Plant Biotechnology 34, 51–55 (2017)
DOI: 10.5511/plantbiotechnology.16.1226a

Note

5′-non-transcribed flanking region and 5′-untranslated region play distinctive roles in sulfur deficiency induced expression of *SULFATE TRANSPORTER 1;2* in *Arabidopsis* roots

Akiko Maruyama-Nakashita[1,2,*], Akiko Suyama[1], Hideki Takahashi[2,3]

[1] Graduate School of Agricultural Science, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan; [2] RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan; [3] Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA
* E-mail: amaru@agr.kyushu-u.ac.jp Tel & Fax: +81-92-642-2848

Received August 30, 2016; accepted December 26, 2016 (Edited by Y. Chiba)

Abstract Plants increase sulfate uptake activity under sulfur deficiency (−S). In *Arabidopsis*, *SULTR1;2* is the major high-affinity sulfate transporter induced in epidermis and cortex of roots for mediating sulfate uptake under −S. Though it is known that transcript levels of *SULTR1;2* increase under −S largely due to the function of 5′-upstream region, contributions of 5′-non-transcribed flanking region and 5′-untranslated region (UTR) to transcriptional and post-transcriptional regulations have not yet been individually verified. To investigate the roles of 5′UTR of *SULTR1;2* in −S responses, transcript levels and activities of firefly luciferase (Luc) were analyzed in transgenic plants expressing *Luc* under the control of the 2,160-bp long 5′-upstream region of *SULTR1;2* with (PL2160) or without (PL2160ΔUTR) the 154-bp 5′UTR. Both transgenic plants expressed similar levels of *Luc* mRNAs that showed significant accumulations under −S relative to +S regardless of presence of the 5′UTR. In contrast, Luc activities were detected only in PL2160 plants, suggesting presence of 5′UTR of *SULTR1;2* being necessary for translational initiation while its absence impairing translation of functional Luc protein in PL2160ΔUTR. These results indicate an essential role of the 5′-non-transcribed flanking region of *SULTR1;2* at positions −2160 to −155 in −S-responsive transcriptional regulation.

Key words: sulfate transporter, SULTR1;2, −S-inducible expression, translation, 5′UTR.

Sulfur is an essential macronutrient for all organisms. It is taken up by plants as sulfate, which is activated, reduced, and assimilated into an amino acid cysteine. Following cysteine biosynthesis, a wide variety of sulfur-containing compounds, such as glutathione, methionine, proteins, lipids, coenzymes, vitamins, and various secondary metabolites are synthesized in plants (Leustek et al. 2000; Saito 2004; Takahashi et al. 2011). Thus, sulfur in these essential compounds derives from sulfate which is taken up from the soil environment through the function of plasma membrane-localizing sulfate transporters.

The initial uptake of sulfate is facilitated by two high-affinity sulfate transporters, SULTR1;1 and SULTR1;2, expressed in epidermis and cortex of roots in *Arabidopsis* (Maruyama-Nakashita et al. 2003; Shibagaki et al. 2002; Takahashi et al. 2000; Vidmar et al. 2000; Yoshimoto et al. 2002, 2007). Sulfate uptake activity in plants is enhanced by sulfur deprivations concomitantly with increase in transcript and protein levels of *SULTR1;1* and *SULTR1;2* (Maruyama-Nakashita et al. 2003; Shibagaki et al. 2002; Takahashi et al. 2000; Vidmar et al. 2000; Yoshimoto et al. 2002, 2007). In both sulfur sufficient (+S) and deficient (−S) conditions, the transcript levels of *SULTR1;2* are higher than those of *SULTR1;1* (Maruyama-Nakashita et al. 2003; Rouached et al. 2008; Shibagaki et al. 2002; Yoshimoto et al. 2002, 2007). In addition, the growth phenotypes and sulfate uptake activity as well as the sulfate, cysteine, and GSH levels of knockout lines deficient in *SULTR1;1* and *SULTR1;2* indicate that SULTR1;2 is the main contributor determining sulfate uptake capacity of Arabidopsis roots under both +S and −S conditions (Maruyama-Nakashita et al. 2003; Shibagaki et al. 2002; Yoshimoto et al. 2002, 2007). The −S-induced expression of *SULTR1;1* and *SULTR1;2* depends on the promoter activities of their 5′-upstream regions (Maruyama-Nakashita et al. 2004a, 2004b). Further studies have indicated that both SULTR1;1 and SULTR1;2 are controlled by the activity of a transcription factor, SLIM1, which coordinates

