Short Communication

Induction of SULTR1;1 Sulfate Transporter in Arabidopsis Roots Involves Protein Phosphorylation/Dephosphorylation Circuit for Transcriptional Regulation

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SULTR1;1 high-affinity sulfate transporter is highly regulated by sulfur deficiency (–S) in the epidermis and cortex of Arabidopsis roots. The regulatory mechanism of SULTR1;1 expression was studied using inhibitors for transcription, translation, protein phosphorylation and dephosphorylation. The induction of SULTR1;1 mRNA during –S was blocked by the addition of actinomycin D in the medium, suggesting that SULTR1;1 is transcriptionally regulated. Cycloheximide repressed the –S induction of SULTR1;1, but enhanced the basal mRNA level of SULTR1;1 under sulfur replete (+S) condition. In addition, the induction of SULTR1;1 by –S was significantly blocked by okadaic acid (OKA) and calyculin A (CalyA). Regulation of SULTR1;1 was further confirmed in transgenic plants expressing green fluorescent protein (GFP) under the control of SULTR1;1 promoter. Accumulation of GFP during –S was dependent to SULTR1;1 promoter, and the effects of OKA and CalyA were reproducible in the SULTR1;1 promoter-GFP plants. These results suggested that the up-regulation of SULTR1;1 by –S requires protein phosphatase as an upstream regulatory factor.

Keywords: Arabidopsis thaliana — Okadaic acid — Sulfate transporter — Sulfur signaling — SULTR1;1 — Transcriptional regulation.

Abbreviations: ActD, actinomycin D; CalyA, calyculin A; CHX, cycloheximide; GFP, green fluorescent protein; RT-PCR, reverse transcription-PCR; SULTR, sulfate transporter; OKA, okadaic acid.

Sulfur is an essential macro-nutrient for plants, and its deficiency significantly affects the growth. Sulfate is the major form of sulfur that plants can utilize in the assimilatory pathways. Sulfate is taken up from the soil, and is primarily metabolized through the reductive sulfur assimilation to form cysteine and methionine that are the essential constituents of proteins (Leustek and Saito 1999, Leustek et al. 2000, Saito...
The mRNA level of SULTR1;2 was induced by –S, but was also abundantly expressed under +S conditions (Yoshimoto et al. 2002). With respect to the regulation of sulfur assimilation, transcription factors controlling the expression of –S responsive genes are identified in yeast and filamentous fungi (Marzluf 1997). The 26S proteasome is suggested to control the stability of the transcription factor involved in the regulation of Met genes in response to sulfur availability in yeast (Kaiser et al. 2000). Sac3, a snf1-like serine/threonine kinase, was isolated from Chlamydomonas as a positive regulator of high-affinity sulfate transport system (Davies et al. 1999). In contrast to these micro-organisms, regulatory mechanisms of +S and –S responses have not been well characterized in higher plants.

Recently, transcriptome analysis of –S response in Arabidopsis revealed numbers of candidate genes for signaling molecules and transcription factors (Nikiforova et al. 2003, Hirai et al. 2003). However, none of these molecules has been reported to play roles in sensing of sulfate levels or in the subsequent signaling pathways. To study these micro-organisms, regulatory mechanisms of +S and –S responses has been reported to play roles in sensing of sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability. In this study, we examined the effects of inhibitors on the expression of SULTR1;1 that is significantly modulated by sulfur availability.

To analyze the mechanisms of the early response of SULTR1;1 expression during –S, the mRNA levels of SULTR1;1 were quantified in the presence of transcription and translation inhibitors. Thirteen-day-old plants grown on agar medium containing 1,500 µM sulfate (Inaba et al. 1994) were transferred to the same medium (HH) or to the medium without sulfate (–S) and incubated for 24 h with or without the inhibitors. SULTR1;1 mRNA was accumulated about 10 times more abundantly in HL than in HH (Fig. 1). The up-regulation of SULTR1;1 in HL was completely blocked by the addition of actinomycin D (ActD) in the medium, suggesting that SULTR1;1 is controlled at the level of transcription under –S. The HL to HH ratio of SULTR1;1 mRNA was 0.36 in the presence of CHX (Fig. 1). These results suggested that de novo protein synthesis is required both for the up-regulation and down-regulation of SULTR1;1 in HL and HH, respectively.

