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BRIEF COMMUNICATION

The influence of sulfur limitation on expression levels of an *o*-acetylserine (thiol) lyase gene cloned from *Vicia sativa*

A.U. NOVERO* and R. FORD

Melbourne School of Land and Environment, University of Melbourne, Parkville, Victoria, 3010 Australia

Abstract

The *o*-acetylserine (thiol) lyase (OAS-TL) gene is part of the plant sulfate assimilation pathway. The synthesis of cysteine, the first product of the pathway containing organic sulfur, has been previously observed to be dependent on the availability of sulfur and activity of OAS-TL. In this study, the ability of *Vicia sativa* L. to alter its metabolism to compensate for deficiencies brought about by sulfur stress was utilized to elucidate the functionality of *Voas-tl5* (GenBank Accession No. DQ456491), a gene cloned from *Vicia sativa*. The transcription levels of *Voas-tl5* increased in response to sulfur deficiency, indicating that the gene was active in the cysteine biosynthetic pathway.

Additional key words: cysteine biosynthesis, sulfate assimilation, vetch.

Sulfur is an essential element in plant growth and plays several roles in plant cells (Leustek and Saito 1999). It is a macronutrient used for synthesis of primary metabolites such as cysteine, methionine, vitamins, coenzymes and storage proteins. It plays a role in redox cycle in cells or stress mitigation as a part of glutathione, thioredoxin, phytochelatins, choline sulfate, *etc*. Several important secondary metabolites contain sulfur that is contributed for their characteristic biological activities. In plant cells, sulfate is reduced to sulfite (SO_3^{2-}) by adenosine-5'-phosphosulfate reductase (APS), and then to sulfide (S^{2-}). Cysteine is synthesized by condensation of sulfide with *o*-acetylserine (OAS), which is a product of a reaction between serine and *o*-acetyl coenzyme A. The two major enzymes involved in the final step of cysteine biosynthesis are serine acetyltransferase (SAT) and *o*-acetylserine (thiol) lyase (OAS-TL). These two enzymes form a SAT/OAS-TL bi-enzyme complex, also known as the "cysteine synthase complex" which regulates cysteine synthesis (Leustek *et al.* 2000). Both enzymes are present in the chloroplasts, cytosol and mitochondria (Hofgen *et al.* 2001). Cysteine is an important precursor of many important compounds that require reduced sulfur (Saito 2004, Droux 2004). Thus, it is important to uncover the characteristics of genes coding enzymes such as OAS-TL.

The expression of genes involved in the sulfate assimilation pathway is influenced by external environmental factors (Nakamura *et al*. 1999). The expression levels of individual OAS-TL isoforms differ in response to varying sulfur content, irradiance or pH (Nakamura *et al*. 1999, Burandt *et al*. 2001, 2002, Hesse *et al*. 2004). Hell *et al*. (2002) suggested that OAS is a mediator of plant sulfur status because it accumulates during sulfur deficiency and affects the expression of genes involved in sulfur assimilation. In transcriptome analyses, Hirai *et al*. (2003) confirmed the role of OAS as a regulator of gene expression.

The isoform distribution pattern and OAS-TL activity in leaf and root extract of sulfur-deficient *Spinacia medania* plants have been studied by Schneider *et al*. (1997). They showed that the activity of the two OAS-TL isoforms decreased by a factor of two in leaves whereas the enzyme activity in roots increased by the same factor. Thus, total enzyme activity remained constant. Warrilow and Hawkesford (1998) found that sulfur deprivation had little effect on the abundance of the *Spinacia oleracea*

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Abbreviations: APS - adenosine phosphosulfate reductase; OAS - *o*-acetylserine; OAS-TL - *o*-acetylserine (thiol) lyase; QRT-PCR - real-time quantitative reverse transcription polymerase chain reaction; SAT - serine acetyltransferase.

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* Corresponding author present address: College of Science and Mathematics, University of the Philippines, Mindanao, Davao City 8002, Philippines; fax: (+63) 822930312, e-mail: anovero@upmin.edu.ph

OAS-TL root isoforms. However, there was a substantial decrease in the abundance of the leaf chloroplastic isoform B in response to sulfur deficiency.

