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Invited Review

Transcriptional regulation of genes involved in sulfur assimilation in plants: Understanding from the analysis of high-affinity sulfate transporters

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Abstract Sulfur is one of the essential macronutrients required for plant growth. Since the expression of several sulfur-assimilatory genes is stimulated under the condition of sulfur deficiency (−S), transcriptional regulation of these genes is considered to be critical for the control of sulfur assimilation. In the last several years, the author and coworkers have been investigated molecular mechanisms of −S-inducible expression of high-affinity sulfate transporters, *SULTR1;1* and *SULTR1;2* in *Arabidopsis thaliana*. SULTR1;1 and SULTR1;2 facilitate sulfate uptake in roots. This review summarizes the recent progress about the transcriptional regulation of these sulfate transporters by focusing on three major topics. 1) Identification of a *cis*-acting element involved in the −S-inducible expression of *SULTR1;1*. 2) Cytokinin-dependent repression of *SULTR* expression and sulfate uptake. 3) Central transcription regulator SLIM1 controlling −S responsive gene expression including sulfur assimilation and metabolism.

Key words: *Arabidopsis thaliana*, Glucosinolate, Sulfur assimilation, Sulfate transporter, SULTR1;1, SULTR1;2, SLIM1.

Sulfur is one of the essential macronutrients required for plants. Plants use sulfate as the major sulfur source and synthesize sulfur-containing amino acids cysteine and methionine (Crawford et al. 2000; Leustek et al. 2000; Saito 2004). Animals, including humans, require sulfur-containing amino acids, mostly methionine, and proteins as dietary sulfur sources because of their inability to assimilate sulfate. Thus the plant ability to assimilate sulfate is essential for the global sulfur cycle in nature. In addition to amino acids and proteins, plants synthesize a variety of metabolites, which critical for biological processes and beneficial for humans (Crawford et al. 2000; Leustek et al. 2000; Saito 2004). Considering the essential roles of sulfur assimilation for both plant growth and quality, understanding the regulatory systems of plant sulfur assimilation is important for improvement of sulfur utilization capacity and nutritional values of plants. Improvement of sulfur utilization capacity would also contribute to plant remediation of excess sulfate found in particular environments.

In 1990's, enzymes and sulfate transporters involved in the sulfur assimilatory pathway were identified by the efforts of many researchers (Leustek et al. 2000; Saito 2004). Sulfur assimilation starts from the uptake of external sulfate by the activity of sulfate transporters in roots. Sulfate taken up by plant roots are activated by ATP sulfulyrase and then reduced by two-step reactions catalyzed by APS reductase (APR) and sulfite reductase to produce sulfide. Then with cysteine synthase, sulfide reacts with *O*-acetyl-L-serine and turned into cysteine, the first organic form of sulfur in the plant sulfur assimilatory pathway. Glutathione (GSH), methionine and many kinds of sulfur containing compounds are produced from cysteine.

During the process of enzyme gene hunting for the enzymes in the sulfur assimilatory pathway in late 1990's, Takahashi et al. (1997) found that mRNA levels of several sulfur assimilatory genes, sulfate transporter (SULTR), APR and serine acetyl transferase, were up-regulated by sulfur deficiency (−S) in *A. thaliana*. In 2000's transcriptional regulation of sulfur assimilatory pathway responding to sulfur starvation has been extensively studied with the combination of omics-based approach (Hirai et al. 2003, 2004, 2005; Maruyama-Nakashita et al. 2003, 2005, 2006; Nikiforova et al. 2003) and the dissection of molecular machinaries

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Abbreviations: APR, APS reductase; GFP, green fluorescent protein; GSH, glutathione; SLIM1, sulfur limitation1; SULTR, sulfate transporter; GSL, glucosinolate
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underlying −S response of each gene responsible to −S, such as APR, β-subunit of β-conglycinin (a seed storage protein of soybean), NIT3 and SULTR (Awazuhara et al. 2002; Koprivova et al. 2000; Kutz et al. 2002, Maruyama-Nakashita et al. 2004ab; 2005; 2006; Ohkama et al. 2002). Recent transcriptomics studies suggested that activation of sulfate assimilation and repression of glucosinolate (GSL) production may occur in parallel in response to −S (Hirai et al. 2003; 2004; 2005; Maruyama-Nakashita et al. 2003; 2005; 2006; Nikiforova et al. 2003). The entire network of sulfur metabolism is coordinately regulated under −S. This review focuses on the recent progress of transcriptional regulation of sulfur assimilation, which has been lead by the study on high affinity sulfate transporters in *A. thaliana*.

