Coordinated expression of sulfate transporters and its relation with sulfur metabolites in *Brassica napus* exposed to cadmium

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(Received March 7, 2006; Accepted June 5, 2006)

ABSTRACT. Full-length cDNAs encoding a putative low-affinity sulfate transporter (LAST), designated *BnSultr2;2* (accession number DQ091257) were isolated from *Brassica napus* 72 h after sulfate deficiency. *BnSultr2;2* shows significant similarity at an amino acid level with the available LASTs from the dicotyledonous *Arabidopsis* and *Brassica juncea*. It displays the typical twelve-membrane spanning domains of other plant species' sulfate transporters. Examination of steady-state mRNA levels reveals that *BnSultr2;2* transcripts were enhanced in leaves of sulfate-deficient plants. The leaf *BnSultr2;2* expression was also up-regulated under 20-120 μM Cd exposure, but under the same conditions, the *BnSultr2;2* expression in roots was severely suppressed. To understand the relationship between sulfate uptake and Cd stress, we simultaneously isolated another cDNA encoding a known high-affinity sulfate transporter (*BnSultr1;1*, accession number AJ416460) from roots. RT-PCR analysis demonstrates that *BnSultr1;1* was expressed only in roots, and its expression was upregulated by both sulfate-deficiency and Cd exposure. To link up the expression of sulfate transporters to sulfur accumulation and assimilation, the concentrations of sulfate, sulfide, glutathione, and thiol-containing compounds in plant tissues were measured. Elevating Cd concentrations in the culture medium up to 40 μM increased the accumulation of sulfate, sulfide, glutathione, and non-protein thiols in roots. These results suggest that a coordinated regulation of sulfate transporters enables plants to tolerate Cd stress via an efficient sulfate uptake and assimilation.

Keywords: *Brassica napus*; Cadmium; Sulfate transporter; Transcriptional expression.

INTRODUCTION

Cadmium (Cd) is one of the most toxic of heavy metals and is widely released into agricultural soils. Due to its high mobility in soil, cadmium is readily taken up and accumulated by plants. Excess Cd²⁺ accumulation in plants leads to impaired growth and development and even death (Sanità di Toppi and Gabbrielli, 1999). Several mechanisms have been proposed for Cd toxicity in plants (Clemens, 2001). Cd-induced generation of hydrogen peroxide and other reactive oxygen species has been correlated with the damage to plasma membrane lipids and alteration of secondary metabolism (Schützendübel et al., 2001; Shah et al., 2001; Kuo and Kao, 2004). Plasma membrane H⁺-ATPase activity and other enzymes related to nitrogen metabolism are also sensitive to cadmium (Astolfi et al., 2004, 2005). In addition, cadmium has been shown to influence the sulfate uptake and assimilation in plants (Lee and Leustek, 1999; Heiss et al., 1999; Nocito et al., 2002).

Although most of plant species can not thrive under the excessive heavy metal environment, some other species have evolved sophisticated strategies for tolerating high levels of heavy metals. Plants possess a range of cellular mechanisms for detoxification of heavy metals (Rauser, 1999; Clemens, 2001). Cd-induced synthesis of metal chelating compounds like glutathione (GSH), phytochelatins (PCs), and other sulfide-enriched compounds is considered one of the important mechanisms allowing plants to tolerate higher levels of Cd in cells (Zenk, 1996; Cobbett and Goldsborough, 2002). Cd-induced PCs alleviate Cd toxicity by formation of Cd-PC complexes, known as low-molecular-weight (LMW) Cd complexes (Speiser et al., 1992; Cobbett, 2000). The Cd-PC complexes are formed in cytosol and actively
transported from cytosol to the vacuole, where more complicated and stable Cd-PC complexes are formed (Erika and Laszlo, 2002; Hrstioger, 2000). Substantial evidence shows that Cd-induced PC synthesis and metal detoxification mechanisms involve a variety of molecular and biochemical processes (Meuwly et al., 1995; Keltjens and van Beusichem, 1998; Clemens, 2001). The process of metal-induced synthesis of PCs most often leads to a short-term depletion of the glutathione pool, one of the major sources of thiol groups responsible for biological redox processes and a precursor for the synthesis of PCs (Leustek and Saito, 1999; Cobbett, 2000; Wang et al., 2004). However, recovery in the glutathione pool up to the level of or above controls has been demonstrated after a prolonged exposure of plants to Cd (Vögeli-Lange and Wagner, 1996; Schützendübel et al., 2001). Enzymes involved in GSH synthesis such as γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) and GSH synthetase (EC 6.3.2.3) have been examined in plants under metal stress, and the activity of γ-ECS was found to be enhanced upon Cd exposure (Schäfer et al., 1998; Cobbett, 2000). Molecular responses to Cd exposure have also been identified in plants, such as Brassica juncea (Zhu et al., 1999), Arabidopsis (Xiang and Oliver, 1998), and the other plant species in which Cd exposure induced a coordinated transcriptional regulation of genes encoding γ-ECS, GSH synthase, and PC synthase (Schäfer et al., 1998; Zhu et al., 1999; Vatamaniuk et al., 2000; Sun et al., 2005).