In *Oryza sativa* cv. Nipponbare, four cDNA clones (*rcs1, rcs2, rcs3* and *rcs4*) encoding OAS-TL were isolated (Nakamura et al. 1999). The transcription level of *rcs1* was increased in both roots and shoots within one day of sulfur starvation. The increase was up to two times greater after 6 d in comparison with the controls, with the response more pronounced in the roots than in the shoots. Transcript accumulations of *rcs2* and *rcs3* were dependent on irradiance and nutritional deprivation whereas transcript accumulation of *rcs4* was too low to quantify. Increased transcript accumulation as a response to sulfur starvation was also observed for other genes of the sulfate assimilation pathway such as ATP sulfurylase in canola (Lappartient and Touraine 1996) and *Arabidopsis thaliana* (Logan *et al.* 1996) and APS reductase in *A. thaliana* (Gutierrez-Marcos *et al.* 1996). These observations suggested that the effective synthesis of cysteine requires the induction of a particular OAS-TL isoform along with other genes involved in the cysteine synthase pathway (Nakamura *et al*. 1999).

This study determined the effects of sulfur limitation on the transcription levels of *Voas-tl5* (GenBank Accession No. DQ456491), a gene cloned from *Vicia sativa,* an important pasture crop in Australia (Novero *et al*. 2008). The gene has a cDNA length of 871 bp with its open reading frame coding for a polypeptide of 68 amino acid residues. A better understanding of the genes involved in the cysteine biosynthetic pathway may help breeding efforts toward vetch plants with improved sulfur utilization efficiency. Over the years, many research groups have been investigating the potential of plants for increased sulfate assimilation by studying the effects of elevated SAT and OAS-TL activities.

Vicia sativa cv. Blanche Fleur seeds were obtained from the Department of Primary Industries, Horsham, Victoria, Australia. The seeds were germinated in a Petri dish lined with moist filter paper. After 5 d, when the radicles were about 0.5 - 1.0 cm long, all of the seedlings were transferred to blocks of rock wool irrigated twice a week with MGRL nutrient solution formulated by Fujiwara *et al*. (1992). This nutrient formulation has been shown to supply normal plant nitrogen requirement in sulfur stress experiments. Normal sulfate concentration was provided to the seedlings until the four-leaf stage. The 10-d-old seedlings were then transferred into fresh solutions with (1.5 mM $MgSO_4$) or without sulfate (where an equimolar concentration of $MgCl_2$ was used) in a completely randomized design with three replications. They were grown in the glasshouse at 22 °C with 16-h photoperiod, irradiance of 1 000 μmol m^{-2} s^{-1} at noon and air humidity of 60 % and watered twice a week with MGRL nutrient medium.

RNA was extracted from leaf and root tissues using *TRIzol* reagent (*Invitrogen*, CA, USA) following the manufacturer's protocol. The RNA pellets were eluted in 0.015 cm^3 RNAse-free water (*Promega*, WI, USA) then stored at -70 °C until used. The RNA content was assessed using a spectrophotometer (*Gene Quant Pro*, *Amersham Pharmacia Biotech*, Uppsala, Sweden) at 260 nm and the quality was assessed by running 0.005 cm^3 of the re-suspended pellet on a 1.4 % agarose gel, post staining with 0.05 μg cm^{-3} of ethidium bromide and viewing under UV-radiation. The RNA stock solutions were subsequently diluted to a working concentration of 500 μg cm^{-3}. These were treated with DNAse I (*Invitrogen*) to eliminate contaminating DNA. Working stocks were stored at -70°C until use.

Real-time quantitative reverse transcription polymerase chain reactions (QRT-PCR) were performed in the *Mx3000P* real-time PCR (*Stratagene*, CA, USA) machine using the *Brilliant SYBR Green Single-Step QRT-PCR Master Mix* (*Stratagene*) protocol. The reaction mixture contained 0.0125 cm^3 of 2 × SYBR QRT-PCR master mix, forward and reverse primers (each at a final concentration of 0.1 μM), 0.0625 mm^3 of *Stratascript* RT/RNase block enzyme mixture, 100 ng of RNA template and nuclease-free water to a final volume of 0.025 cm^3. The gene-specific primers for *Voas-tl5* were forward 5'-TCCTCTACCGGAACGAAATC-3' and reverse 5'-GAACTAAACAGAGCCGTTGTG-3'. The putative actin gene *Vs-actin2* was used as an internal control standard. The thermal cycling profile was performed with a three-step cycling program: 50 °C for 30 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s.

