusion protein, *SBP3* cDNA was first amplified with *Xma*I restriction sites at the 5′ and 3′ ends and subcloned into the pGEM-T Easy vector before cloning in the GFP-JFH1 vector, kindly provided by J. Harper (The Scripps Research Institute).

All resulting expression vectors were introduced in the *Agrobacterium tumefaciens* C58 strain by electroporation. Arabidopsis flowers were then transformed following the protocol described in Clough and Bent (1998).

RNA Isolation and RT-PCR Analyses

Total RNA was extracted from Arabidopsis shoot and root samples using Trizol reagent as described by the manufacturer (Invitrogen). Thirty micrograms of total RNA were treated with ultrapure DNAseI for 30 min at 37°C to eliminate any DNA and further purified on a column using the RNeasy kit (Qiagen). Three to 5 μg of purified total RNA were then used for RT using the first-strand cDNA synthesis kit (Amersham) and a *Not*I primer. cDNA was 10-fold diluted and PCR was performed using titanium Taq polymerase (Ozyme) and gene-specific primers (Supplemental Table S1) amplifying a DNA fragment size of 500, 520, and 440 bp for *SBP1*, *SBP2*, and *SBP3*, respectively. To check the specificity of each couple of primers, PCR products were digested using restriction enzymes specific to each isoform, namely, *Bgl*II for *SBP1*, *Bam*HI for *SBP2*, and *Pst*I for *SBP3*. The efficiency of each couple of primers was identical based on amplification performed on genomic DNA. *ACTIN2* expression was used as a control. Quantifications, when provided, were performed with Quantity One software using the local subtraction background.

LUC Imaging

LUC imaging was performed as described (Welsh et al., 2005) using a CCD camera (Princeton Instrument) linked to a dark chamber. Data were analyzed using Metavue software (Bio-Rad). Plants were sprayed with a fresh solution of 1 mM luciferin (Promega) prepared in 0.02% (v/v) Triton X-100 and luminescence was recorded after 5-min incubation in the dark chamber. LUC activity was recorded for 5 to 10 min. Experiments were performed on three independent *SBP1::LUC* lines and showed similar results.

Overexpression of SBP1 in *Escherichia coli* and Purification of the Recombinant Proteins

The *SBP1* cDNA contained in the entry clone (U15803) was cloned into the destination vectors pET 16b gateway engineered as described in Belin et al. (2006) for the production of His-tagged SBP1 protein. Due to the His tag and the gateway technology, the recombinant SBP1 protein contains an additional

49 amino acids at the N terminus. The pGEX-3X gateway vector kindly provided by Lionel Gissot (INRA) was used for the production of GST-SBP1 protein, which carries an additional 250 amino acids at the N terminus compared to SBP1. The recombinant plasmids were used to transform *E. coli* strain BL21. In standard conditions, cell cultures were done at 37°C in 8 × 800 mL Luria-Bertani medium until the culture OD 600 nm reached 1.3. After 2 h at 20°C, expression of the recombinant proteins was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside to the cultured cells for 17 h. Cells were then centrifuged and disrupted by sonication (6 × 1 min, using the Branson Sonifier 250) proteins in a buffer containing 20 mM sodium phosphate, pH 7.7, 0.5 M NaCl, 0.05% Triton X-100, 1 mM dithiothreitol, 5% glycerol, 50 mM imidazole, and an antiprotease cocktail (Roche). For GST-SBP1 production, imidazole was omitted. After centrifugation, the recombinant protein SBP1 carrying a 10-His tag was purified by chromatography through a nickel Sepharose high-performance column according to the manufacturer's instructions (GE Healthcare). The purified His-SBP1 protein was then used to immunize rabbits (Charles River Laboratories). The recombinant GST-SBP1 protein was purified using a GSH Sepharose 4B resin according to the manufacturer's instructions (GE Healthcare) and was used for Cd-binding assay. GST alone was produced using the pGEX-4T vector (Amersham) and was kindly provided by Jeremy Gaillard (LPCV, CEA).