Identification of a *cis*-acting element involved in the −S-inducible expression of high affinity sulfate transporter, *SULTR1;1*

The entire process of uptake, distribution and remobilization of sulfate has gradually been clarified by the recent reverse genetic approaches about SULTRs (Takahashi et al. 2006). Among the 12 members of SULTR in *A. thaliana*, two high-affinity sulfate transporters, SULTR1;1 and SULTR1;2, facilitate the initial uptake of sulfate in roots (Takahashi et al. 2000; Vidmar et al. 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002). They are expressed in the epidermis and cortex of roots, and their transcripts accumulation is elevated under −S conditions to maximize sulfate uptake for efficient utilization of limited amount of sulfate in the soil (Takahashi et al. 2000; Vidmar et al. 2000; Shibagaki et al. 2002; Yoshimoto et al. 2002).

Inhibitor studies demonstrated that −S response of *SULTR1;1* is controlled at the level of transcription and requires protein phosphatase activity (Figure 1; Maruyama-Nakashita et al. 2004a). It was likely that a specific sulfur-responsive *cis*-element was present in the *SULTR1;1* promoter and elucidation of the element is important for understanding of the molecular mechanism of −S responsive gene expression. The promoter region of *SULTR1;1* was dissected for deletion and gain-of-function analysis using a firefly luciferase gene as a reporter in transgenic *A. thaliana*. The 16-bp sulfur-responsive element from −2777 to −2762 of *SULTR1;1* promoter was identified as a sufficient and necessary *cis*-acting element for the −S-responsive expression (Maruyama-Nakashita et al. 2005). The element was also found sufficient for repression by application of cysteine or glutathione (GSH). Base substitution analysis further identified the core sequence GGAGACA (Figure 1). This core sequence and its homologous sequence were found in the several −S-inducible gene promoters, including

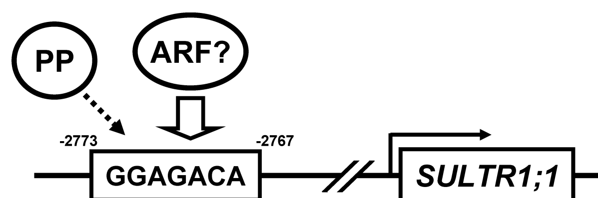

Figure 1. −S inducible expression of SULTR1;1
PP: protein phosphatase, ARF: auxin response factor

the −S responsive region of *NIT3* (Kutz et al. 2002) and the β-subunit gene promoter of β-conglycinin (Awazuhara et al. 2002), suggesting that a common regulatory mechanism induces the expression of a gene set required for adaptation to the −S conditions.

−S responsive *cis*-acting element of *SULTR1;1* contains an auxin response factor (ARF) binding sequence (GAGACA). However, *cis*-acting element of *SULTR1;1* was not responsive to exogenous auxin. There are some reports suggesting that increase of auxin in sulfur-starved plant roots mediate the signals for the regulation of −S-responsive genes (Kutz et al. 2002; Nikiforova et al. 2003). Connection between −S and auxin signal is still controversial, but the identification of ARF-like transcription factor could bind to the *cis*-acting element and induce −S-responsive expression of *SULTR1;1* may explain the relationship.

