Amino Acids (2010) 39:1077–1086
DOI 10.1007/s00726-010-0694-0

ORIGINAL ARTICLE

Enzymes of cysteine synthesis show extensive and conserved modifications patterns that include N^{α}-terminal acetylation

Markus Wirtz · Corinna Heeg · Arman Allboje Samami · Thomas Ruppert · Rüdiger Hell

Received: 3 March 2010 / Accepted: 9 July 2010 / Published online: 24 July 2010

Abstract Biosynthesis of cysteine is a two-step process in higher plants subsequently catalyzed by serine acetyltransferase (SAT) and *O*-acetylserine (thiol) lyase (OAS-TL) which are present in cytosol, plastids and mitochondria. Recently, the distribution of SAT and OAS-TL in these subcellular compartments was shown to be crucial for efficient cysteine synthesis in *Arabidopsis thaliana*. In this study, the abundances of OAS-TLs were quantified independently by immunological detection in crude protein extracts and by SAT affinity purification (SAP) of OAS-TL. OAS-TL A and B were evidenced to be the most abundant isoforms in all analyzed tissues, which is consistent with micro array-based transcript analyses. Application of SAP to Arabidopsis revealed significant modification of the major OAS-TL isoforms present in cytosol, plastids and mitochondria into up to seven subspecies. Specific OAS-TL isoforms were found to be differentially modified in the leaves, roots, stem and cell culture. Sulphur deficiency did not alter modification of OAS-TL proteins purified from cell culture that showed the highest complexity of OAS-TL modifications. However, the pattern of OAS-TL modification was found to be stable within an analyzed tissue, pointing not only to a high reproducibility of SAP but likely biological significance of each subspecies. The most abundant OAS-TL subspecies in cytosol and plastids were subject of N-terminal processing followed by acetylation of the newly originated N-terminus. The mode of N^{α}-terminal acetylation of OAS-TL and its possible biological function are discussed.

Keywords Sulphur metabolism · Cysteine synthesis · Post-translational modification · Co-translational modification

Introduction

Cysteine synthesis in bacteria and plants is catalyzed by *O*-acetylserine (thiol) lyase (OAS-TL; EC 2.5.1.47). It uses the substrates sulphide from sulphate assimilation or other sources and *O*-acetylserine that is formed by serine acetyltransferase (SAT; EC 2.2.1.30), forming the metabolic cysteine synthase complex with the latter (Hell and Wirtz 2008). In contrast, fungi and animals synthesize cysteine from methionine via homocysteine. OAS-TL belongs to the superfamily of pyridoxal phosphate-dependent β-substituted alanine synthases (Hatzfeld et al. 2000; Watanabe et al. 2008a). Bacteria mostly carry two genes encoding OAS-TLs. While CysK is a true OAS-TL enzyme and forms a cysteine synthase complex with CysE, CysM exists only in free form, uses thiosulphate and is assumed to function under anaerobic conditions (Kredich 1996; Zhao et al. 2006). OAS-TL diversity is much higher in plants which are thus the system of choice to study cysteine synthesis with respect to biochemical variability and

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0694-0) contains supplementary material, which is available to authorized users.

M. Wirtz · C. Heeg · A. A. Samami · R. Hell (✉)
Heidelberg Institute for Plant Sciences (HIP),
University of Heidelberg, Im Neuenheimer Feld 360,
69120 Heidelberg, Germany
e-mail: rhell@hip.uni-heidelberg.de

T. Ruppert
Center for Molecular Biology Heidelberg (ZMBH),
Im Neuenheimer Feld 282, 69120 Heidelberg, Germany

physiological aspects such as human nutrition. The genome of *Arabidopsis thaliana* and other plants reveals about nine genes encoding OAS-TL-like proteins, which form together the family of β-substituted alanine synthases (Bsas, Hatzfeld et al. 2000; Hell et al. 2002; Patron et al. 2008). In Arabidopsis, OAS-TL A (At4g14880, Bsas1;1), B (At2g43750, Bsas2;1), C (At3g59760, Bsas2;2), D1 (At3g04940, Bsas4;1), D2(At5g28020, Bsas4;2) have been shown to be enzymatically true OAS-TLs (Yamaguchi et al. 2000; Wirtz et al. 2004; Heeg et al. 2008). However, AtCysC1 (At3g61440, Bsas3;1) is a β-cyanoalanine synthase that preferentially catalyzes the formation of β-cyanoalanine from cyanide and cysteine in mitochondria, and CS-like protein (At5g2803; Bsas1;3) in the cytosol cleaves cysteine to form sulphide, ammonia and pyruvate (Hatzfeld et al. 2000; Alvarez et al. 2010). Recently; CS26 (At3g03630, Bsas5;1) have been evidenced to act as a *S*-sulphocysteine synthase accepting thiosulphate instead of sulphide as donor of reduced sulphur for incorporation into OAS (Bermudez et al. 2010). All true OAS-TL proteins are assumed to associate with SAT in the cysteine synthase complex, but not AtCysC1, CS26 and CS-like.

Functional diversity of OAS-TL-like proteins is further enhanced by compartment-specific roles. The major isoforms OAS-TL A, B and C are distributed between cytosol, plastids and mitochondria, respectively (Saito 2000). Lowest OAS-TL activity is found in mitochondria where OAS-TL C seems to act mostly as an activator of SAT activity, while the cytosol and not the chloroplast was described a major compartment of cysteine synthesis (Heeg et al. 2008; Watanabe et al. 2008a; Krueger et al. 2009). According to uneven distribution of OAS-TL activities the abundance of OAS-TL proteins is expected to vary greatly. Indeed, a SAT affinity purification (SAP) approach using immobilized SAT as anchor and separation by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) suggested highly variable abundances of OAS-TL proteins in leaves of Arabidopsis (Heeg et al. 2008). Surprisingly however, multiple proteins of each OAS-TL A, OAS-TL B and OAS-TL C were observed, suggesting post-translational modifications. Processing and modification of proteins are generally known to be important for activity, stability and function of many proteins (Zybailov et al. 2008; Hwang et al. 2010).

Here we report the molecular identity of the major cytosolic and plastidic OAS-TL proteins as de-methionated and subsequently N-acetylated. The OAS-TL protein profiles in different tissues are analysed and the uneven distribution of OAS-TL proteins between the cytosol and organelles in leaves of Arabidopsis is quantified by SAP and immunological detection.