Abbreviations: Luc, firefly luciferase; SULTR, sulfate transporter; UTR, untranslated region.
This article can be found at http://www.jspcmb.jp/
Published online March 8, 2017

the expression of a wide range of −S-responsive genes in *Arabidopsis* (Maruyama-Nakashita et al. 2006). However, the *cis*-acting elements in 5′-upstream regions responding to sulfate availabilities appear to be different between *SULTR1;1* and *SULTR1;2*. A putative auxin response factor binding sequence, SURE11, is present in the *SULTR1;1* promoter region to control its sulfur response, while the identical sequences have not been identified in the *SULTR1;2* promoter region (Maruyama-Nakashita et al. 2005). These previous findings implicate the importance of transcriptional regulation of *SULTR1;1* and *SULTR1;2*, although the regulatory pathways may involve slightly different mechanisms. In addition to regulation at mRNA levels, yet unknown post-transcriptional mechanisms can be essential for the maintenance of SULTR1;1 and SULTR1;2 protein abundance under −S (Yoshimoto et al. 2007). Thus, multiple mechanisms are involved in regulation of sulfate uptake systems in roots in order to obtain adequate amount of sulfate under −S conditions.

We previously reported that transgenic plants expressing GFP under the control of a 2160-bp 5′-upstream region of *SULTR1;2* accumulated GFP concomitantly with an increase in endogenous *SULTR1;2* mRNA under −S (Maruyama-Nakashita et al. 2004b). The 2160-bp 5′-upstream region used in our previous study, however, includes the 5′UTR of *SULTR1;2* flanking 154-bp upstream of the translational start codon according to the sequences deposited in the TAIR database (http://www.arabidopsis.org; Shibagaki et al. 2002; Yoshimoto et al. 2002). Several studies provide evidence that presence of 5′UTR can substantially contribute to regulation of mRNA stability and translational efficiency in plants (Bailey-Serres and Dawe 1996; Gutierrez et al. 1999; Hulzink et al. 2002; Kawaguchi and Bailey-Serres 2002; Mardanova et al. 2008). In addition, the existence of two splicing variants in *SULTR1;2* cDNA, which comprised of 60 bp 5′UTR containing one splicing site between −107 and −12 bp (At1g78000.1) and with 55 bp 5′UTR (At1g78000.2) without splicing site (http://www.arabidopsis.org; Figure 1), seems to be suggestive for the regulatory role of 5′UTR. To verify the function of 5′UTR in transcriptional and post-transcriptional regulations of *SULTR1;2* in response to sulfate availabilities, we analyzed the transcript levels and the activities of firefly luciferase (Luc) reporter in transgenic plants expressing the *Luc* gene under the control of the 5′upstream region of *SULTR1;2* with or without the 5′UTR.

The chimeric gene constructs named PL2160 and PL2160ΔUTR were designed to contain the 2,160 bp 5′-upstream region of *SULTR1;2* or the same region with a deletion of the 154-bp 5′UTR respectively fused to the coding sequence cassette of Luc and nopaline synthase terminator (Figures 1, 2A). For these constructs,