Cytokinin-dependent repression of *SULTR* expression and sulfate uptake

To setup a simple and traceable system for detection of SULTR expression, a GFP reporter system that generally displays the expression of *SULTR1;2* was constructed (Figure 2; Maruyama-Nakashita et al. 2004b). In the $P_{SULTR1;2}$-*GFP* plants, carrying a −2160 bp fragment of the 5′-region of *SULTR1;2* fused to the GFP coding sequence, GFP accumulation showed typical sulfur responses that correlate with the changes in *SULTR1;2* mRNA levels; accumulation of GFP was induced by −S, but was repressed in the presence of reduced sulfur compounds, cysteine and GSH (Figure 2). The significant correlation between the GFP accumulation and the *SUTR1;2* expression ensured the authenticity of GFP expression in $P_{SULTR1;2}$-*GFP* plants for monitoring promoter-dependent regulation of *SULTR1;2* (Figure 2).

By monitoring *SULTR1;2* expression using $P_{SULTR1;2}$-*GFP*, it was demonstrated that cytokinin negatively regulates sulfate uptake in roots (Figure 3A; Maruyama-Nakashita et al. 2004b). Exogenous application of cytokinin repressed the uptake of sulfate, accompanied with a drastic reduction of *SULTR1;1* and *SULTR1;2* mRNA contents in roots (Figure 3B, C). The effect of cytokinin on sulfate uptake was less prominent in the *cre1-1* mutant (Figure 3C), lacking the CRE1/WOL/

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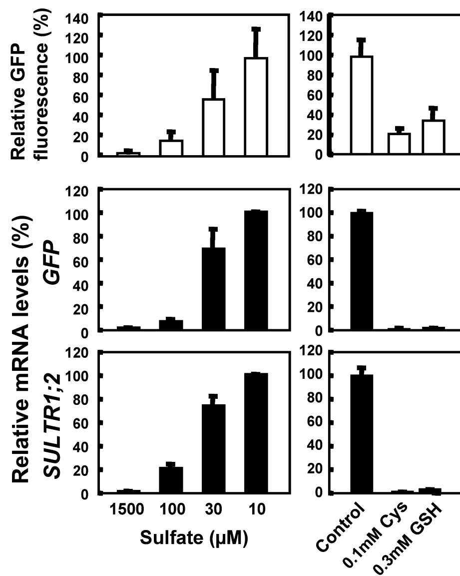

Figure 2. $P_{SULTR1;2}$-*GFP* transgenic plants can trace sulfur dependent regulation of SULTR1;2 Relative quantification of GFP fluorescent intensities and internal *SULTR1;2* and *GFP* expression in the root of $P_{SULTR1;2}$-*GFP* plants. Plants were visualized using FluorImager 595 and the GFP fluorescence was quantified using Image Quant software. The mRNA contents were calculated using ubiquitin as an internal standard. Left: Plants were grown for 11 days on the agar medium containing 1500, 100, 30 and 10 μM of sulfate, respectively. The relative values of GFP and mRNA levels indicate comparisons with those determined under 10 μM sulfate. Right: Effects of cysteine and GSH. Plants were grown for 9 days on −S agar medium (10 μM sulfate), and then incubated for 2 days on the −S medium (Control) or on the −S medium containing 0.1 mM Cysteine or 0.3 mM GSH. The relative values of GFP and mRNA levels indicate comparisons with those determined under −S condition (Control).

AHK4 cytokinin receptor (Inoue et al. 2001). However, −S inducibility of sulfate uptake is not altered by cytokinin or in the *cre1-1* mutant (Figure 3C; Maruyama-Nakashita et al. 2004b), suggesting that the cytokinin-dependent repression of sulfate uptake is independent from the sulfur-responsive regulation.

The repression of nutrient uptake in roots by cytokinin application has also been reported for the phosphate transporter, PT1 (Martin et al. 2000; Karthikeyan et al. 2002; Franco-Zorrilla et al. 2002), and for the nitrate uptake of *Fagus sylvatica* (Collier et al. 2003). Cytokinin and its receptor CRE1/WOL/AHK4 possibly play an essential role in regulation of nutrient uptake in general. The downstream components in cytokinin signal transduction, which directly regulate nutrient uptake, i.e. transcription factors, remains to be determined.