Materials and methods

Growth of plant material

All experiments were performed using *Arabidopsis thaliana* ecotype Col-0. For in-depth analysis of OAS-TL subspecies from leaves, Arabidopsis Col-0 was grown on soil, in order to obtain sufficient material for purification of OAS-TL via SAP (see below). Seeds were stratified for 3 days at 4°C in the dark and transferred for germination to climate chambers. After 2 weeks, seedlings were transferred to individual pots and grown for another 6–7 weeks at short-day conditions (8.5 h light period). The light intensity was set to 100 µE $m^{-2}s^{-1}$, while the relative humidity was kept at 50%. The temperature during day and night cycle changed from 22 to 18°C.

The abundance of OAS-TL isoforms in different organs (e.g., roots, stem and rosette leaves) was analyzed with Arabidopsis Col-0 plants that were grown in hydroponic cultures. Seeds were germinated in Eppendorf tubes and placed on small boxes (0.25 l) as described in Tocquin et al. (2003). After 11 days, seedlings were transferred to large pots containing 5 l of the same medium and grown for additional 31 days. Growth media were exchanged every week. Hydroponically grown plants were kept at long day (14 h light period) and otherwise same conditions as soil-grown plants. At day 42 roots, leaves and stem were harvested separately and immediately frozen in liquid nitrogen. The distribution of OAS-TL subspecies in different tissues of the wild type was determined in two individual plants and confirmed the results of which are shown in Fig. 2. Heterotrophic *A. thaliana* cell suspensions cultures were grown in 0.3 l Erlenmeyer flasks at 90 rpm (Multitron, Infors, US) and 24°C in the dark. 1 g of cells were used to inoculate 50 ml 1 × MS-Medium pH 5.7 (Murashige and Skoog 1962) supplemented with 0.1 g l^{-1} ampicillin (Sigma–Aldrich, Germany). To analyze the impact of sulphur deficiency on modification of OAS-TL proteins, cells were transferred after 4 days to sulphur-deficient or sulphur-containing MS-Medium (Duchefa, Netherlands). Sulphur-deficient medium was produced by replacing sulphate (1.5 mM) containing MS-salts with their corresponding chloride salts (Sigma–Aldrich, Germany). Samples were taken 1 day after transfer and analysed for modification of OAS-TL proteins after separation of affinity purified OAS-TL proteins via 2D PAGE. The cell culture was subjected to sulphur starvation independently two times.

Purification and separation of OAS-TL proteins

For affinity purification of OAS-TL proteins, total soluble proteins were extracted from 100 g of leaf material from

8-week-old soil-grown Arabidopsis plants that were ground in liquid nitrogen. The resulting leaf powder was stirred for 30 min in 350 ml of ice cold extraction buffer B [50 mM Tris–HCl pH 8.0, 250 mM NaCl, 80 mM imidazole, 0.5 mM phenylmethylsulphonylfluoride (PMSF), 1 mM dithio-DL-threitol (DTT)]. The crude plant extract was filtered through a Miracloth™ tissue (Calbiochem, Germany) and the residual cell debris was collected by centrifugation for 20 min at 27,500*g* and 4°C. For purification of OAS-TL isoforms from roots, stem and heterotrophic cell culture the amount of sample varied between 4 and 5 g of material. The amount of buffer was adjusted accordingly.

The resulting supernatant was fractionated by successive precipitation of proteins with 20 and 75% ammonium sulphate on ice for 30 min, followed by centrifugation as described above to collect the proteins. The sediment was dissolved in extraction buffer A and desalted by size exclusion chromatography using PD-10™ columns in the same buffer (GE-Healthcare, Germany) according to the manufacturer's instructions. The desalted proteins were quantified as described in Bradford (1976) using bovine serum albumin as a standard and subjected to purification of OAS-TLs by their interaction with recombinant AtSAT5 (At5g56760) according to Heeg et al. (2008). The purified OAS-TLs species were separated by 2D PAGE in order to analyze for modifications of proteins by MALDI-TOF. Separation in the first dimension was achieved by isoelectric focusing (IEF) of proteins between pH 5 and 8 in a Protean IEF Cell (Bio-Rad, Germany) according to the manufacturer's instructions. The proteins were separated in the second dimension by SDS-PAGE as described below after reduction of proteins in the IEF gel by incubation with equilibration buffer [50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerin, 2% (v/v) SDS, 0.0025% (w/v) bromophenol blue and 65 mM DTT) for 10 min.

Recombinant expression of OAS-TL A

Bacterial strain HMS 174(DE3) was transformed with the vector pET3d-OAS-TL A (Wirtz et al. 2004) and grown at 37°C in 1 l of Luria–Bertani medium [1% (w/v) trypton, 0.5 (w/v) yeast extract, 1% (w/v) NaCl] supplemented with 0.1 mg ml^{-1} ampicillin, 10 µM pyridoxine and 15 µM thiamine. Expression of recombinant proteins was induced by adding 1 mM isopropyl *β*-thiogalactopyranoside at an optical density of 0.8. Cells were grown for additional 4 h and harvested by centrifugation for 15 min at 6,000*g*. The collected bacteria were resuspended in buffer A [10 mM Tris–HCl pH 7.5, 1 mM DTT, 1 mM phenylmethanesulphonyl fluoride (PMSF)] and disrupted by sonication. After centrifugation at 4°C and 48,000*g* for 15 min the soluble bacterial protein of the supernatant was subjected to SAT affinity purification.