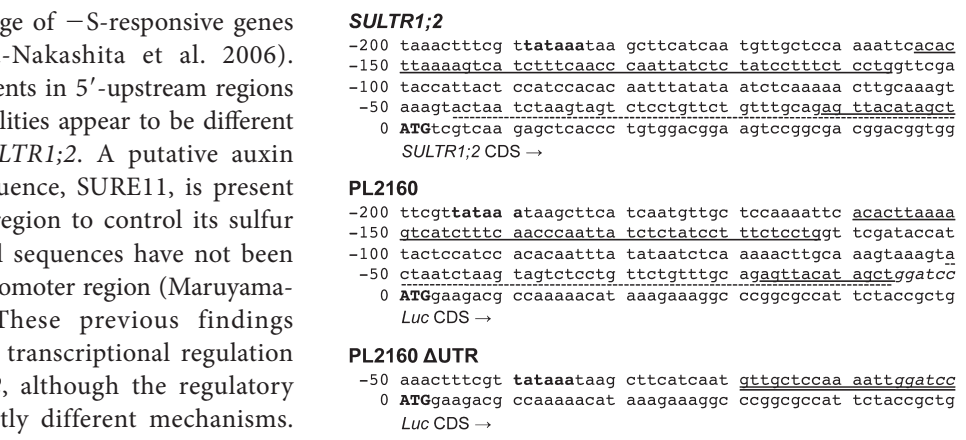

Figure 1. Sequences around the translational start codons of *SULTR1;2* and *Luc* in the fusion gene constructs. The translational start codon (bold and capital), putative TATA box (bold), 5′UTR sequence of *SULTR1;2* (At1g78000.1, solid underlined; At1g78000.2, dotted underlined), *Bam*HI site used for the vector construction (italicized), and the experimentally determined 5′UTR sequence of *Luc* in PL2160ΔUTR plants (double underlined), were shown in the sequences. Numbers on the left of the sequences show the distance in nucleotides from the translational start codons.

the 5′-regions of *SULTR1;2*, starting from the positions −2160 and terminating before the translational start codon or the 5′-end of the 5′UTR of *SULTR1;2*, were amplified from genomic DNA of *Arabidopsis thaliana* (Col-0 accession) by polymerase chain reaction (PCR) using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and primer combinations comprised of the forward primer 1;2ProFSal: 5′-GTC GAC TTG ATT TGG AGC CAG TGG CAT TGT CGT-3′ paired with either 1;2ProRBam: 5′-GGA TCC AGC TAT GTA ACT CTG CAA ACA GAA CAG GAG A-3,′ or 1;2ProRBam(−UTR): 5′-GGA TCC AAT TTT GGA GCA ACA TTG ATG AAG CT-3′ as the reverse primer. Following cloning of PCR fragments into pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA, USA) and sequencing, the *Sal*I-*Bam*HI fragments of *SULTR1;2* promoter region were cloned between the *Sal*I-*Bam*HI sites of pBI101-Luc (Maruyama-Nakashita et al. 2005; Figures 1, 2A). The resultant binary plasmids were transferred to *Agrobacterium tumefaciens* GV3101 (pMP90) (Koncz and Schell 1986), and used for the transformation of *Arabidopsis* plants (Clough and Bent 1998). The transgenic plants were selected on GM media (Valvekens et al. 1988) containing 50 mg l^{-1} kanamycin sulfate.

The T_2 progenies of PL2160 and PL2160ΔUTR transgenic lines were grown for 10 days on the agar medium (Hirai et al. 1995) supplied with 1500 μM (+S) or 50 μM (−S) sulfate. Agar plates were set vertically in a growth chamber controlled at 22°C and 16h/8h light and dark cycles, and the root tissues from the 10-day-old seedlings were used for the analysis. The transcript levels of *Luc* were determined by real-time PCR using SYBR Green Perfect Real Time kit (Takara) and Thermal Cycler