Actual experiments were run using the PCR conditions stated previously after a series of primer and template dilutions were carried out to determine ratios that would obtain optimum target amplifications. Samples were analyzed twice with three replicates each. Amplification products were visualized in agarose gels stained with ethidium bromide to rule out spurious products and check for primer dimer formation. C_T values were obtained using the *Mx3000P* version 2 software (*Stratagene*).

OAS-TL activity was measured according to the acid-ninhydrin method of Gaitonde (1967). Leaf or root tissue (100 mg) was ground in a chilled mortar and pestle with 1 cm^3 of cold extraction buffer (200 mM potassium-phosphate, pH 8.0, 250 mM sucrose, 0.5 mM EDTA and 10 mM β-mercaptoethanol), and centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was collected for the subsequent OAS-TL assay and determination of protein concentration. There were three replicates per assay.

Protein contents were determined in crude plant extracts using the *Bio-Rad* protein assay kit (*Bio-Rad Laboratories,* Hercules, USA), and bovine gamma globulin (IgG) as a standard. Absorbance was measured at 595 nm using the *DU530 Life Science UV/VIS* spectrophotometer (*Beckman*, Coulter, USA).

Vetch plants exhibited the first obvious visual symptoms of sulfur deficiency after 8 d in medium without sulfur. Leaves were stunted, newly emerging shoots were chlorotic and root growth was significantly

reduced. This may be an indication that insufficient sulfur supply caused a reduction of cysteine production at the growing regions.

Messenger RNA levels and OAS-TL enzyme activities were detectable in both leaves and roots of seedlings grown in hydroponic solution with or without 1.5 mM $MgSO_4$ (Fig. 1). Transcription levels were higher in sulfur-starved plants and they were higher in the leaves than in the roots. The increase in transcription level was detected from day 2, reaching a maximum of about 2 000-times greater than the control at day 4. The transcription level dropped on day 8 but increased again by day 10 and remained high until day 14. When transcription level in the shoots was at its lowest on day 8, it was at its highest in the roots (about 500 times greater than in the control roots). As a general trend, when mRNA transcript level was abundant in the leaves, it was scarce in the roots except when the trend was reversed on day 8 of the sulfur stress experiment. Higher gene expression levels in the leaves than in the roots supported previous observations that plant OAS-TL genes are generally expressed in the leaves because reductive sulfate assimilation takes place in the chloroplasts. This has been reported for ATP-sulfurylase (Lunn *et al*. 1990) and OAS-TL (Lunn *et al*. 1990, Saito *et al*. 1993) in *Spinacea oleracea*.

The OAS-TL enzyme activity in sulfur-starved seedlings was higher over the control. The activity was highest in the leaves with the peak occurring on day 6. OAS-TL activity of sulfur-starved roots was almost constant during the stress period and only slightly lower on day 6. Total crude protein content was higher in sulfur-starved plants, with the greatest amount measured in leaves. Peak leaf protein contents were observed from 4 to 12 d of the stress period. Protein content in sulfur-starved roots changed very little during the stress period and was not much higher than in the control.

In potato, Maruyama *et al*. (2001) detected the highest OAS-TL enzyme activity in leaves, being 1.6 times greater than in tubers and buds.

Increases in transcription levels of OAS-TL genes of other plants such as rice, spinach and *Arabidopsi*s in response to sulfur starvation have been reported (Warrilow and Hawkesford 1998, Nakamura *et al*. 1999). Expression of other genes of the sulfate assimilation pathway such as APS reductase and ATP sulfurylase was also recorded (Hesse *et al*. 2004).