Immunological detection and quantification of OAS-TL isoforms

Total soluble proteins were isolated with 1.2 ml 50 mM *N*-(2-hydroxyethyl)-piperzine-*N*′-ethanesulphonic acid (HEPES), pH 7.4, 10 mM KCl, 1 mM ethylenedioxy-bis-(ethylenenitrilo)-tetraacetic acid (EDTA), 10% glycerine, 30 mM DTT and 0.5% PMSF, from 0.2 g leaf material that was ground to a fine powder in liquid nitrogen. Cell debris was removed by centrifugation at 16,000*g* and 4°C for 10 min. The supernatant was desalted in 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF with a PD-10™-column (GE-Healthcare, Germany) according to the manufacturer's protocol. The proteins were separated according to Laemmli (1970) by discontinuous sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in Mini-Protean™ II cells (Bio-Rad, Germany). The monomer concentration (%T) and crosslinker concentration (%C) in focussing and resolving gels were 4%T/0.026%C and 12.5%T/0.026%C, respectively. Prior to separation proteins were denatured by incubation for 10 min at 65°C in 20 mM Tris pH 7.0, 0.1% SDS, 0.7% *β*-mercaptoethanol, 4% glycerol. Separated soluble proteins (1–30 µg) were transferred to nitrocellulose in a transfer cell (Bio-Rad, Germany) according to the manufacturer's protocol. After blocking of nitrocellulose (AppliChem, Germany) with 5% bovine serum albumin an alkaline phosphatase–anti rabbit IgG conjugate (1:10,000) in combination with NBT/BCIP substrate system (Roche) was used to detect primary polyclonal rabbit antisera against *OAS-TL A, OAS-TL B and OAS-TL C* (Jost et al. 2000; Heeg et al. 2008) that were diluted 1:5,000, 1:5,000 and 1:1,000, respectively. Quantification of protein abundance was achieved by comparison with external standards for each OAS-TL isoform (1–50 ng) transferred to the same nitrocellulose. Densitometry of signals was recorded by the histogram function of Adobe Photoshop v. CS (Adobe, Germany) after digitalization of nitrocellulose with the Perfection 2400 scanner (Epson, Germany). An area of the same size with no protein was defined as background and subtracted from the protein signal.

Identification and analysis of proteins by ESI Q-TOF and MALDI-TOF

Molecular weight determination of proteins was done using ESI Q-TOF hybrid mass spectrometer (QStar, Applied Biosystems) as described in Rist et al. (2005). Briefly, proteins were trapped on a porous R1 reversed phase column, washed with 0.1% formic acid, eluted with 80% acetonitrile, 0.1% formic acid and ionized by electrospray. Acquired spectra were deconvoluted using the software Bayesian Protein Reconstruct (Applied Biosystems).

For protein identification individual spots were excised after 2D PAGE, reduced with DTT, alkylated with iodoacetamide and digested with trypsin (Catrein et al. 2005) using a Digest pro MS liquid handling system (Intavis AG, Germany). Following digestion tryptic peptides were extracted from the gel pieces with 50% acetonitrile/0.1% TFA and concentrated. For MALDI-TOF mass spectrometry (Ultraflex, Bruker) sample was desalted using ZipTip and spotted onto a steel target using a-cyano-4-hydroxy cinnamic acid as matrix and the peptide mass fingerprint (PMF) was acquired after external calibration (peptide calibration standard II, Bruker). For protein identification by PMF peptide masses were labeled manually without smoothing and background substraction using a sample from an empty area of the same gel as background control. The PMF was searched against the NCBInr database using the Mascot software (Matrix Science, US). The algorithm was set to use trypsin as enzyme, allowing at maximum for one missed cleavage site and assuming carbamidomethyl as a fixed modification of cysteine and oxidized methionine as a variable modification. Mass tolerance was set to 100 ppm. Protein hits were taken as identified if the mascot score exceeded the significance level ($p < 0.05$).

Results

Identification of OAS-TL isoforms and subspecies

All OAS-TL-like proteins which function in cysteine synthesis so far have been found to closely interact with SAT in the cysteine synthase complex. This property was exploited to construct an affinity purification column with bound recombinant SAT5 from Arabidopsis cytosol as described in Heeg et al. (2008) to isolate OAS-TL proteins from leaf extracts. Bound OAS-TLs were specifically eluted from the column by *O*-acetylserine (OAS) that is known to efficiently dissociate the cysteine synthase complex (Berkowitz et al. 2002). In extension of the work by Heeg et al. (2008) OAS-TL populations from several tissues were analysed by various 2D PAGE conditions followed by ESI Q-TOF and MALDI-TOF mass spectrometry with the aim to detect as many OAS-TL proteins as possible.

In-depth analysis of leaf protein extracts identified up to four spots of OAS-TL A, seven spots of OAS-TL B, three spots of OAS-TL C and one spot of OAS-TL D1 and OAS-TL D2 each (Fig. 1a). The identification of each OAS-TL A and B subspecies in the 2D-gel was based on sequencing by MALDI-TOF analysis of respective spots in combination with the molecular mass and abundance of signals in ESI Q-TOF analysis. The sequence coverage following tryptic proteolysis and separation of peptides by MALDI-TOF was between 39 and 58% (Suppl. Fig. 1). ESI Q-TOF analyses of OAS-TL A and B subspecies allowed further confirmation of sequence identity (94% for OAS-TL B), determination of molecular weight of each subspecies and its relative abundance (Fig. 1b, c). Furthermore, all subspecies were found to be shifted in the spectra by a mass of 229 Da, indicating the presence and the absence of the lysine-bound cofactor pyridoxal phosphate in these polypeptides. Using smaller amounts of total leaf protein for 2D-gels yielded higher resolution but less spots, underlining the wide span of abundances between OAS-TL isoforms and within one isoform such as OAS-TL A. Even at very high protein loading conditions no other members of the OAS-TL-like protein family were detectable, either because they do not functionally interact with SAT or were still of too low abundance. Further analyses focussed