The results of ActD treatment indicated transcriptional regulation of SULTR1;1 under –S (Fig. 1). It is suggested that SULTR1;1 promoter may play a regulatory role for the expression of SULTR1;1 mRNA. Regulation of SULTR1;1 gene expression was further examined using transgenic Arabidopsis plants expressing SULTR1;1 promoter-GFP fusion constructs. The SULTR1;1 promoter-GFP plants previously constructed with the −1,944 bp 5′-region exhibited specific expression of GFP in root hairs, root epidermis and cortex (Takahashi et al. 2000). However, the −1,944 bp promoter region was not sufficient to provide –S responsiveness of SULTR1;1 in transgenic plants (data not shown). To solve this problem, we made a new fusion construct with −3,031 bp promoter region that confers both the cell-type specificity and –S inducibilities of SULTR1;1 (Fig. 2). Transgenic plants were grown on 1,500 µM sulfate (+S) or 15 µM sulfate (–S) medium and accumulation of GFP was observed under an image analyzer (Fig. 2A). Accumulation of GFP was increased by –S in six independent transgenic lines (Fig. 2A). As has been indicated previously (Takahashi et al. 2000), GFP driven by the SULTR1;1 promoter was primarily expressed in root tissues (Fig. 2B), and was detected in the

Fig. 2 –S responsive induction of GFP expression in SULTR1;1 promoter-GFP transgenic Arabidopsis. Transgenic plants were constructed as follows. SULTR1;1 promoter fragment that starts from the position −3,031 and terminates at the translation initiation codon was amplified from genomic DNA of Arabidopsis by PCR using Pfu turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.), and was fused with the GFP coding sequence. Primers used for the PCR amplification are as follows: P3031F (5′-GGTGTCGACATGTAAAACGATATCAAACTAAACTAC-3′), PR (5′-CCATGGGACTATGTAACCTTGCAAGACAGAGGG-3′). The PCR amplified Salt–NcoI fragment of the SULTR1;1 promoter region, and the NcoI–EcoRI fragment containing the GFP coding sequence (Chiu et al. 1996) and the NOS terminator, were cloned in the Salt–EcoRI sites of pBI101 (Clontech, Palo Alto, CA, U.S.A.). The resultant binary plasmids were transferred to Agrobacterium tumefaciens GV3101 (pMP90) (Koncz and Schell 1986) and used for transformation of Arabidopsis plants according to the floral dip method (Clough and Bent 1998). (A) GFP fluorescence and vascular formation of the major veins of transgenic lines. Plants were grown for 11 d on agar medium containing 1,500 µM of sulfate (+S) or 15 µM of sulfate (–S). Expression of GFP was visualized using an image analyzer Fluorimag R950 with a S90DF30 filter that provides the emission spectra at 515–545 nm under 488 nm excitation (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Auto fluorescence of plants was detected in the same measurement with a 610RG filter that provides the emission spectra at 610 nm. (B) Emission of GFP from plant roots (Line 8). +S and –S corresponds to the conditions in (A). (C) Cell type-specific localization of GFP. Basal (a, b) and apical (c, d) parts of roots from the plants grown under +S (a, c) or –S (b, d) were observed under a Fluoview 500 confocal laser scanning microscopy (Olympus, Tokyo, Japan) equipped with a 505–525 nm band-pass filter. Panels, a, b, c, d, correspond to the regions displayed in (B). (D) GFP accumulation in the transgenic plants. Emission of GFP from 16 plantlets in each well in (E) was quantified, and compared between –S and +S. Ratios of the fluorescent intensities between –S and +S are shown above the columns. (E) mRNA levels of SULTR1;1 and GFP in the transgenic plants. The gene-specific primers for SULTR1;1 (Fig. 1) and for GFP (204F 5′-AGTGCTTCAGCCGCTTACCC-3′ and 345R 5′-CCCTGCAGCCTTCACCTCGGG-3′) were used for the analysis. Equality of RNA preparation was confirmed by constitutive expression of ubiquitin (UBQ2, accession no. J05508). Ratios of mRNA levels between –S and +S are shown above the columns.
epidermis, cortex and root hairs, and was preferentially expressed in the cortex of the mature parts of roots (Fig. 2C). The abundance of GFP was 8.3-fold higher in –S than in +S (Fig. 2D). The mRNA levels of SULTR1;1 and GFP were both increased by –S, exhibiting 10.5- and 11.5-fold higher expression in –S than in +S, respectively (Fig. 2E). We got similar results with luciferase as a reporter gene (data not shown).