Central transcription regulator SLIM1 controlling −S responsive gene expression including sulfur assimilation and metabolism

The finding of the *cis*-regulatory element is indicative of the existence of *trans*-acting regulatory proteins for the induction of transcripts for high-affinity sulfate transporter. Recently, we identified SLIM1 transcription factor as the key regulatory proteins controlling the upstream signaling cascades of sulfur metabolism through a forward genetics approach (Maruyama-Nakashita et al. 2006). From the ethyl-methanesulfonate-mutagenized population of $P_{SULTR1;2}$-*GFP* plants, seedlings showing reduced levels of GFP were screened under −S conditions. *sulfur limitation 1*, *slim1*, was obtained as a family of allelic mutants with recessive, single-gene segregations. The *slim1* mutants mostly lacked the −S induced GFP accumulation and endogenous *SULTR1;2* expression observed in the parental plants (Figure 4A). In addition, high-affinity sulfate uptake and the plant growth were reduced in the *slim1* mutants under the −S condition (Figure 4B). Transcriptome and metabolite analysis revealed that SLIM1 coordinately controlled activation of sulfur assimilation and degradation of glucosinolates (GSL) and repression of GSL synthesis (Figure 4C; Grubb and Abel 2006; Halkier and Gershenzon 2006).

Mutations causing the −S-response-less phenotypes of *slim1* mutants were identified in a putative EIL-family transcription factor, *ETHYLENE-INSENSITIVE3-LIKE3* (*EIL3*). The −S-response-less phenotype of *slim1* mutants could be restored by the expression of *A. thaliana* SLIM1 and its rice homologues, but not by other EIL proteins including EIN3 (Chao et al. 1997; Guo and Ecker 2004), suggesting the uniqueness of the SLIM1/EIL3 sub-group members in EIL family as sulfur response regulators.

SLIM1 was the first transcription factor identified as the regulatory protein that affects the whole sulfur assimilatory pathway in higher plants. Findings obtained from the SLIM1 analysis will lead to in-depth analysis of the downstream regulatory elements and further facilitate general improvement of sulfur use efficiencies and engineering of beneficial GSL in cruciferous plants.

Concluding remarks

This review documented the recent findings about transcriptional regulation of the high affinity sulfate transporters and sulfur assimilatory pathway in *A. thaliana* (Figure 5). Identification of SLIM1 working upstream of −S signal transduction pathway and the *cis*-acting element which directly controls the −S responsive expression of high affinity sulfate transporter may allow

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Figure 3. Cytokinin repressed *SULTR* expression and sulfate uptake. (A) GFP accumulation in $P_{SULTR1;2}$-*GFP* plants is down-regulated by cytokinin. $P_{SULTR1;2}$-*GFP* plants were grown for 7 days on −S agar medium (10 μM sulfate), and then incubated for 2 days on the control −S medium (−Zeatin) or on the −S medium containing 10^{-6} M of *t*-zeatin (+Zeatin). (B) Down-regulation of high-affinity sulfate transporters by cytokinin. Wild type plants (Ws) were grown for 8 days on +S agar medium (1500 μM sulfate), and then incubated for 2 days on the +S medium containing 10^{-7} M *t*-zeatin (+Zeatin) or on the control +S medium (−Zeatin). The mRNA contents of *SULTR1;2* and *SULTR1;1* in roots were determined. The mRNA contents were calculated using ubiquitin as an internal standard. (C) Down-regulation of sulfate uptake by cytokinin is moderated in the *cre1-1* mutant. *Ler* and *cre1-1* plants (n=8) were grown for 8 days on +S agar medium (1500 μM sulfate), and then incubated for 2 days on the +S media containing 10^{-7} M t-zeatin (+Zeatin) or on the +S control medium (−Zeatin). P-values of the comparisons of *t*-zeatin-treated *Ler* (black bars) and *t*-zeatin-treated *cre1-1* plants (shaded bars) are indicated.