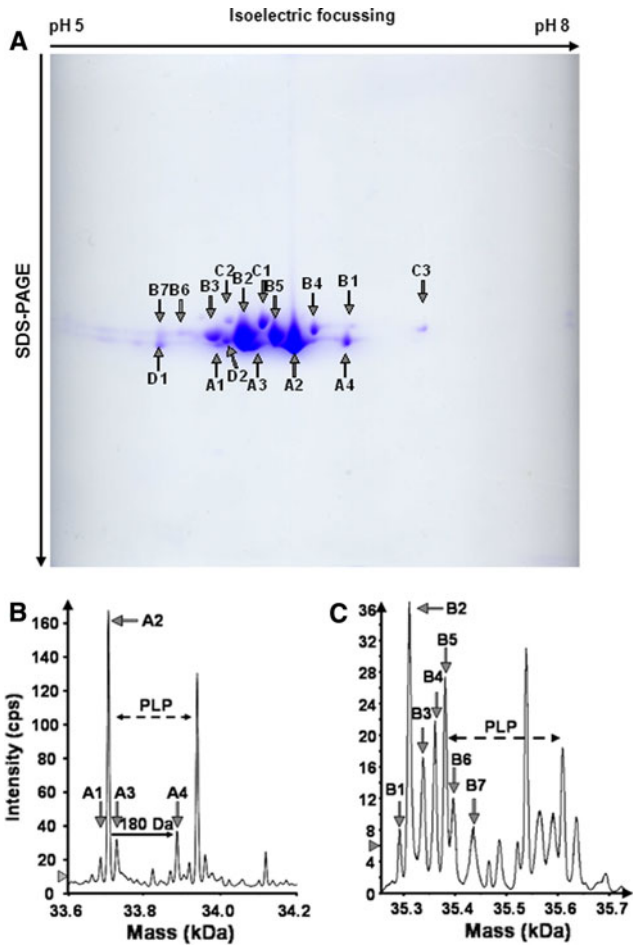

Fig. 1 Identification of cytosolic and plastidic OAS-TL species in leaves of *A. thaliana ecotype Col-0*. The mix of purified OAS-TL isoforms (*A*, *B*, *C D1* and *D2*) was subjected to 2D PAGE (**a**) in order to separate modified OAS-TL species of each isoform. The molecular weight of OAS-TL A species (**b**) and OAS-TL B species (**c**) was determined in solution by ESI Q-TOF analysis. The shift caused by the absence of pyridoxal-5′-phosphate (*PLP*) is only shown exemplary for one of the subspecies in each spectrum

therefore on the dominating cytosolic OAS-TL A and plastidic OAS-TL B isoforms.

The presence of several subspecies of a protein can be caused by several isogenes, gene models or protein modification. However, genomic sequence analysis confirms the presence of only nine *OAS-TL*-like genes of which OAS-TL A2 is a pseudogene (Jost et al. 2000) and the others being represented by distinctly different isogenes (Initiative 2000). Alternative splicing products are another way to produce several protein subspecies from one gene. Annotated gene models predicted from EST sequencing in database, The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org), indicate two models for OAS-TL A that give rise to the same protein sequence, one for OAS-TL B and three for OAS-TL C that result in three predicted protein species (Suppl. Fig. 2). The determined molecular weights of OAS-TL A and B subspecies (Fig. 2a, b; Table 1) were smaller and showed no agreement with the annotated molecular weights of the gene model-derived sequences of OAS-TL A and B. In contrast, the molecular weight of the dominant OAS-TL C subspecies C1 (At5g59760.3; Suppl. Fig. 2) was determined as 35.135 Da. It was thus larger than the predicted mature size of 35,058 Da after cleavage of the mitochondrial sorting peptide at position ala^{101} using the PSORT software package. This does not exclude the possibility that the observed subspecies OAS-TL C2 and C3 correspond to the other two OAS-TL gene models. However, in case of OAS-TL A and B extensive protein processing and modification appears much more likely to explain the various subspecies.

Modifications of OAS-TL A and OAS-TL B proteins

All four OAS-TL A subspecies numbered A1 to A4 (Fig. 1a) differed from the predicted molecular weight of the translated gene models (Suppl. Fig. 1). Further differences in molecular masses of 179 Da between subspecies A2 and A4 and of 42 Da between subspecies A1 and A3 were observed. N$^{\alpha}$-terminal acetylation is generally indicated by a mass of 42 Da and corresponded exactly to the difference between OAS-TL variants A1 and A3. Further modifications downstream in the sequence must therefore be responsible for the deviation from predicted molecular weights. Amino-terminal sequencing of the dominant subspecies OAS-TL A2 by Edman degradation was attempted but yielded no positive result even after heat treatment in the presence of trifluoroacetic acid before sequencing. This strongly suggests that the N-terminus was blocked. However, the mass of subspecies OAS-TL A2 matched exactly the predicted molecular weight (Suppl. Fig. 2) if removal of the starting methionine followed by N$^{\alpha}$-terminal acetylation of ala^{2} was assumed. Still, the total weights of subspecies OAS-TL A1, A3 and A4 did not correspond to the derived peptide sequences, suggesting probably two or more modification processes downstream in the polypeptides that summarized to the observed molecular weights. The removal of the N-terminal methionine of OAS-TL A was verified by comparison with recombinant full length OAS-TL A in *E. coli* (Suppl. Fig. 3).

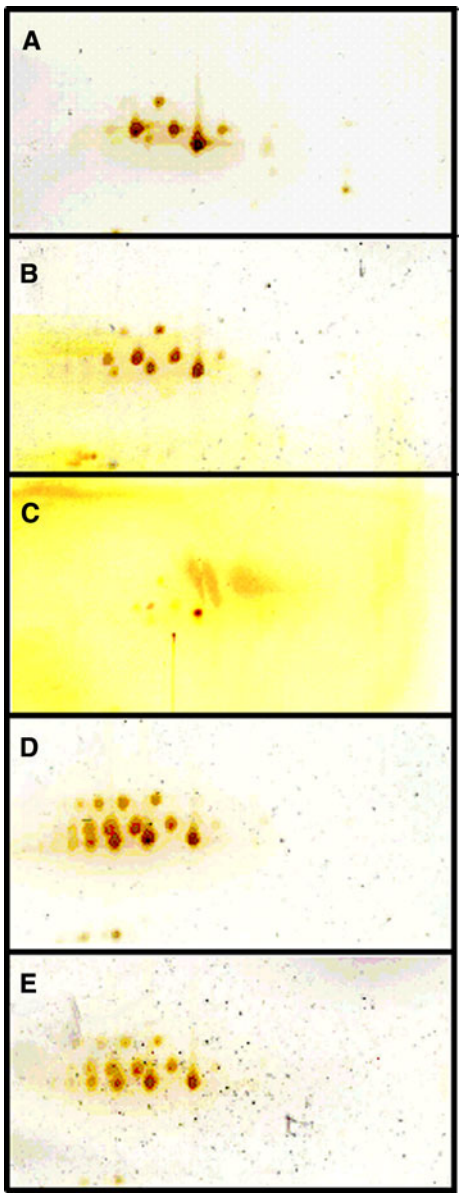

Fig. 2 Abundance of OAS-TL proteins in different tissues of *A. thaliana ecotype Col-0.* Native OAS-TL proteins were purified as described in Heeg et al. (2008) from rosette leaves (**a**), stem (**b**) and roots (**c**) of 10 weeks *Arabidopsis thaliana* plants grown hydroponically under short-day conditions. The same method was applied to purify OAS-TL proteins from cell cultures that were grown under normal sulphur supply (**d**) or subjected to sulphur deficiency for 24 h (**e**). The mix of purified OAS-TL isoforms (A, B, C, D1 and D2) was subjected to 2D PAGE (**a**) in order to separate modified OAS-TL species of each isoform