These results clearly indicated that induction of SULTR1;1 mRNA under –S is regulated in a promoter dependent manner.

Protein phosphorylation and dephosphorylation play crucial roles in various aspects of plant life (Smith and Walker 1996, Luan 1998). To determine whether protein phosphorylation is involved in the regulatory circuit of SULTR1;1 expression, the effects of inhibitors for protein kinase and phosphatase were analyzed under different environmental sulfur conditions (Fig. 3). Thirteen-day-old plants were treated with the inhibitors for 24 h in the presence or absence of sulfate in the medium. The induction of SULTR1;1 mRNA during sulfur starvation (HL) was blocked by protein phosphatase inhibitors, calyculin A (CalyA) and okadaic acid (OKA), whereas the addition of the inhibitors for protein kinase (K252a, staurosporine) exhibited no effects (Fig. 3A). The HL to HH ratios of the SULTR1;1 mRNA levels were markedly decreased by the CalyA and OKA treatments; however, those with K252a and staurosporine were not changed significantly (Fig. 3A). The inhibitory effect of OKA on SULTR1;1 mRNA expression became significant from 0.05 µM and was completed over 0.3 µM (Fig. 3B). To determine whether OKA is able to suppress SULTR1;1 from the beginning of mRNA induction, fluctuations of mRNA levels were monitored during a –S time course with or without OKA (Fig. 3C). Plants were treated with OKA at the lowest limit (0.1 µM), and the SULTR1;1 mRNA levels were quantified. During –S, the increment of SULTR1;1 mRNA levels in the control plants became significant between 4 and 8 h after the onset of the –S treatment. The inhibitory effect of OKA was clearly observed from these early phases of SULTR1;1 induction (Fig. 3C). The results indicated that the presence of 0.1 µM of OKA in the medium is sufficient to down-regulate the –S response of SULTR1;1 from the early stage of mRNA induction. The dose dependency and the time course of the regulation of SULTR1;1 by OKA were similar to those observed for other genes regulated by protein phosphatases (Kuo et al. 1996, Rojo et al. 1998, Hirose and Yamaya 1999). The results presented here strongly suggest that induction of SULTR1;1 mRNA under –S in Arabidopsis roots may require the action of an OKA-sensitive protein phosphatase as a regulatory factor.