Protein Extraction and Western-Blot Analysis

Proteins were extracted from Arabidopsis tissues in 100 mM Tris buffer, pH 7.5, supplemented with an antiprotease cocktail (Roche). After centrifugation, protein concentration in the supernatant was determined using the Bio-Rad protein assay reagent. Five to 10 μg of total soluble proteins were separated on an acrylamide gel and transferred to a nitrocellulose membrane. Western-blot analyses were performed using the polyclonal SBP antibody at a 1:20,000 dilution, the anti-enhanced GFP antibody (Euromedex), at a 1:5,000 dilution. Quantifications, when provided, were performed using Quantity One software (Bio-Rad) using the local subtraction background.

Yeast Strains, Growth, and Cd Application

The wild-type yeast (*Saccharomyces cerevisiae*; DTY165) and the Cd-sensitive mutant (Δ*ycf1*, DTY167; Szczypka et al., 1994) kindly provided by J. Thiele (Duke University Medical Center) were used for heterologous expression of SBP protein and phenotype characterization. *SBP1* and *SBP2* cDNAs contained in the entry clones (U15803 and U15274) were cloned in the destination vector pFL61 (A.G. Desbrosses-Fonrouge, unpublished data) for the production of hemagglutinin-tagged SBP. These vectors were used to transform yeast DTY165 and DTY167 strains according to standard procedure (Invitrogen) using an overnight culture grown in yeast peptone dextrose medium. Transformed cells were cultured in the selective synthetic dextrose-Ura medium, at 28°C with shaking. To test the sensitivity of yeast cells on Cd, cultured cells in the exponential phase (OD around 1.0) were diluted to OD = 0.02 in synthetic dextrose-Ura medium supplemented with (or without) Cd. Yeast cell growth was followed for 48 h by measuring OD at 600 nm. Experiments were always conducted using three independent clones for each construct tested.

Cd-Binding Assay

Binding experiments were conducted with recombinant GST-SBP1 protein in parallel with GST protein alone. One nanomole of recombinant SBP1 protein was incubated for 15 min at 25°C, with 1 to 20 nmol of $CdNO_3$ in a total volume of 25 μL containing 10 mM Tris, pH 7.4, and 150 mM NaCl. Recombinant protein was then separated from free Cd by chromatography through a Sephadex G-25 column (0.5 × 8.5 cm) with an elution rate of 150 μL/min. Fractions of 200 μL were collected. Protein elution was followed using a spectrophotometer at 280 nm and Cd content was assayed by inductively coupled plasma mass spectrometry (ICP-MS; HP4500 ChemStation; Yokogawa Analytical Systems).

Cd Measurements in Plant Extracts and Yeast Cells

Shoots and roots of Cd-treated and untreated plants were dried for 3 d at 50°C and mineralized in 3 mL of HNO_3 65% (Suprapur; Merck) and 1 mL of HCl 30% (Suprapur; Merck) for 3 h at 85°C. After complete evaporation of the mixture, residual material was resuspended in 1% HNO_3. Cd concentrations in the extract was then determined using ICP-MS (HP4500 ChemStation; Yokogawa Analytical Systems).

For yeasts, untreated and treated cells were pelleted, washed twice with water, incubated in 0.1 M NaOH for 5 min, then centrifuged, resuspended in 0.06 M Tris-HCl, pH 6.8, 2% SDS, 4% β-mercaptoethanol, and finally boiled for 3 min. After centrifugation, an aliquot was diluted in 1% nitric acid and analyzed by ICP-MS.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At4g14030 (*SBP1*), At4g14040 (*SBP2*), and At3g23800 (*SBP3*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Specificity of anti-SBP1 serum against SBP protein family.

Supplemental Figure S2. Characterization of T-DNA insertion lines in *SBP2* and *SBP3*.

Supplemental Figure S3. Phenotypes of Arabidopsis T-DNA insertion lines in *SBP* genes.

Supplemental Figure S4. Phenotype of Arabidopsis wild-type seedlings overexpressing *SBP1*.

Supplemental Figure S5. Phenotype of Arabidopsis *cad2-1* seedlings overexpressing *SBP1*.

Supplemental Figure S6. Phenotype of Arabidopsis *cad1-3* seedlings overexpressing *SBP1*.

Supplemental Figure S7. Amino acid sequences of SBP from various organisms.

Supplemental Table S1. List of primers used for PCR.

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