Table 1 Identification of *O*-acetylserine(thiol)lyase species in leaves of Arabidopsis Col-0

ID	Molecular weight (Da)	Abundance	N-terminus	Description corresponds to	Further modifications
A1	33,694	+	Unknown	–	Yes
A2	33,714	+++	ac-ASRIA	1	None
A3	33,736	++	Unknown	–	Yes
A4	33,892	++	Unknown	–	Yes
B1	35,437	+	ac-*CK*AVSIK	1	None
B2	35,399	++	Unknown	–	Yes
B3	35,384	+++	ac-*A*VSIK	2	179 Da
B4	35,363	++	Unknown	–	Yes
B5	35,340	++	Unknown	–	Yes
B6	35,314	+++	ac-VSIK	3	179 Da
B7	35,295	+	Unknown	–	Yes
C1	35,136	++	Unknown	–	Yes
C2	35,160	+	Unknown	–	Yes
C3	35,206	+	Unknown	–	Yes

1 determination of exact molecular weight by ESI Q-TOF, *2* identification of N-terminus by Zybailov et al. (2008), *3* sequencing of N-terminus by ESI Q-TOF, confirmed by Zybailov et al. (2008)

The mature plastidic OAS-TL B protein was predicted to start with val[58] according to TargetP V1,1 (Emanuelsson et al. 2007). Similar to OAS-TL A the pattern of subspecies was repeated in the mass spectrum without PLP cofactor (Fig. 2c). Using ESI Q-TOF coverage of peptide sequence of 94% was achieved (Suppl. Fig. 1). Molecular weights and amino-terminal peptides of subspecies OAS-TL B1, B3, and B6 were analysed (Table. 1) and compared to recent proteomics data were available (Zybailov et al. 2008). The observed data corresponded exactly to starting amino acids cys[59], ala[61], val[62] of the mature proteins if each of the N-termini was acetylated. Further modifications from predicted molecular weights referred to 179 Da for subspecies B3 and B6 as observed for OAS-TL A1/A3. The determined molecular weights of subspecies OAS-TL B4, B5 and B7 could not be resolved but indicated further, possibly multiple modifications.

OAS-TL protein and expression patterns in different tissue types

Investigation of OAS-TL protein profiles so far had focussed on rosette leaves of wild type and OAS-TL A, OAS-TL B and OAS-TL C deficient mutant plants (Heeg et al. 2008). Using SAP about 85% of total OAS-TL activity of leaf, stem and root tissue from hydroponically grown plants and Arabidopsis dark grown cell culture was retained on the affinity column (Suppl. Fig. 4). Best OAS-TL yield was observed for leaf tissue with a purification factor of 287-fold compared to crude protein (data not shown). For all tissues analysed the entire eluted OAS-TL protein fraction was concentrated for 2D PAGE followed by silver staining (Fig. 3). Total protein and OAS-TL protein fractions varied considerably as can be expected from the different functions of these tissues.

Comparison of leaf and stem fractions revealed similar OAS-TL patterns, with the exception that the most abundant subspecies of each isoform observed in leaf were less dominating, i.e. several subspecies were of almost equal abundance. In contrast, comparison of photosynthetic tissues with root detected only four subspecies in total. They correspond to OAS-TL A2, OAS-TL B2 and B5 and OAS-TL C2 which were found to be most abundant in leaf. It cannot be excluded that further OAS-TL subspecies in roots were present but below detection limit. Since the specific activity of affinity purified root OAS-TLs was three times higher (0.14 ± 0.04 U g^{-1} protein compared to 0.05 ± 0.01 U g^{-1} protein) compared to leaf OAS-TL fraction, the lower representation of OAS-TL subspecies in 2D PAGs is a consequence of strongly different protein composition of root and leaf.

Analysis of the OAS-TL profile of dark grown cell culture resembled more leaf than root composition. All 14 subspecies found in leaf (Fig. 1) were observed and in some but not all 2D PAGs a fifth OAS-TL A and a fourth OAS-TL C subspecies, both with lower isoelectric points, showed up, suggesting the presence of possibly even more modifications in OAS-TL proteins. Cell cultures in mid log phase were transferred to sulphate-free medium for 24 h and compared to control treated cells, but the OAS-TL pattern remained constant as did total OAS-TL activity (not shown).

The abundance of OAS-TL like proteins provides the opportunity for comparison with steady-state levels of transcripts. The summarized intensities of OAS-TL subspecies proteins in 2D gels corresponded well with transcription patterns found in public microarray databases