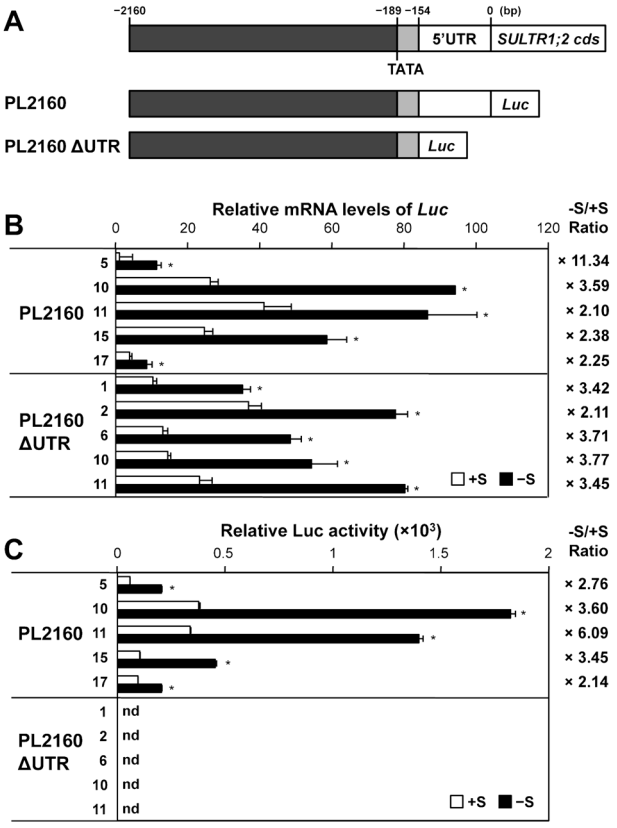

Figure 2. The 5′-upstream flanking sequence of *SULTR1;2* affects mRNA and protein expression in response to sulfate availabilities. (A) Schematic presentation of the constructs used in this study. The diagram shown on the top indicates the structure of 5′-upstream sequence of *SULTR1;2*. The lower two diagrams show the fusion gene constructs, PL2160 and PL2160ΔUTR, used for plant transformation. (B) Effect of deletion of 5′UTR on transcript levels of *Luc*. Average values of mRNA levels and their ratios between −S and +S (−S/+S ratio) are presented. (C) Effect of deletion of 5′UTR on Luc activities. Average values of Luc activities and the ratios between −S and +S (−S/+S ratio) are presented. The Luc activities are shown as relative luminescence units per mg protein. The Luc activities and protein concentration were determined as described previously (Bradford 1976; Maruyama-Nakashita et al. 2005, 2015). nd, not detected. In (B) and (C), T_2 progenies of five independent PL2160 and PL2160ΔUTR transgenic lines were grown for 10 days on agar medium containing 1500 μM (+S, white bar) or 50 μM of sulfate (−S, black bar) as described previously (Maruyama-Nakashita et al. 2005, 2015). Root tissues from 20 plantlets were pooled as one sample and used for determining the *Luc* mRNA levels by real-time PCR or for assaying the Luc activities. Error bars denote the standard error of the mean (SEM, n=5). Asterisks indicate significant differences (Student's t-test; * $p<0.01$) between +S and −S conditions in each plant line.

Dice Real Time System (Takara) using the gene-specific primers for *Luc*, Luc-552F: 5′-GTC CTT CGA TAG GGA CAA GAC A-3′ and Luc-674R: 5′-GGA TCT CTG GCA TGC GAG AAT CT-3,′ and for *UBQ2*, UBQ2-144F: 5′-CCA AGA TCC AGG ACA AAG AAG GA-3′ and UBQ2-372R: 5′-TGG AGA CGA GCA TAA CAC TTG C-3′, as reported previously (Maruyama-Nakashita et al. 2004a, 2004b). The results indicated that both PL2160 and PL2160ΔUTR plants express similar levels of *Luc* mRNA showing significantly increased accumulations under −S relative to +S conditions (Figure 2B). The −S/+S ratios of *Luc* mRNAs ranged from 2.10 to 11.34 in PL2160, and from 2.11 to 3.77 in PL2160ΔUTR, respectively, which were similar and equally significant between PL2160 and PL2160ΔUTR (Figure 2B). Luc activities were also determined using the roots of these transgenic lines grown under +S and −S conditions according to the methods described previously (Maruyama-Nakashita et al. 2005, 2015; Figure 2C). In contrast to the mRNA levels, the Luc activities were detected only in PL2160 but not in PL2160ΔUTR plants. The Luc activities were consistently higher under −S relative to +S in five independent PL2160 lines with a range of −S/+S ratios being 2.14 to 6.09. These trends of increase in Luc activities under −S well reflected the *Luc* mRNA accumulations in PL2160 transgenic lines (Figure 2B, C).

The differences shown between PL2160 and PL2160ΔUTR plants indicated that 5′UTR of *SULTR1;2* was not necessary for the control of transcription. The *Luc* mRNA levels were consistently elevated under −S to a similar extent in PL2160 and PL2160ΔUTR, suggesting that the −S-induced expression of *SULTR1;2* is controlled through the function of the −2160 to −155 region of the 5′-upstream sequence that may serve as an enhancer for transcriptional activation under −S (Figure 2B). The −S/+S ratios were similar between *Luc* mRNA levels and Luc activities in PL2160 plants (Figure 2B, C), implicating that 5′UTR of *SULTR1;2* was not involved in the control of mRNA stability.