Cd-induced GSH and PC synthesis have a well-established relationship with sulfate uptake, transport, and assimilation in plants (Lee and Leustek, 1999; Nocito et al., 2002; Astolfi et al., 2004). For example, under Cd stress a large amount of sulfate accumulates and the level of thiol-containing compounds surges (Leustek and Saito, 1999; Nocito et al., 2002). Plants have a specific group of root carriers for uptake of sulfate from soil (Leustek and Saito, 1999; Hawkesford, 2003; Mendoza-Cózatl et al., 2005). Equally, translocation of sulfate or thiol-containing metabolites between the root and shoot is performed by an array of sophisticated transport systems (Hawkesford, 2000; Howarth et al., 2003). In higher plants, sulfate transporters are subdivided into five groups. Group 1 sulfate transporters have a high affinity for sulfate, and normally facilitate primary uptake of sulfate from soils into roots. AtSultr1;1, AtSultr1;2, and AtSultr1;3, isolated from Arabidopsis (Vidmar et al., 2000), HVST1 from barley (Smith et al., 1997) and SHST1, SHST2 from Stylosanthes hamata (Smith et al., 1995) are typical high affinity sulfate transporters, and they express mainly in roots. Group 2 members such as AtSultr2;1 (AST56) and AtSultr2;2 (AST68) from Arabidopsis and SHST3 from S. hamata have a low affinity to sulfate and are expressed in both roots and shoots (Smith et al., 1995; Takahashi et al., 1996; Takahashi et al., 1997; Takahashi et al., 2000). Suggestions have been made that this group of transporters are principally involved in translocation, distribution or reallocation of sulfate in plants (Smith et al., 1995; Takahashi et al., 1997). Group 3 sulfate transporters are localized in root, stem, leaf and even in root nodules of Lotus japonicus (Takahashi et al., 1999; Buchner et al., 2004a; Krusell et al., 2005), and the group 4 transporters are positioned in tonoplast, suggesting that they facilitate the efflux of sulfate from vacuoles (Kataoka et al., 2004). Compared to Groups 1-4, the sequence divergence of group 5 suggests that these may be functionally distinct transporters. So far, genes encoding sulfate transporters from a variety of higher plants have been identified and a majority of these transporters are up- or down-regulated at the transcriptional level under sulfate starvation and restored (Hawkesford, 2003). However, a fine and coordinated expression pattern of these genes in response to heavy metal (e.g. Cd) stress has not been described.

Brassica napus falls into the same family as Brassica juncea, which as a heavy metal accumulator has the potential to remove excessive heavy metals from contaminated soils (Banuelos and Meek, 1990; Salt et al., 1995). Some genotypes of B. napus exhibit a comparable or even stronger tendency to accumulate heavy metals (Ebbs and Kochian, 1997; Su and Huang, 2002). Since B. napus produces as much high biomass as B. juncea, it is, therefore, suitable for use in phytoremediation. The use of higher plants in the phytoremediation of heavy metal-contaminated soils is not only based on their ability to take up and accumulate the metals, but also on mechanisms able to alleviate their toxic effect (Salt et al., 1998; Nocito et al., 2002). However, little is known about the process of Cd tolerance in B. napus. Understanding of the molecular and physiological regulatory mechanisms of plant sulfate transporters may provide a better insight into our understanding of Cd tolerance in plants. The present study examined the Cd-induced transcriptional expression of BnSultr2;2 and BnSultr1;1 and its correlation with the sulfur accumulation and assimilation in B. napus.