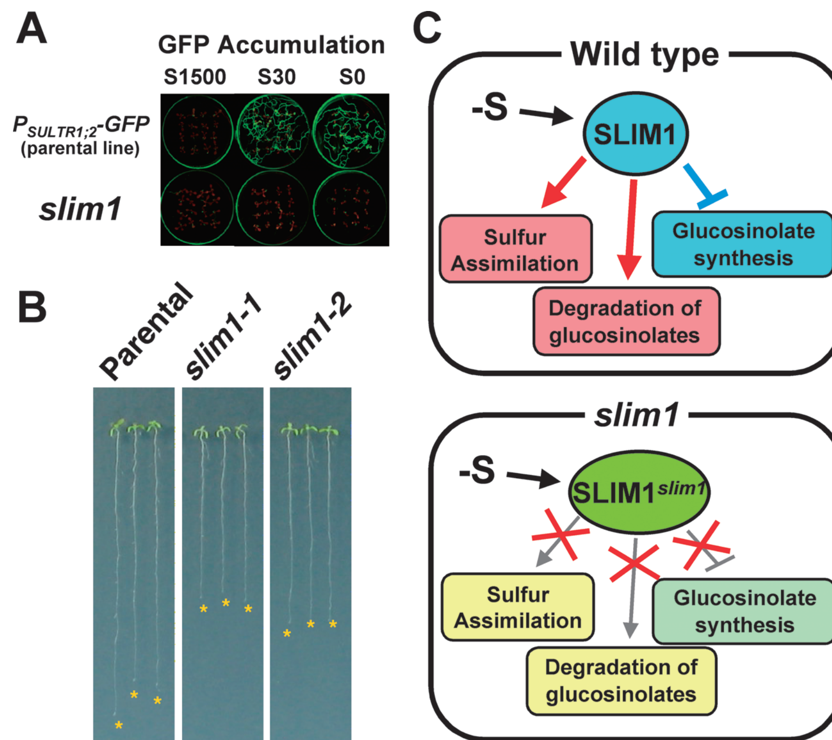

Figure 4. SLIM1 controls plant growth and gene expression of S-assimilatory pathway and GSL metabolism. (A) *slim1* mutant unable to induce *SULTR1;2* gene expression under −S. $P_{SULTR1;2}$-*GFP* and *slim1* mutant were grown for 11 days with 1500 or 30 μM of sulfate (S1500 and S30), or with no sulfate (S0). GFP fluorescence was visualized with FluorImager 595. (B) Root elongation of parental plants ($P_{SULTR1;2}$-*GFP*) and *slim1* mutants under −S. Plants were vertically grown for 11 days on S0 agar medium. Asterisks indicate the positions of root tips. (C) The schematic presentation of the metabolic process regulated by SLIM1.

us to take the reverse genetics approach to identify the regulatory components working between them, e.g. transcription factor that directly controls sulfate uptake or a molecule that may control GSL synthsesis and degradation. The SLIM1 localization was in the vascular tissues, similary to the case of CRE1/WOL/AHK4 existing in the procambium and pericycle in roots (Mähönen et al. 2000), contrary to the fact that the nutrient uptake is conducted at the root surface. This apparent contradiction of the localization of regulators

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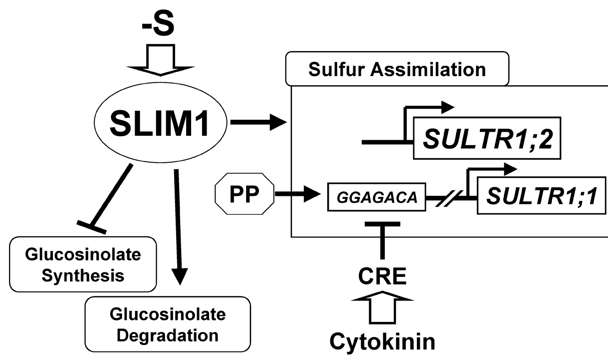

Figure 5. Transcriptional control of sulfur assimilation and metabolism responding to −S

and downstream transporters might indicate the importance of the inter-organ signaling in the control of sulfate uptake as previously described by Lappartient et al. (1999), and poses an interesting question concerning cell-to-cell communication of the regulatory signals. Moreover, research on upstream signals that regulate SLIM1 function is needed to further understand how plants sense environmental and internal sulfur status and control sulfur metabolism of themselves. I believe that these basic researches will contribute to improve crop value and plant use in phytoremediation.

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