It was intriguing to find the absence of Luc activities in PL2160ΔUTR (Figure 2C), because the sequences starting from the position −7 bp of the first ATG to the end of the *Luc* coding sequence as well as the sequence context around that translational start codon, which has been reported to be important for translation of mRNAs (Luetcke et al. 1987; Lukaszewicz et al. 2000; Rangan et al. 2008), were identical between the two constructs (Figure 1). As there was the possibility that the first AUG appeared at −22 bp of the first ATG of *Luc* in PL2160ΔUTR (Figure 1), which has different frame from Luc, could recruit ribosome and inhibit the translation of Luc by the translational overlap (Jackson et al. 2010; von Arnim et al. 2014), 5′UTR sequence of *Luc* in PL2160ΔUTR plants were determined by 5′RACE as described previously (Maruyama-Nakashita et al. 2015). In brief, following the RNA preparation from roots of PL2160ΔUTR plants grown on the +S and −S media, reverse transcription and RT-PCR was carried out using SMART RACE cDNA Amplification Kit (Clontech-Takara Bio) and the primers, Universal Primer A mix (Short) and Luc-5′RACE-1 (5′-ACG AAC ACC ACG GTA GGC TGC GA-3′), then the amplified fragments were sequenced. The determined 5′UTR did not contain the first AUG appeared at −22 bp of

Luc coding sequence, excluding the possibility that the translation of Luc is inhibited by the translational overlap with upstream open reading frame (Jackson et al. 2010; von Arnim et al. 2014). The 5′UTR of *SULTR1;2* can be necessary for the recruitment of translational initiation factors to couple the 5′-cap structure with the 3′-poly(A) tail, which may help the pre-initiation complex to start translation (Kawaguchi and Bailey-Serres 2002; Sonenberg and Hinnebusch 2009; Wilkie et al. 2003). Given the assumption that transcription of *Luc* occurs in the same manner and efficiency determining *Luc* mRNA levels in PL2160 and PL2160ΔUTR, it is unlikely that *SULTR1;2* 5′UTR controls *Luc* mRNA stability. With regard to the absence of Luc activities in PL2160ΔUTR, we cannot exclude a possibility that the first ATG in the *Luc* coding sequence could have been read through and another ATG used for the translation of non-functional Luc proteins.

In summary, we demonstrated that the −S-responsive accumulation of *Luc* mRNA is controlled by the 5′-upstream non-transcribed region of *SULTR1;2* independent of the function of 5′UTR. Since the sulfur-responsive element found in *SULTR1;1* promoter region is not present in the 5′-upstream region of *SULTR1;2*, novel elements responsible for −S-responsive transcriptional regulation of *SULTR1;2* probably exist in the −2160 to −155 region. Precise determination of these elements would reveal the transcriptional molecular machinery involved in regulation of −S-induced expression of sulfate uptake systems that are required for plant survival under sulfur deprived conditions.

Acknowledgements

We thank Yukiko Okuo, Atsumi Onoda, Kazue Nakabayashi, Chieko Komori and Chie Uchiyama for growing plants. This work was supported by JSPS KAKENHI Grant Number 24380040 (for AMN) and in part by the National Science Foundation (MCB-1244300) and AgBioResearch (MICL02313) (for HT).

References

Bailey-Serres J, Dawe RK (1996) Both 5′ and 3′ sequences of maize *adh1* mRNA are required for enhanced translation under low-oxygen conditions. *Plant Physiol* 112: 685–695

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254

Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735–743

Gutiérrez RA, MacIntosh GC, Green PJ (1999) Current perspectives on mRNA stability in plants: Multiple levels and mechanisms of control. *Trends Plant Sci* 4: 429–438

Hirai MY, Fujiwara T, Chino M, Naito S (1995) Effects of sulfate concentrations on the expression of a soybean seed storage protein gene and its reversibility in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol* 36: 1331–1339

Hulzink RJ, de Groot PF, Croes AF, Quaedvlieg W, Twell D, Wullems GJ, Van Herpen M (2002) The 5′-untranslated region of the *ntp303* gene strongly enhances translation during pollen tube growth, but not during pollen maturation. *Plant Physiol* 129: 342–353

Jackson RJ, Hellen CUT, Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* 11: 113–127