MATERIALS AND METHODS

Plant material and treatment

Seeds of B. napus L. (cv. Youyan No. 9) were sterilized with 5% NaClO for 10 min, washed several times with distilled water, and germinated for 3 d in the dark on floating plastic net. After germination, nine young seedlings were transferred to 1 L polyethylene containers containing 1/4-strength modified Hoagland nutrient solution (0.7 mM Ca^{2+}, 1.5 mM K^{+}, 0.5 mM Mg^{2+}, 0.25 mM NH_{4}^{+}, 2.9 mM NO_{3}^{-}, 0.25 mM H_{2}PO_{4}^{-}, 0.5 mM SO_{4}^{2-}, 4.75 μM Fe^{2+}, 0.32 μM Cu^{2+}, 0.2 μM Zn^{2+}, 1.25 μM Mn^{2+}, 11.5 μM H_{2}BO_{3}, 0.025 μM MoO_{3}). Seedlings were grown at 22±1°C, with a light intensity of 200 μmol m^{-2} s^{-1} and a 14-h photoperiod. After growing for 14 days, plants were treated with 0, 20, 40, 80, and 120 μM Cd as CdCl\textsubscript{2} in the 1/4-strength nutrient solution as indicated above for 72 h, or with 0 and 40 μM Cd for 0, 6, 12, 24, 48, 72 and 96 h. In the experiment of sulfate deficiency, a sulfur-free solution was prepared using MgCl\textsubscript{2} instead of MgSO_{4}. The pH of culture as well
as of treatment solutions was adjusted to 5.6. Treatment solutions were renewed with freshly prepared nutrient solution each day. After treatment, root and leaf tissues were separately harvested and immediately frozen in liquid nitrogen and stored at -80°C in a freezer.

RNA isolation and cDNA synthesis

Total RNA was extracted from root or leaf tissues using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Reverse transcription was carried out at 37°C in 25 μL reaction mixture containing 3 μg of RNA, 0.5 μg oligo (dT) primer, 12.5 nmol dNTPs, 25 units of RNase inhibitor and 200 units of M-MLV reverse transcriptase (Promega, Madison, WI). The first strand cDNA was then used as a template for polymerase chain amplification.

Cloning of a putative low-affinity sulfate transporter

The amplification primers for partial BnSultr2;2 in B. napus were 5′-AAC CCT AAT CGT GTA TTT-3′ (sense) and 5′-GGA CAA TAT ATC ATT TAT TCT G-3′ (antisense) based on Indian mustard (Brassica juncea) partial low affinity sulfate transporter mRNA (accession number AJ223495). The amplification product was cloned into the vector pGEM-T (Promega, Madison, WI) and sequenced (SEQUAB, Göttingen, Germany). The resulting partial cDNA sequence containing 3′ UTR showed a 93% identity with Brassica juncea and an 86% identity with Arabidopsis thaliana putative sulfate transporter mRNA (accession number ATY74516). Another pair of primers was designed to amplify 5′-region to obtain the full-length cDNA. The sense primer based on Arabidopsis thaliana was 5′-CAC TTC AAT AAC CCA CAA-3′, and the antisense primer based on partial BnSultr2;2 was 5′-GCA AGA AGT TAA AGA GCC-3′. The amplified 1.2 kb fragment was cloned into the vector pGEM-T and sequenced. To obtain 5′ UTR, the gene-specific primers for 5′-RACE were: RACE-5A: 5′-GCA AGA AGT TAA AGA GCC-3′, RACE-5B: 5′-CGA AAC TAC AGC CAC AGG ACC A-3′ and RACE 5C: 5′-ACC GTA CTC TGG ATC AAC TG-3′.

In order to create the full-length cDNA of BnSultr2;2 from B. napus by RT-PCR, a 2144 bp fragment was amplified with ExTaq DNA polymerase (Takara) and cloned into the vector pGEM-T for sequencing.

Determination of BnSultr1;1 and BnSultr2;2 gene expression by RT-PCR

The first-strand cDNAs derived from the total RNA of different treatments were used to analyze transcripts of BnSultr1;1 (accession number AJ416460, Bucher et al., 2004a) and BnSultr2;2 (accession number DQ091257, this study). PCR was carried out using primers: Hast-S (5′-AGA TGA TCG CAT TGG GTA-3′) and Hast-A (5′-CCT TTC TCG GAC ATA GTT G-3′) for BnSultr1;1; Last-S (5′-TAC TTC ACA AAT TGA AAC GAG-3′) and Last-A (5′-GGA CAA TAT ATC ATT TAT TCT G-3′) for BnSultr2;2. Expression of EF-1α gene (sense 5′-AGACCAACAGTACTGAC-3′ and antisense 5′-CCACCAATCTTTGTACATCC-3′) was used as a control. RT-PCR products were obtained after 32 PCR cycles. The PCR products were applied to 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The strength of the fluorescent signal derived from ethidium bromide in each lane was determined by the software GIS Gel-ID produced by the Tanon Company.