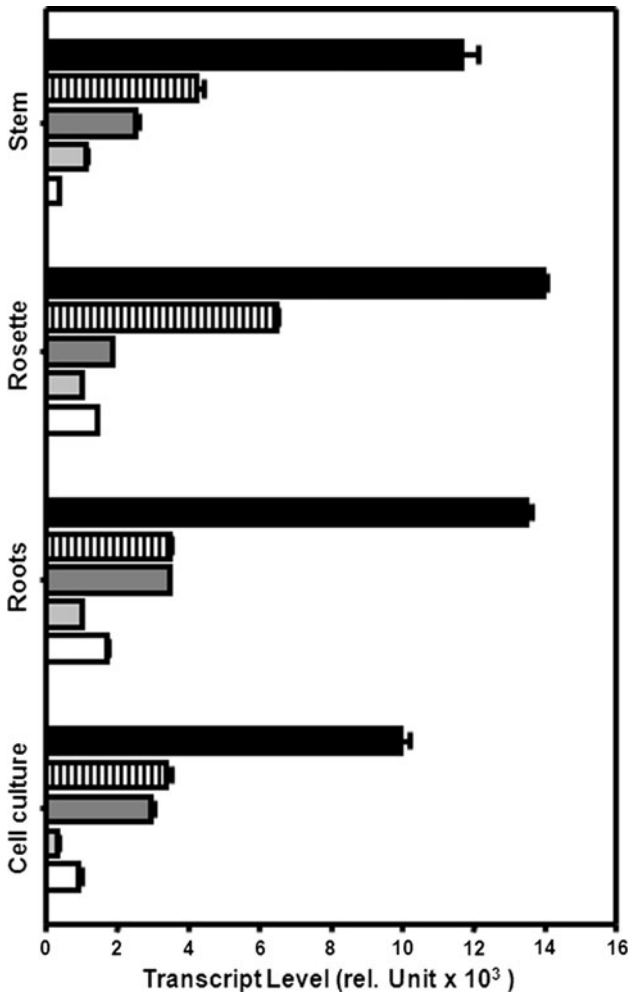

Fig. 3 Transcript levels of OAS-TL isoforms in different tissues of *A. thaliana ecotype Col-0*. Transcript levels of OAS-TL A (*black*), OAS-TL B (*white dashed*), OAS-TL C (*dark grey*), OAS-TL D1 (*light grey*) and OAS-TL D2 (*white*) in different tissues of *A. thaliana* were analysed with the publicly available data analysis tool kit 'Genevestigator' (Zimmermann et al. 2004). The total number of analyzed arrays for stem, rosette leaves, roots, and cell culture was 46, 1,220, 390 and 130, respectively

(Fig. 3; Zimmermann et al. 2004). This strongly suggests that expression and translation of the major OAS-TLs in different tissues are in a linear relationship, despite the extensive post-translational modifications. Apart from very few stress situations OAS-TL genes are semi-constitutively expressed as is also documented for development from seedling to silique (Suppl. Fig. 5).

Quantification of compartment-specific OAS-TL isoforms by immunological detection

In addition to determination of relative abundances of OAS-TL isoforms and their subspecies, an absolute quantification was carried out using polyclonal antibodies that had been raised against Arabidopsis OAS-TL A, B and C. Specificities had been verified (Jost et al. 2000; Heeg et al. 2008) and cross-reactivities were tested (Fig. 4). Loading of increasing amounts of whole leaf protein extracts and purified recombinant antigens on the same immuno-blot allowed direct quantification by intensity scanning. Using the staining signals from recombinant OAS-TL proteins the following concentrations were determined: OAS-TL A, 3.17 ± 0.62 ng OAS-TL μg^{-1} soluble protein ($n = 3$); OAS-TL B, 1.04 ± 0.53 ng OAS-TL μg^{-1} soluble protein ($n = 4$); OAS-TL C, 0.29 ± 0.18 ng OAS-TL μg^{-1} soluble protein ($n = 3$). In terms of relative abundance this is confirmed by the sum of subspecies intensities of the isoforms A to C in 2D PAGs.

Discussion

The importance of subcellular localization of cysteine synthesis in higher plants came into focus of research in the last 2 years. The major sites for production of cysteine precursors, OAS and sulphide, were evidenced to be separated during evolution of higher plants, which draws attention to the importance of OAS-TL, which needs both OAS and sulphide for production of cysteine, in different subcellular compartments (Haas et al. 2008; Heeg et al. 2008; Watanabe et al. 2008a, b). In this study the frequent modification of major OAS-TL isoforms reported by Heeg et al. (2008) was confirmed and further characterized. The detailed analysis revealed that the major subspecies of cytosolic and plastidic OAS-TLs are subject to N-terminal processing followed by acetylation of the newly originated N-terminus. The cleavage of methionine followed by acetylation of the penultimate alanine is in agreement with the subsequent action of methionine amino peptidase (MAP) and the NAT-A complex of yeast (Tsunasawa et al. 1985; Mullen et al. 1989). The presence of cytosolic MAP has been demonstrated in higher plants and is essential for normal development (Giglione et al. 2000; Ross et al. 2005). N-terminal methionine excision by MAP is supposed to be conserved between prokaryotes and eukaryotes (Ross et al. 2005), while N-terminal acetylation occurs post-translationally in prokaryotes but co-translationally in eukaryotes (Polevoda and Sherman 2003a). Both hypotheses are in good agreement with the determined molecular weight of the recombinant OAS-TL A expressed in the prokaryote *E. coli*, which corresponds to an N-terminus that is de-methionylated but non-acetylated.

In contrast to MAP, NAT-A activity has not been evidenced up to now in plants. Nonetheless, co-translational N^{α}-terminal acetylation by the three major NAT complexes A, B and C is conserved in eukaryotes, and supposed to be even more frequent in higher eukaryotes like mammals than in the lower eukaryote yeast (Polevoda and Sherman