Fig. 3 Effects of protein kinase and phosphatase inhibitors on mRNA accumulation of SULTR1;1. (A) Plant roots were harvested after 24 h of treatments. One µM of K252a and staurosporine (Stauro) were used as a protein kinase inhibitor, and 0.5 µM of calyculin A (CalyA) and okadaic acid (OKA) were used as protein phosphatase inhibitors. K252a was purchased from Calbiochem (Darmstadt, Germany). Staurosporine, CalyA and OKA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ratios between HL and HH are shown above the columns. Bars = SDs (n = 3). The conditions of HH and HL were described in the legends of Fig. 1. (B) Dose dependency of the inhibition of SULTR1;1 mRNA accumulation by OKA. Bars = SDs (n = 3). (C) Time course inhibition of SULTR1;1 mRNA by 0.1 µM OKA. Bars = SDs (n = 3). The mRNA levels were quantified as described in Fig. 1.
To further examine the effects of protein kinase and phosphatase inhibitors on SULTR1;1, accumulation of GFP in the SULTR1;1 promoter-GFP plants was quantified in the presence of inhibitors in the medium (Fig. 4). The induction of GFP became significant 2 d after transfer to the –S medium (Fig. 4, HL). A three-fold increase in GFP levels was observed in HL in all four independent lines of transformants. The inhibitor treatments were performed for 2 d taking degradation of inhibitors into account. The induction of GFP by HL was blocked in the presence of protein phosphatase inhibitors, OKA and CalyA. In contrast, the protein kinase inhibitors, K252a and staurosporine, did not affect the accumulation of GFP under –S. The results were consistent with the regulation of SULTR1;1 RNA (Fig. 3A). It is suggested that the up-regulation of SULTR1;1 by –S requires protein phosphatase as a key factor in the upstream regulatory circuit. OKA and CalyA are known as potent inhibitors of type 1 and type 2A protein phosphatase, and have been used to identify the roles of reversible protein phosphorylation in various processes in plant tissues (Kuo et al. 1996, Smith and Walker 1996, Luan 1998, Hirose and Yamaya 1999). Induction of SULTR1;1 by –S was inhibited both by OKA and CalyA to a same extent (Fig. 3A). OKA preferentially inhibits type 2A protein phosphatase, whereas CalyA is 10- to 100-fold more active than OKA for the inhibition of type 1 protein phosphatase (Cohen et al. 1990). The spectrum differences between OKA and CalyA suggest that type 2A protein phosphatase may potentially regulate SULTR1;1 in Arabidopsis.

A Ser/Thr kinase is involved in the regulation of arylsulfatase and sulfate transport system in Chlamydomonas (Davies et al. 1999); however, the inhibitors of protein kinase did not show significant effects on the expression of SULTR1;1 in Arabidopsis (Fig. 3, 4). The 26S proteasome plays important roles in the regulatory circuit of –S responsive genes in yeast and filamentous fungi (Kaiser et al. 2000, Marzluf 1997). The inhibitors of 26S proteasome (MG-132 and clasto-lactacystin-β-lactone) showed no effects on the level of SULTR1;1 mRNA (data not shown). Ineffectiveness of these inhibitors suggested that higher plants may have unique signal transduction pathways for the regulation of sulfate transporter.

In higher plants, in addition to SULTR1;1, the promoter sequences of NIT3 nitrilase (Kutz et al. 2002) and β-subunit gene of β-conglycinin (Hirai et al. 1995, Awazuhara et al. 2002) have been reported to be responsive to –S. NIT3 encodes nitrilase that catalyzes the conversion of indole-3-acetonitrile to indole-3-acetic acid. It is suggested that induction of NIT3 is related to the degradation of glucosinolates that allows recycling of sulfur under –S. β-Conglycinin is a soybean seed storage protein specifically expressed under –S (Naito et al. 1994). Expression of this subunit substitutes the S-rich storage proteins under –S and reduces the cost of losing sulfur from the primary metabolism. Apparently, induction of NIT3 and β-conglycinin is part of the adaptive response during prolonged –S stress. Compared to the roles of these two genes, the initial uptake of sulfate carried out by SULTR1;1 sulfate transporter may affect the rate of primary sulfur assimilation. Presumably, plants require acute response of SULTR1;1 for acquisition of sulfate. These facts suggest the importance of quick induction of SULTR1;1 during the early stage of adaptation to the –S environment. The –S responsive regions of SULTR1;1, NIT3 and β-conglycinin promoters contained numerous conserved

Fig. 4  Effects of protein kinase and phosphatase inhibitors on GFP accumulation in SULTR1;1 promoter-GFP plants. Nine-day-old plants grown on agar medium containing 1,500 µM sulfate were transferred to the medium containing the same amount of sulfate (1,500 µM, HH) or no sulfate (HL), with or without protein kinase and phosphatase inhibitors. The concentration of each inhibitor was same with the experiment in Fig. 3A. Two d after the transfer, accumulation of GFP in the roots was visualized and quantified as described in Fig. 2. Four independent lines were used for the analysis. Bars = SDs (n = 12). Averages of the ratios between HL and HH are shown above the columns.
motifs for the putative cis-acting elements. Further studies of the promoter sequences may define the machineries involved in the –S response, and may clarify the differences between these three –S responsive genes.

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References


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