Although exposure to elevated levels of some metals

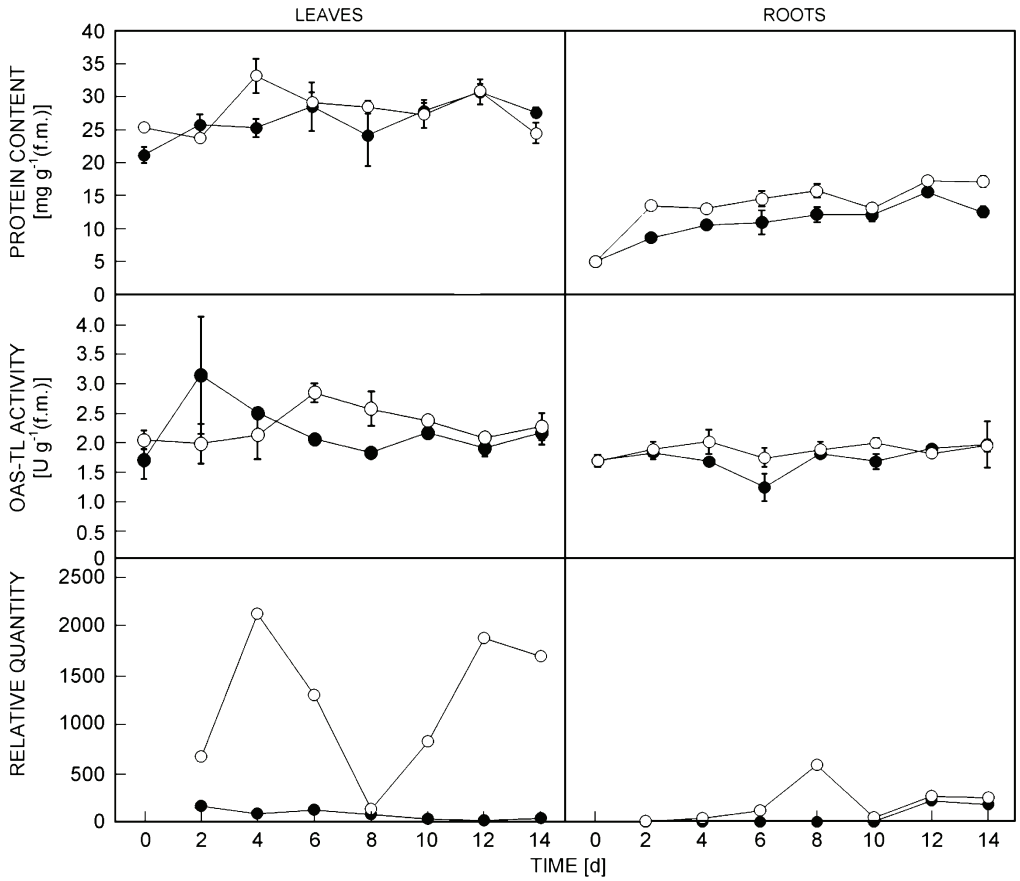

Fig. 1. Total protein content, OAS-TL enzyme activity and relative mRNA transcription levels of the *Voas-tl5* gene in *Vicia sativa* leaf and root tissues in response to sulfur stress. Control plants (*closed circles*) were supplied with 1.5 mM $MgSO_4$ while sulfur-starved plants (*open circles*) were supplied with an equimolar concentration of $MgCl_2$. *Bars* represent standard error (n = 3).

such as zinc (500 μM) increased the activity of antioxidant enzymes in *Hydrilla verticillata* plants (Srivastava *et al.* 2009), in this study, there was a lack of correlations among the amount of mRNA transcription, OAS-TL activity and protein content. Such observation has also been noted in other genes of the sulfate assimilation pathway such as those coding sulfate transporters (Hopkins *et al*. 2005), suggesting that control of sulfate uptake may be at the transcriptional level.

References

Anderson, J.: Assimilation of inorganic sulfate into cysteine. - In: Miflin, B. (ed.): The Biochemistry of Plants. Vol. 5. Pp. 203-223. Academic Press, New York 1980.

Burandt, P., Schmidt, A., Papenbrock, J.: Cysteine synthesis and cysteine desulfuration in *Arabidopsis* plants at different developmental stages and light conditions. - Plant Physiol. Biochem. **39**: 861-870, 2001.

Burandt, P., Schmidt, A., Papenbrock, J.: Three *o*-acetyl-L-serine (thiol) lyase isoenzymes from *Arabidopsis* catalyze synthesis and cysteine desulfuration at different pH values. - J. Plant Physiol. **159**: 111-119, 2002.

Droux, M.: Sulfur assimilation and the role of sulfur in plant metabolism: a survey. - Photosynthesis Res. **79**: 331-348, 2004.

Fujiwara, T., Hirai, M.Y., Chino, M., Komeda, Y., Naito, S.: Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia.- Plant Physiol. **99**: 263-268, 1992.

Gaitonde, M.K.: A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. - Biochemistry **104**: 627-633, 1967.