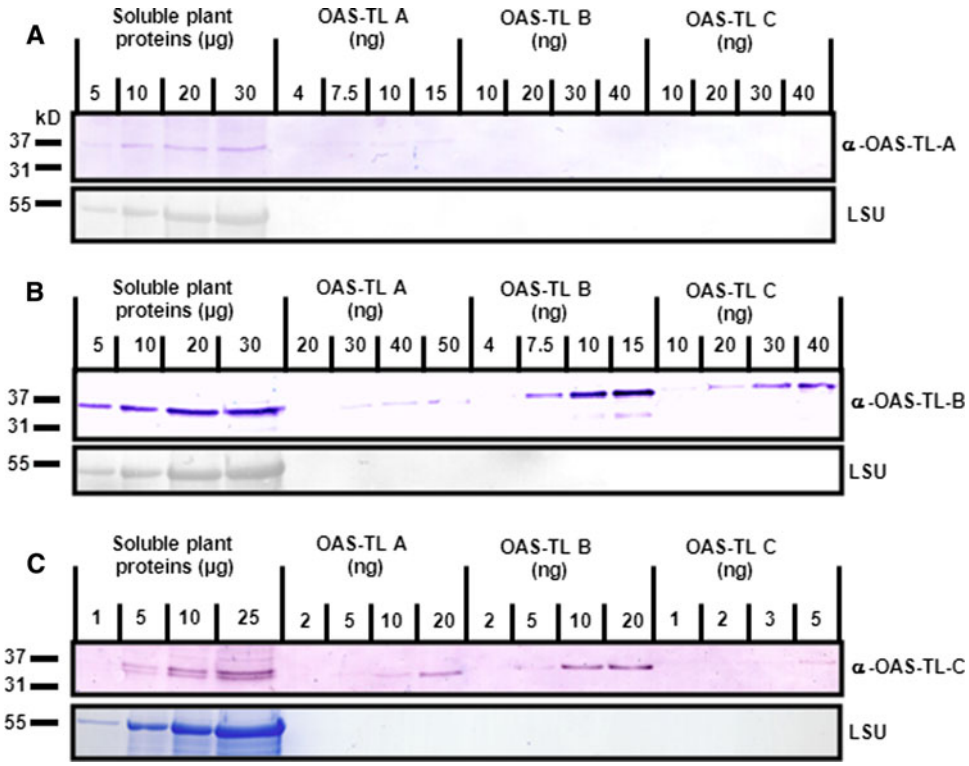

Fig. 4 Quantification of abundances of OAS-TL proteins in leaves of *A. thaliana ecotype Col-0* by immunological detection. The abundance of major OAS-TL isoforms in leaves of 8-week-old *A. thaliana* plants grown on soil was determined with polyclonal immuno-sera against cytosolic (**a**), plastidic (**b**) and mitochondrial OAS-TL (**c**). The serum raised against mitochondrial OAS-TL C showed the highest cross-reactivity with other OAS-TL isoforms and was used to confirm results obtained with immuno-sera against OAS-TL A and B. The large subunit of Rubisco (*LSU*) is shown as loading control for leaf protein

2003a). A homology search with the amino acids sequences of yeast NATs revealed candidates for each catalytically active subunit of NAT-A, -B and-C in Arabidopsis (Polevoda and Sherman 2003b), suggesting a conserved substrate specificity of these complexes also in higher plants (not shown). In agreement with the determined molecular weight of native OAS-TL A from Arabidopsis, OAS-TL A was identified (score, 0.476) as a substrate of NAT-A by the NetAcet 1.0 server (Kiemer et al. 2005), which is optimized for identification of NAT-A substrates in yeast and humans.

Analysis of OAS-TL B allowed the determination of the mature protein in the chloroplast stroma. In addition to two OAS-TL B subspecies identified by Zybailov et al. (2008) in a large scale analysis of the chloroplast proteome by nanoLC-Q-TOF and nanoLC-LTQ-Orbitrap mass spectrometry, the abundances of both acetylated subspecies were quantified and a third acetylated subspecies of OAS-TL B was identified. The number of different N-termini could be explained by a rather floppy cleavage of the plastid transit peptide by stromal processing peptidase (SPP) or precise recognition of cleavage site by SPP followed by subsequent degradation of the OAS-TL B during maturation in the stroma. A rather floppy cleavage by SPP is conceivable, since SPP recognizes the cleavage site rather by physicochemical properties of the sequence in the vicinity of cleavage than by specific amino acid residues (Rudhe et al. 2004). Also a specific maturation of the N-terminus of OAS-TL B by subsequent degradation of single amino acids is possible, since all identified acetylated subspecies were C-terminal of the predicted cleavage site for SPP. In addition, very recently N^{α}-terminal acetylation of proteins was described to create specific degradation signals in yeasts that allow to speculate on maturation of OAS-TL B by action of exo-peptidases (Hwang et al. 2010). In both cases the newly originated N-terminus of OAS-TL B must be acetylated in the stroma of the plastids, which demonstrates that acetylation can occur in the plastids of higher plants in a post-translational manner by a so far unknown process. This result is unexpected since N^{α}-terminal acetylation of proteins is supposed to occur exclusively in a co-translational manner in the cytosol of yeast and mammalian cells (Polevoda and Sherman 2003a, b). Very recently, the significance of post-translational ε-acetylation of lysine residues for regulation of carbon metabolizing proteins was described in bacteria and humans (Wang et al. 2010; Zhao et al. 2010), suggesting a possible regulatory function also for post-translational N^{α}-terminal acetylation.

All known true OAS-TLs (A, B, C, D1 and D2) were purified from leaves by SAP and their abundances after separation by 2D PAGE reflect their contribution to total extractable OAS-TL activity of leaves determined by T-DNA insertion lines of each gene (Heeg et al. 2008; Watanabe et al. 2008a). The public available transcript data of OAS-TLs for different tissues are also consistent with

the amount of purified OAS-TL proteins from the tissues analysed in this study. No unspecific purification of other members of the Bsas protein family (e.g. CS26, CS-like and AtCysC1) with functions other than synthesis of cysteine have been reported in the analyzed tissues, although these members are known to be expressed (Hatzfeld et al. 2000; Alvarez et al. 2010; Bermudez et al. 2010). Taken together these data suggest that SAP is a sensitive and specific biochemical approach to address composition of OAS-TL protein family independently of the availability of genomic sequence of a plant species.

Quantification of OAS-TL proteins in addition to qualitative 2D PAG analysis confirmed that cytosolic OAS-TL A is the dominating isoform, while the mitochondrial OAS-TL C isoform at the site of major OAS formation is minor (Heeg et al. 2008). In fact, OAS-TL A was about ten times more abundant in leaves compared to OAS-TL C. Only trace amounts of cytosolic OAS-TL D1 and D2 were detected, questioning their significant contribution to cysteine synthesis in general. It is intriguing that, apart from tissue-specific differences in abundance, the overall modification patterns were very similar under all conditions tested, which confirms the conserved patterns found in OAS-TL A, B and C knock-out mutants (Heeg et al., 2008). Each subspecies of OAS-TL is therefore reliably produced and thus appears to have an independent physiologically relevant property. These specific properties could change affinities or specificities towards substrates or interaction partners like SAT. All modified OAS-TL subspecies, evidenced in this study, are still able to interact with SAT in principle, since they were purified by SAP. Nonetheless, differences in affinity towards SAT of particular OAS-TL subspecies in planta cannot be excluded. The specific activities of native plant OAS-TL subspecies were in the same range as the specific activities of the same unmodified recombinant OAS-TLs (Wirtz et al. 2004), indicating that the modification of OAS-TLs in planta did not alter significantly the substrate affinity and specificity. It seems more likely that the N^{α}-terminal acetylation of cytosolic and plastidic OAS-TL modifies the turn-over rate of the target proteins, possibly by creation of degradation signals (Hwang et al. 2010).

Acknowledgments We are indebted to Dr. T. Brugger, BASF SE, for support with Edman sequencing. C.H. was generously supported by Landesgraduiertenkolleg BioQuant and the Schmeil Stiftung, Heidelberg.