Sequence analysis

Protein sequence homologies were calculated by the DNAMAN program. Two protein alignments were performed by the same program. The phylogenetic tree of sulfate transporters was drawn using TreeView32 (Page, 1996) based on the multiple alignments performed by Clustal X (Thompson et al., 1997). The transmembrane analysis was carried out using TopRed (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html).

Determination of non-protein thiols and glutathione

Non-protein thiols (NPT) were extracted by homogenizing 0.5 g plant material in 1 mL ice-cold 5% (w/v) sulfosalicylic acid solution. After centrifugation at 10,000 g and 4°C for 30 min, the supernatants were collected and immediately assayed for determination of NPT. NPT levels in samples were measured with Ellman’s reagent (Ellman, 1959). Briefly, 300 μL of the supernatant was mixed with 1.2 mL of 0.1 M phosphate buffer solution (pH 7.6). After reaching a stable absorbance at 412 nm, 25 μL of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) solution (6 mM DTNB dissolved in 5 mM EDTA and 0.1 M phosphate buffer, pH 7.6) was added, and the increase in absorbance at 412 nm was recorded.

Reduced glutathione (GSH) contents were determined fluorimetrically (Hissin and Hilf, 1976). Plant material (0.5 g) was ground in 0.5 ml 25% H3PO4 and 1.5 ml of 0.1 M sodium phosphate-EDTA buffer (pH 8.0). The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was further diluted 5 times with sodium phosphate-EDTA buffer (pH 8.0). 100 μL of diluted supernatant was incubated with 1.8 ml of phosphate-EDTA buffer and 100 μL O-phthalaldehyde (1 mg ml−1) for 15 min at room temperature to interact with the GSH in the solution. Finally, the solution was transferred to quartz cuvette, and the fluorescence at 420 nm was measured after excitation at 350 nm.

Determination of total sulfate and sulfide

For determination of sulfate content in plant tissues, plant samples were digested using HNO3:HClO4 (8:1) solution and turbidimetry was used to determine sulfate according to method of Tababai and Bremner (1970).

Total sulfide in fresh plant tissues was assayed by the methylene blue spectrophotometric method as described by Huang and Wang (1997) with a few modifications.
Plant samples were homogenized in an iced-cold 10 mM NH$_4$-NH$_4$ buffer (pH 10). The homogenate was centrifuged at 15,000 g at 4°C for 10 min. Supernatants from the root sample were directly used to measure sulfide. To avoid the interference of chlorophylls and pigments with measurements, the supernatant from leaf tissues was mixed with the CCl$_4$:CHCl$_3$ (3:1) extraction solution to remove these pigments. The proportion of the supernatant to extraction solution was 1:1. The supernatant was used for sulfide determination. Sulfide content was spectrophotometrically measured at 412 nm in a reaction mixture containing 2 mL of 1% p-amino-N, N-dimethylaniline (dissolved in 2 M H$_2$SO$_4$), 1 mL of 10 mM Fe$^{3+}$ and 1 mL of supernatant.

Cadmium measurement

Root and leaf tissues were thoroughly rinsed with deionized water and blotter dried. Samples were dried at 70°C in a forced-air oven, weighted, and digested with a solution containing nitric acid and perchloric acid (1:1, v/v). Cd in solution was determined by atomic absorption spectrometry (Yang et al., 2001).

Statistical analysis

Each result shown in a table and figure was the mean of at least three replicated treatments. Each treatment included at least nine seedlings for determination. The significance of differences between treatments was statistically evaluated by standard deviation and Student’s t-test methods.

RESULTS

Cloning and analysis of a putative BnSultr2;2 from B. napus

The Arabidopsis genome contains at least 14 members of sulfate transporter genes that are assumed to function independently for the uptake and distribution of sulfate in various cell types (Hawkesford, 2003). In Brassica napus, five different sulfate transporter genes have been cloned (accession number: AJ311389, AJ311388, AJ581745, AJ416461, AJ416460). In this study, a cDNA encoding a putative B. napus low-affinity sulfate transporter, designated as BnSultr2;2, was cloned by RT-PCR and 5´ RACE. The BnSultr2;2 cDNA is 2150 bp long with an open reading frame (ORF) of 1959 bp, a 35-nucleotide(nt)-5´ untranslated region (UTR), and a 153-n 3´ UTR. The ORF encodes a 653-amino acid protein with a predicted molecular weight of 72 kDa and an isoelectric point of 9.5.