Gutierrez-Marcos, J.F., Roberts, M.A., Campbell, E.I., Wray, J.L.: Three members of a novel small gene family from *Arabidopsis thaliana* able to complement functionally as *Escherichia coli* mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and 'APS reductase' activity. - PNAS **99**: 13377-13382, 1996.

Hell, R., Jost, R., Berkowitz, O., Wirtz, M.: Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant *Arabidopsis thaliana*. - Amino Acids **22**: 245-257, 2002.

Hesse, H., Nikiforova, V., Gaklere, B., Hofgen, R.: Molecular analysis and control of cysteine biosynthesis: integration of nitrogen and sulphur metabolism. - J. exp. Bot. **55**: 1283-1292, 2004.

Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noj, M., Saito, K.: Global expression profiling of sulfur-starved *Arabidopsis* by DNA microarray reveals the role of *o*-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. - Plant J. **33**: 651-663, 2003.

Hofgen, R., Kreft, O., Willmitzer, L., Hesse, H.: Manipulation of thiol contents in plants. - Amino Acids **20**: 291-299, 2001.

Hopkins, L., Parmar, S., Blaszczyk, A., Hesse, H., Hoefgen, R.: *O*-acetylserine and the regulation of expression of genes encoding components for sulfur uptake and assimilation in potato. - Plant Physiol. **138**: 433-440, 2005.

Lappartient A., Touraine B.: Demand-driven control of root ATP sulfurylase activity and sulfate uptake in intact canola. - Plant Physiol. **111**: 147-157, 1996.

Leustek, T., Martin, M.N., Bick, J.A., Davies, J.P.: Pathways and regulation of sulfur metabolism revealed through molecular studies. - Plant mol. Biol. **51**: 141-166, 2000.

Leustek, T., Saito, K.: Sulfate transport and assimilation in plants. - Plant Physiol. **120**: 637-643, 1999.

Logan, H.M., Cathala, N., Grignon, C., Davidian, J.C.: Cloning of a cDNA encoded by a member of the *Arabidopsis thaliana* ATP sulfurylase multigene family. Expression studies in yeast and in relation to sulfur nutrition. - J. biol. Chem. **271**: 12227-12223, 1996.

Lunn, J.E., Droux, M., Martin, J., Douce, R.: Localization of ATP-sulfurylase and *o*-acetylserine (thiol) lyase in spinach leaves. - Plant Physiol. **94**:1345-1352, 1990.

Nakamura, T., Yamaguchi, Y., Sano, H.: Four rice genes encoding cysteine synthase: isolation and differential responses to sulfur, nitrogen and light. - Gene **229**: 155-161, 1999.

Ng, B., Anderson, J.: Chloroplast cysteine synthases of *Trifolium repens* and *Pisum sativum*. - Phytochemistry **17**: 879-885, 1978.

Novero, A.U., Taylor, P.W.J., Ford, R.: Isolation and characterization of *o*-acetylserine (thiol) lyase, an enzyme of the cysteine biosynthetic pathway of vetch (*Vicia sativa* L.). - Aust. J. Crop Sci. **2**: 96-104, 2008.

Saito, K.: Sulfur assimilatory metabolism, The long and smelling road. - Plant Physiol. **136**: 2443-2450, 2004.

Saito, K., Tatsuguchi, K., Murakoshi, I., Hirano, H.: cDNA cloning and expression of cysteine synthase B localized in chloroplasts of *Spinacea oleracea*. - FEBS Lett. **324**: 247-252, 1993.

Schneider, A., Bowsher, C.G., Hawkesford, M.J.: Purification of two tissue-specific isoforms of *o*-acetylserine (thiol) lyase from spinach (*Spinacia medania*) and influence by sulfur nutrition. - In: Cram, W.J. (ed.): Sulphur Metabolism in Higher Plants. Pp 239-242. Backhuys Publishers, Leiden 1997.

Srivastava, S., Mishra, S., Dwivedi, S., Tripathi, R.D., Tandon, P.K., Gupta, D.K.: Evaluation of zinc accumulation potential of *Hydrilla verticillata*. - Biol. Plant. **53**: 789-792, 2009.

Warrilow, A.G.S., Hawkesford, M.J.: Separation, subcellular location and influence of sulphur nutrition on isoforms of cysteine synthase in spinach. - J. exp. Bot. **327**: 1625-1636, 1998.