References

Alvarez C, Calo L, Romero LC, Garcia I, Gotor C (2010) An *O*-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis. Plant Physiol 152:656–669

Berkowitz O, Wirtz M, Wolf A, Kuhlmann J, Hell R (2002) Use of biomolecular interaction analysis to elucidate the regulatory mechanism of the cysteine synthase complex from *Arabidopsis thaliana*. J Biol Chem 277:30629–30634

Bermudez MA, Paez-Ochoa MA, Gotor C, Romero LC (2010) Arabidopsis *S*-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control. Plant Cell 22:403–416

Bradford MM (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

Catrein I, Herrmann R, Bosserhoff A, Ruppert T (2005) Experimental proof for a signal peptidase I like activity in *Mycoplasma pneumoniae*, but absence of a gene encoding a conserved bacterial type I SPase. FEBS J 272:2892–2900

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2:953–971

Giglione C, Serero A, Pierre M, Boisson B, Meinnel T (2000) Identification of eukaryotic peptide deformylases reveals universality of N-terminal protein processing mechanisms. EMBO J 19:5916–5929

Haas FH, Heeg C, Queiroz R, Bauer A, Wirtz M, Hell R (2008) Mitochondrial serine acetyltransferase functions as a pacemaker of cysteine synthesis in plant cells. Plant Physiol 148:1055–1067

Hatzfeld Y, Maruyama A, Schmidt A, Noji M, Ishizawa K, Saito K (2000) β-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis. Plant Physiol 123:1163–1172

Heeg C, Kruse C, Jost R, Gutensohn M, Ruppert T, Wirtz M, Hell R (2008) Analysis of the Arabidopsis *O*-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis. Plant Cell 20:168–185

Hell R, Wirtz M (2008) Metabolism of cysteine in plants and phototrophic bacteria. In: Hell R, Dahl C, Leustek T (eds) Sulfur metabolism in phototrophic organisms. Springer, Dordrecht, pp 61–94

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

Hwang CS, Shemorry A, Varshavsky A (2010) N-terminal acetylation of cellular proteins creates specific degradation signals. Science 327:973–977

Initiative TAG (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815

Jost R, Berkowitz O, Wirtz M, Hopkins L, Hawkesford MJ, Hell R (2000) Genomic and functional characterization of the *oas* gene family encoding *O*-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. Gene 253:237–247

Kiemer L, Bendtsen JD, Blom N (2005) NetAcet: prediction of N-terminal acetylation sites. Bioinformatics 21:1269–1270

Kredich NM (1996) Biosynthesis of cysteine. In: Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger E (eds) *Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology. ASM Press, Washington, DC, pp 514–527

Krueger S, Niehl A, Lopez Martin MC, Steinhauser D, Donath A, Hildebrandt T, Romero LC, Hoefgen R, Gotor C, Hesse H (2009) Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in Arabidopsis. Plant Cell Environ 32:349–367

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Mullen JR, Kayne PS, Moerschell RP, Tsunasawa S, Gribskov M, Colavito-Shepanski M, Grunstein M, Sherman F, Sternglanz R (1989) Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. EMBO J 8:2067–2075

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Patron NJ, Durnford DG, Kopriva S (2008) Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers. BMC Evol Biol 8:39

Polevoda B, Sherman F (2003a) Composition and function of the eukaryotic N-terminal acetyltransferase subunits. Biochem Biophys Res Commun 308:1–11

Polevoda B, Sherman F (2003b) N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. J Mol Biol 325:595–622

Rist W, Mayer M, Andersen J, Roepstorff P, Jørgensen T (2005) Rapid desalting of protein samples for on-line microflow electrospray ionization mass spectrometry. Anal Biochem 342:160–162

Ross S, Giglione C, Pierre M, Espagne C, Meinnel T (2005) Functional and developmental impact of cytosolic protein N-terminal methionine excision in Arabidopsis. Plant Physiol 137:623–637

Rudhe C, Clifton R, Chew O, Zemam K, Richter S, Lamppa G, Whelan J, Glaser E (2004) Processing of the dual targeted precursor protein of glutathione reductase in mitochondria and chloroplasts. J Mol Biol 343:639–647

Saito K (2000) Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Curr Opin Plant Biol 3:188–195

Tocquin P, Corbesier L, Havelange A, Pieltain A, Kurtem E, Bernier G, Perilleux C (2003) A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of *Arabidopsis thaliana*. BMC Plant Biol 3:2

Tsunasawa S, Stewart JW, Sherman F (1985) Amino-terminal processing of mutant forms of yeast iso-1-cytochrome c. The specificities of methionine aminopeptidase and acetyltransferase. J Biol Chem 260:5382–5391

Wang Q, Zhang Y, Yang C, Xiong H, Lin Y, Yao J, Li H, Xie L, Zhao W, Yao Y, Ning ZB, Zeng R, Xiong Y, Guan KL, Zhao S, Zhao GP (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science 327:1004–1007

Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K (2008a) Physiological roles of the β-substituted alanine synthase gene family in Arabidopsis. Plant Physiol 146:310–320

Watanabe M, Mochida K, Kato T, Tabata S, Yoshimoto N, Noji M, Saito K (2008b) Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in Arabidopsis. Plant Cell 20:2484–2496

Wirtz M, Droux M, Hell R (2004) *O*-Acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in *Arabidopsis thaliana*. J Exp Bot 55:1785–1798

Yamaguchi Y, Nakamura T, Kusano T, Sano H (2000) Three Arabidopsis genes encoding proteins with differential activities for cysteine synthase and β-cyanoalanine synthase. Plant Cell Physiol 41:465–476

Zhao C, Kumada Y, Imanaka H, Imamura K, Nakanishi K (2006) Cloning, overexpression, purification, and characterization of *O*-acetylserine sulfhydrylase-B from *Escherichia coli*. Protein Expr Purif 47:607–613

Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, Yao J, Zhou L, Zeng Y, Li H, Li Y, Shi J, An W, Hancock SM, He F, Qin L, Chin J, Yang P, Chen X, Lei Q, Xiong Y, Guan KL (2010) Regulation of cellular metabolism by protein lysine acetylation. Science 327:1000–1004

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136:2621–2632

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS ONE 3:e1994