The deduced protein includes twelve putative membrane-spanning domains (Figure 1), consistent with the general features of sulfate transporters (Smith et al., 2000). The predicted protein was compared with a sulfate transporter from A. thaliana (Figure 1) (Takahashi et al., 1996) and several other higher plant sulfate transporters (Table 1). The comparison shows a higher protein sequence similarity to low-affinity sulfate transporters than to high-affinity sulfate transporters. To further analyze the relationship between the B. napus sulfate transporter and other higher plant sulfate transporters cloned so far, a phylogenetic tree was constructed based on Clustal X (Figure 2). The phylogenetic relationships among the plant sulfate transporters indicated that the predicted protein of BnSultr2;2 falls into the group 2 together with BjSultr2;2 from B. juncea (Heiss et al., 1999), BoSultr2.1 from B. oleracea (Buchner et al., 2004a), AtSultr2;1 (Takahashi et al., 1997) and AtSultr2;2 (Takahashi et al., 1996) from A. thaliana, and SHST3 from Stylosanthes hamata (Smith et al., 1995), respectively. This group of sulfate transporters is clearly different from others, particularly the high-affinity sulfate transporters of group 1, like SHST1 and SHST2 in S. hamatus (Smith et al., 1995). Therefore, the putative sulfate transporter BnSultr2;2 cloned from B. napus should encode a low-affinity sulfate transporter.

Table 1. Comparison of protein sequence similarity between BnSultr2;2 cDNA and other higher plant sulfate transporters.

<table>
<thead>
<tr>
<th>Sulfate transporters accession number (species)</th>
<th>Sequence identity (%) with BnSultr2;2 protein</th>
<th>Sulfate transporter (ST) type</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ223495 (B. juncea)</td>
<td>93</td>
<td>Partial low-affinity ST (putative)</td>
</tr>
<tr>
<td>D85416 (A. thaliana, AtSultr2;2)</td>
<td>87</td>
<td>Low-affinity ST</td>
</tr>
<tr>
<td>AB003591 (A. thaliana, AtSultr2;1)</td>
<td>58</td>
<td>Low-affinity ST</td>
</tr>
<tr>
<td>X82454 (S. hamata, SHST3)</td>
<td>60</td>
<td>Low-affinity ST</td>
</tr>
<tr>
<td>X82455 (S. hamata, SHST1)</td>
<td>46</td>
<td>High-affinity ST</td>
</tr>
<tr>
<td>X82456 (S. hamata, SHST2)</td>
<td>46</td>
<td>High-affinity ST</td>
</tr>
</tbody>
</table>

Plant growth and Cd accumulation under the Cd stress

Two-week-old plants of B. napus grown in the presence of 10, 20, 40, 80 and 120 µM Cd$^{2+}$ for 7 d showed a concentration-dependent inhibition of growth (Figure 3A and 3B). A significant decline in root biomass was observed at a 40 µM external concentration of Cd, under which the root dry weight decreased by 46% as compared to the control. Shoot growth appeared to be more sensitive to Cd as a relatively low concentration (20 µM) of Cd lead
Figure 1. Alignment of the predicted amino acid sequences of BnSultr2;2 and AtSultr2;2. Nucleotide sequences are deposited with GenBank under accession number DQ091257 (BnSultr2;2) and D85416 (Arabidopsis AtSultr2;2), respectively. The 12 membrane-spanning domains (1-12) were identified by the boxes. The alignment was performed using the DNAMAN program.