Kawaguchi R, Bailey-Serres J (2002) Regulation of translational initiation in plants. *Curr Opin Plant Biol* 5: 460–465

Koncz C, Schell J (1986) The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel types of *Agrobacterium* binary vector. *Mol Gen Genet* 204: 383–396

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. *Annu Rev Plant Physiol Plant Mol Biol* 51: 141–165

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codons differs in plants and animals. *EMBO J* 6: 43–48

Lukaszewicz M, Feuermann M 1, Jerouville B, Stas A, Boutry M (2000) In vivo evaluation of the context sequence of the translation initiation codon in plants. *Plant Sci* 154: 89–98

Mardanova ES, Zamchuk LA, Skulachev MV, Ravin NV (2008) The 5′ untranslated region of the maize alcohol dehydrogenase gene contains an internal ribosome entry site. *Gene* 420: 11–16

Maruyama-Nakashita A, Inoue E, Watanabe-Takahashi A, Yamaya T, Takahashi H (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effect on sulfur nutrition on multiple metabolic pathways. *Plant Physiol* 132: 597–605

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006) Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. *Plant Cell* 18: 3235–3251

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. *Plant J* 42: 305–314

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Yamaya T, Takahashi H (2004a) Induction of SULTR1;1 sulfate transporter in *Arabidopsis* roots involves protein phosphorylation/dephosphorylation circuit for transcriptional regulation. *Plant Cell Physiol* 45: 340–345

Maruyama-Nakashita A, Nakamura Y, Yamaya T, Takahashi H (2004b) A novel regulatory pathway of sulfate uptake in *Arabidopsis* roots: Implication of CRE1/WOL/AHK4-mediated cytokinin-dependent regulation. *Plant J* 38: 779–789

Maruyama-Nakashita A, Watanabe-Takahashi A, Inoue E, Yamaya T, Saito K, Takahashi H (2015) Sulfur-responsive elements in the 3′-nontranscribed intergenic region are essential for the induction of *SULFATE TRANSPORTER 2;1* gene expression in Arabidopsis roots under sulfur deficiency. *Plant Cell* 27: 1279–1296

Rangan L, Vogel C, Srivastava A (2008) Analysis of context sequence surrounding translation initiation site from complete genome of model plants. *Mol Biotechnol* 39: 207–213

Rouached H, Wirtz M, Alary R, Hell R, Arpat AB, Davidian JC, Fourcroy P, Berthomieu P (2008) Differential regulation of the expression of two high-affinity sulfate transporters, SULTR1.1

and SULTR1.2, in Arabidopsis. *Plant Physiol* 147: 897–911

Saito K (2004) Sulfur assimilatory metabolism. The long and smelling road. *Plant Physiol* 136: 2443–2450

Shibagaki N, Rose A, Mcdermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify *SULTR1;2*, a sulfate transporter required for efficient transport of sulfate into roots. *Plant J* 29: 475–486

Sonenberg N, Hinnebusch AG (2009) Regulation of translation initiation in eukaryotes: Mechanisms and biological targets. *Cell* 136: 731–745

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: Molecular functions and regulations of transporters and assimilatory enzymes. *Annu Rev Plant Biol* 62: 157–184

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K (2000) The role of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana. Plant J* 23: 171–182

Valvekens D, Montagu MV, Lijsebettens MV (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci USA* 85: 5536–5540

Vidmar JJ, Tagmount A, Cathala N, Touraine B, Davidian JC (2000) Cloning and characterization of a root specific high-affinity sulfate transporter from *Arabidopsis thaliana. FEBS Lett* 475: 65–69

von Arnim AG, Jia Q, Vaughn JN (2014) Regulation of plant translation by upstream open reading frames. *Plant Sci* 214: 1–12

Wilkie GS, Dickson KS, Gray NK (2003) Regulation of mRNA translation by 5′- and 3′-UTR-binding factors. *Trends Biochem Sci* 28: 182–188

Yoshimoto N, Inoue E, Watanabe-Takahashi A, Saito K, Takahashi H (2007) Posttranscriptional regulation of high-affinity sulfate transporters in Arabidopsis by sulfur nutrition. *Plant Physiol* 145: 378–388

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. *Plant J* 29: 465–473