Figure 2. Phylogenetic relationship of plant sulfate transporters. The phylogenetic tree was based on the alignment of protein sequences of sulfate transporters using the Clustal X program. Accession number: *Arabidopsis thaliana* AtSultr1:1, AB018695; AtSultr1:2, AB042322; AtSultr1:3, AB049624; AtSultr2:1, AB003591; AtSultr2:2, D85416; AtSultr3:1, D98631; AtSultr3:2, AB004060; AtSultr3:3, AB023423; AtSultr3:4, AB054645; AtSultr3:5, AB061739; AtSultr4:1, AB008782; AtSultr4:2, AB052775; AtSultr5:1, NP_178147; AtSultr5:2, NP_180139; *Stylosanthes hamata*: SHST1, X82255; SHST2, X82256; *Brassica oleracea*: BoSultr1:1, AJ633707; BoSultr1:2, A633705; BoSultr3:1, AJ581745; BoSultr3:2, AJ601439; BoSultr3:3, AJ704373; BoSultr3:4, AJ704374; BoSultr4:1, AJ633706; BoSultr4:2, AJ555124; *Brassica napus*: BnSultr1:1, AJ416460; BnSultr1:2, AJ311388; BnSultr2:2, DQ091257 (this study), BnSultr4:1, AJ416461; BnSultr5:1, AJ311389; *Brassica juncea*: BjSultr2:2, AJ223495.
to a significant inhibition of leaf growth. Treatment with Cd at 40 µM decreased the shoot growth by 43%. Since 40 µM Cd triggered a moderate effect on the plant growth, this concentration was selected to examine transcriptional and physiological responses of *B. napus* to Cd stress.

Figure 4 illustrates the time-course of Cd accumulation in the root and shoot of seedlings from nutrient solution containing 40 µM Cd. Cd accumulation in roots began immediately after exposure of seedlings to Cd. The accumulation of Cd in root was rapid and nearly linear during the first 12 h of exposure and then slowed down up to experiment end. A maximum of 3634 mg kg⁻¹ Cd accumulated in roots after a 96-h exposure of seedlings to 40 µM Cd. Meanwhile, less Cd accumulated in shoots than in roots. The highest Cd level in shoots was 371.4 mg kg⁻¹.

### Effects of sulfur deficiency on the transcriptional expression of sulfate transporters

To examine the response of *BnSultr2;2* expression to different sulfur conditions, 2-week-old plants grown on the sulfur-sufficient medium, were transferred into the sulfur-deficient solution for 3 days. Withdrawal of external sulfate supply did not affect the level of root *BnSultr2;2* mRNA (Figure 5A). However, significant *BnSultr2;2* mRNA did accumulate in leaves under the sulfur deficiency. The abundance of *BnSultr2;2* mRNA in sulfur-deficient leaves was 8.8 times higher than in sulfur-sufficient leaves. To understand further the regulation of sulfate uptake and accumulation, we simultaneously isolated another cDNA encoding a known high-affinity sulfate transporter *BnSultr1;1* (accession number AJ416460) from roots. In contrast to *BnSultr2;2*, *BnSultr1;1* appeared to be specifically expressed in roots, and its transcript level there increased by 5.2 fold over the control (Figure 5C).

### Effects of Cd on the expression of *BnSultr2;2* and *BnSultr1;1*

To examine whether *BnSultr2;2* and *BnSultr1;1* expressions were regulated by Cd concentration in nutrient medium, 2-week-old plants were grown continuously for 72 h in hydroponic culture containing 0, 20, 40, 80 and 120 µM Cd. In roots, *BnSultr2;2* transcript level declined progressively with increasing concentration of Cd in media (Figure 6A). At the 40 µM external concentration of Cd, the mRNA transcript level of *BnSultr2;2* was only 12% of the control. The *BnSultr2;2* transcript level in leaves increased with increasing Cd concentration in the nutrient solution, and at a 40 µM external concentration of Cd the highest increase in mRNA level was noted. Further increase in Cd concentration resulted in a decreased expression of *BnSultr2;2* in leaves of *B. napus* (Figure 6B). The level of *BnSultr1;1* transcripts in the root of Cd-treated plants was higher than in the control, and the maximum accumulation of *BnSultr1;1* transcripts was observed at 80 µM of Cd in nutrient solution (Figure 6C).

A time-course experiment was also performed to investigate the transcriptional response of *BnSultr2;2* and *BnSultr1;1* to Cd exposure. The root *BnSultr2;2* expression was inhibited during the time-course of Cd exposure (Figure 7A). *BnSultr2;2* expression in leaf showed a different time-course pattern when compared with root (Figure 7B). Only a small amount of *BnSultr2;2* transcript was detected during the first 12 h of Cd treatment. A rapid induction in level of mRNA transcript level was found after 12 h of exposure to Cd. The *BnSultr2;2* expression in leaf peaked 48 h after Cd treat-
After peaking, however, it sharply decreased and returned to the level of control. For \textit{BnSultr}1;1, treatment with 40 μM Cd did not induce its expression during the first 12 h, but a progressive increase in expression was observed thereafter. The highest expression was reached at 72 h (Figure 7C).

**Effects of Cd on the accumulation and sulfate, sulfide, and non-protein thiols**

To relate expression of the sulfate transporters to sulfur accumulation and assimilation, the content of sulfate, sulfide, and non-protein thiols in plant tissues were measured. Treatments of seedlings with Cd at concentrations ranging from 0-120 μM induced a consistent enhancement in sulfate accumulation in roots (Figure 8A). A significant increase in sulfate content was evident at 40 μM. Time-course study demonstrated that roots treated with 40 μM Cd also exhibited an accumulation of sulfate with the time, but significant increases occurred only after 24 h of the treatment with Cd (Figure 8C). By contrast, sulfate accumulation in leaves consistently decreased as evinced by dose-response (Figure 8B) as well as time-course (Figure 8D) study.
Sulfide ($S_2^-$) is one of the important intermediates in the reductive $SO_4^{2-}$ assimilation pathway, in which the incorporation of sulfide into cysteine is the last step (Leustek and Saito, 1999). Thus, the level of sulfide in plants may reflect their capacity for sulfate reduction and assimilation. Our results show that elevating Cd concentrations from 0 to 80 μM in the culture medium resulted in a massive accumulation (12-fold of control) of sulfide in roots (Figure 9A). However, only a little induction was observed in leaves. Because reduced glutathione (GSH) is thought to play an important role in heavy metal detoxification, the concentration of GSH in Cd-treated plants was measured. Cd exposure (40 and 80 μM) significantly increased the concentrations of GSH in roots. Compared with roots, the content in leaves was not obviously affected by Cd (Figure 9B).

The total non-protein thiols were measured because these thiols represent the compounds of monothiols (e.g. cysteine and glutathione) and polythiols (phytochelatin) (Cobbett, 2000). As shown in Figure 10A, the presence of 20 μM Cd was sufficient to stimulate the production of total non-protein thiols in roots. A concentration-dependent change in thiol abundance was observed. The production of total non-protein thiols in Cd-treated roots also gradually elevated over the time (Figure 10C). However, in the present study, Cd exposure did not induce any leaf non-protein thiol accumulation (Figure 10B and D).

**DISCUSSION**

In this study we isolated and partially identified a novel cDNA ($BnSultr2;2$, accession number DQ091257) encoding *Brassica napus* low-affinity sulfate transporter. Sequence comparison of deduced protein of $BnSultr2;2$ with other sulfate transporters—in particular those of *A. thaliana* (Takahashi et al., 1996, 1997), *S. hamata* (Smith et al., 1995), and *B. juncea* (Heiss et al., 1999)—has shown that the cloned putative LAST should be classified as a member of group-2 sulfate transporters. The
expression of BnSultr2;2 in different tissues indicates that it is localized in the root and leaf of B. napus. This result is consistent with the previous observations in A. thaliana, where a cloned low-affinity sulfate transporter was also expressed in roots and leaves (Takahashi et al., 2000). Although we did not make a further identification of the low-affinity sulfate transporter, the low phylogenetic distance between BnSultr2;2 and AtSultr2;2 suggested that they share a similar function.

Regulation of the sulfate transporter activity that is dependent on S-nutritional status has been investigated in a variety of plants (Hawkesford, 2003). Expression of low-affinity sulfate transporter genes in response to sulfur deficiency has been proposed to be behind improvements in S-utilization efficiency within plants (Hawkesford, 2000). However, little is known about the detailed expression pattern of LAST in response to Cd exposure. It has been demonstrated that a gene for a putative low-affinity sulfate transporter from B. juncea exhibited a significantly reduced expression in roots upon Cd treatment (Heiss et al., 1999). We have likewise found that the root BnSultr2;2 expression was progressively down-regulated by Cd exposure (Figure 6A). In A. thaliana, expression of AtSultr2;2 was detected in root phloem and leaf vascular bundle sheath cells, suggesting that the low affinity sulfate transporter AtSultr2;2 was involved in the release of sulfate from xylem vessels to palisade and mesophyll cells (Takahashi et al., 2000). In the present study, the reduced expression of BnSultr2;2 in Cd-treated roots indicates that the B. napus plants might prevent a sulfate transfer from roots to shoots because synthesis of PCs and other low or high-molecular-weight complexes in roots requires sufficient sulfate (Heiss et al., 1999), thus causing more sulfate to be retained in roots for local assimilation. The result could be supported by the increased sulfate content in root (Figure 8). On the other hand, expression of BnSultr2;2 was up-regulated in leaf in response to Cd exposure (Figure 6B), which is consistent with the expression pattern under sulfur starvation (Figure 5B). The pattern of BnSultr2;2 expression above-ground suggests Cd-induced short-term sulfur starvation, and more sulfate uptake into leaf mesophyll cells may be required for sulfur-containing metabolite synthesis for metal chelating.

To get an insight into the expressional pattern of sulfate transporters in response to Cd stress, we simultaneously isolated and analyzed another gene encoding a high affinity sulfate transporter (BnSultr1;1) in B. napus. BnSultr1;1 was expressed only in roots, suggesting that BnSultr1;1 belongs to the Group-1 high-affinity sulfate transporters involved in the uptake of sulfate into roots at low sulfate availability. BnSultr1;1 in roots showed a base level expression (Figure 5C). However, both sulfur starvation and Cd exposure resulted in a strong expression of BnSultr1;1 in roots (Figure 5C and 6C). Sulfate starvation could lead to a decrease of GSH (Leustek and Saito, 1999), and PC synthesis induced by Cd could also lead to a transient depletion of GSH pools in roots (Scheller, 1987). This depletion may be what increases the uptake of sulfate. It was interesting to find a 12-h lag phase between the Cd treatment and the increased BnSultr1;1 expression (Figure 7C). This suggested that before the onset of a considerable expression of BnSultr1;1 could occur, time for cadmium to induce GSH depletion was required.

It has been proposed that both low- and high-affinity sulfate transporters play key roles in sulfate uptake, translocation, and distribution in higher plants (Buchner et al., 2004b; Sun et al., 2005). However, the mechanism, by which Cd influences the sulfur status and modifies the gene expression of both group-1 and group-2 sulfate transporters is not fully understood. To relate Cd-induced alteration in the expression of BnSultr1;1 and BnSultr2;2 to sulfate uptake, accumulation, and assimilation in B. napus, the total sulfate accumulation and other reduced metabolite content was measured. Our results show that the accumulation of sulfate and sulfide in Cd-treated roots increased significantly. Also, the level of glutathione and non-protein thiols in the roots with Cd was found to increase. The observed modifications of sulfate content and increased metabolite production may reflect the local effects of Cd in the organs or the Cd-driven demand for sulfur nutrients for the whole plant. Indeed, the regulatory signals originated from either the Cd-stressed shoot or the treated roots. We noted that the B. napus plants accumulated Cd rapidly upon Cd exposure (Figure 4). The accumulation long preceded the the enhanced expression of BnSultr2;2, the sulfate accumulation rate, and the synthesis of reduced products of sulfur. Taken together, all of these results suggest an inter-organ signal during Cd exposure responsible for sulfate uptake regulation and reduction within different organs.

Acknowledgements. This study was supported by the National Natural Science Foundation of China (No. 30070447)

LITERATURE CITED


鍋脡迫油菜中硫轉運體的協同表達及其與硫代謝物合成之間的關係

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本研究從油菜植株中克隆到一個編碼低親和硫轉運蛋白基因全長 cDNA (BnSultr2;2, accession number Q091257)。氨基酸序列比對發現該基因編碼的蛋白與擬南芥和印度芥菜中已知的低親和硫轉運蛋白具有很高的相似性，並具有 12 個跨膜結構域。轉錄表達顯示，缺硫處理能誘導油菜植株葉片 BnSultr2;2 基因轉錄水準的提高，而 Cd (20-120 μM) 處理也能使葉片中 BnSultr2;2 轉錄表達呈上升趨勢，但根中的表達則明顯下降。為了探明硫轉運蛋白基因的轉錄表達活性與硫代謝的吸收和同化及其與 Cd 耐性之間的關係，我們還從根中分離到了另一個已知高親和硫轉運蛋白基因 BnSultr1;1 (accession number AJ416460)。研究表明，該基因僅在根中表達，而且其表達水準在缺硫和Cd脡迫時均表現為上調。另外，當處理植株 Cd 的濃度為 40 μM 時，根中的 SO₄²⁻、S⁺⁺、GSH、non-protein thiols 含量均上升。上述結果表明油菜植物可能通過調節硫轉運蛋白的表達來促進油菜植株對硫的吸收和同化，從而達到緩解 Cd 毒害的目的。

關鍵詞：油菜；Cd；硫轉運蛋白；轉錄。