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Research paper

A spinach *O*-acetylserine(thiol)lyase homologue, SoCSaseLP, suppresses cysteine biosynthesis catalysed by other enzyme isoforms

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Abstract

An enzyme, *O*-acetylserine(thiol)lyase (OASTL), also known as *O*-acetylserine sulfhydrylase or cysteine synthase (CSase), catalyses the incorporation of sulfide into *O*-acetylserine and produces cysteine. We previously identified a cDNA encoding an OASTL-like protein from *Spinacia oleracea*, (*SoCSaseLP*), but a recombinant SoCSaseLP produced in *Escherichia coli* did not show OASTL activity. The exon-intron structure of the *SoCSaseLP* gene shared conserved structures with other spinach OASTL genes. The SoCSaseLP and a *Beta vulgaris* homologue protein, KMT13462, comprise a unique clade in the phylogenetic tree of the OASTL family. Interestingly, when the *SoCSaseLP* gene was expressed in tobacco plants, total OASTL activity in tobacco leaves was reduced. This reduction in total OASTL activity was most likely caused by interference by SoCSaseLP with cytosolic OASTL. To investigate the possible interaction of SoCSaseLP with a spinach cytosolic OASTL isoform SoCSaseA, a pull-down assay was carried out. The recombinant glutathione *S*-transferase (GST)-SoCSaseLP fusion protein was expressed in *E. coli* together with the histidine-tagged SoCSaseA protein, and the protein extract was subjected to glutathione affinity chromatography. The histidine-tagged SoCSaseA was co-purified with the GST-SoCSaseLP fusion protein, indicating the binding of SoCSaseLP to SoCSaseA. Consistent with this interaction, the OASTL activity of the co-purified SoCSaseA was reduced compared with the activity of SoCSaseA that was purified on its own. These results strongly suggest that SoCSaseLP negatively regulates the activity of other cytosolic OASTL family members by direct interaction.

Keywords: Cysteine synthesis; Negative regulation; *O*-acetylserine(thiol)lyase; Spinach

1. Introduction

Sulphate is an essential macronutrient that is found in organisms as reduced sulphur. Plants and microorganisms take up sulphate from the soil and reduce it to sulfide via the assimilatory sulphate reduction pathway [1,2]. Sulfide is incorporated into cysteine, the first reduced sulphur organic compound produced in the assimilation of sulphate. The biosynthesis of cysteine is catalysed by two enzymes, serine acetyltransferase (SAT), which produces *O*-acetylserine (OAS) from serine and acetyl-coenzyme A, and *O*-acetylserine(thiol)lyase (OASTL), which catalyses the β-replacement of the acetoxy group of OAS with sulfide to produce cysteine [3]. These two enzymes form the heterooligomeric cysteine synthase complex (CSC). The CSC consists of a dimer of SAT trimers and two homodimers of OASTL. The OASTL dimers in CSC are inactive and act as regulatory subunits for the activation of SAT [4,5]. In contrast, an increased concentration

Abbreviations: CSase, cysteine synthase; CSC, cysteine synthase complex; GST, glutathione *S*-transferase; OAS, *O*-acetylserine; OASTL, *O*-acetylserine(thiol)lyase; SAT, serine acetyltransferase.

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of OAS induces destabilization of the CSC, which results in inactivation of SAT. When released from CSC, OAS is converted into cysteine by the large excess of free OASTL dimers [6].

The enzyme OASTL is also known as *O*-acetylserine sulfhydrylase or cysteine synthase (CSase) [3]. In *Arabidopsis thaliana*, genes encoding nine OASTL isoforms have been identified [2]. These isoforms show distinct subcellular localization. Cytosolic isoforms are CYS-D1, CYS-D2, DES1 and OAS-A1 [7–9]. The OAS-A2 gene does not produce a functional enzyme [8]. The isoforms localized in plastids are CS26 and OAS-B [10,11]. The OAS-C and CYS-C1 isoforms are localized in the mitochondria [12,13]. Several unique functions of these OASTL family members have been uncovered. Cysteine biosynthesis is catalysed in the cytosol, plastids and mitochondria by OAS-A1, OAS-B and OAS-C, respectively. Two other cytosolic isoforms, CYS-D1 and CYS-D2, also have OASTL activity, but their expression levels are very low compared with those of OAS-A1, OAS-B and OAS-C [14]. The mitochondrial isoform CYS-C1 is a β-cyanoalanine synthase [15,16]. The plastid isoform CS26 functions as a *S*-sulfocysteine synthase [11], and the cytosolic isoform DES1 is a cysteine desulfhydrase and degrades cysteine [8]. Therefore these OASTL family members are included in the metabolic regulation of cysteine and its related compounds [2].

In *Spinacia oleracea*, three cDNAs, namely *SoCSaseA*, *SoCSaseB* and *SoCSaseC*, have been found to encode the OASTL proteins localized in the cytosol, plastids and mitochondria, respectively [17–19]. In addition to these major OASTL family members, we previously isolated another cDNA encoding CSase-like protein (SoCSaseLP) [20]. The predicted amino acid sequence of SoCSaseLP showed a high similarity (approximately 70%) to that of SoCSaseA, but the recombinant SoCSaseLP did not show any OASTL activity [20]. In the present study, we produced transgenic tobacco plants in which the *SoCSaseLP* gene was expressed. Interestingly, the total OASTL activity in the transgenic tobacco plants was down-regulated by the ectopic expression of the *SoCSaseLP* gene. The SoCSaseLP interacted with SoCSaseA and reduced the OASTL activity of SoCSaseA *in vitro*. These results suggest that SoCSaseLP acts as a negative regulator of cysteine biosynthesis.

2. Materials and methods

2.1. Plasmid construction

The β-glucuronidase gene of pBI121 was replaced by the insertion of the cDNA fragments encoding *SoCSaseA* and *SoCSaseLP* to produce the overexpression constructs, pBI-CSaseA and pBI-CSaseLP, respectively. For expression of a fusion protein with glutathione *S*-transferase (GST), the *SoCSaseLP* cDNA fragment was inserted into the *Bam*HI/*Eco*RI site of pGEX-2T (GE Healthcare Japan, Tokyo, Japan). The resultant plasmid, pGST-CSLP, was used for production of a GST-fused SoCSaseLP (called GST-CSLP). The plasmid, pET-CSA [20], was used for production of a histidine-tagged SoCSaseA protein (called His-CSA).

2.2. Transgenic plants

Transgenic tobacco plants (*Nicotiana tabacum* cv. SR1) were obtained by infection of *Agrobacterium tumefaciens* cells harbouring pBI-CSaseA or pBI-CSaseLP. Transgenic lines with a single functional T-DNA locus were selected by a 3:1 segregation for kanamycin resistance in seeds obtained from the primary transformants. A transgenic line with an empty vector (EV) was produced as previously described [21]. We selected the null segregants among the offspring of a transgenic line, L6, in which an expression construct for the *SoCSaseLP* gene was segregated away. For detection of T-DNA, the entire region of the *SoCSaseLP* gene in the expression cassette was amplified.

2.3. Isolation of spinach OASTL genes

We designed primers by using the 5′ and 3′ terminal sequences of the spinach OASTL cDNAs [17–20]. The nucleotide sequences of the primers used are shown in Table S1. The spinach total DNA was prepared according to Walbot and Warren [22] and then further purified by CsCl ultracentrifugation. The genomic fragment for each spinach OASTL gene was amplified by using LA Taq DNA polymease (Takara Bio Inc., Shiga, Japan) and sequenced. The genomic nucleotide sequences were deposited in the GenBank data libraries under accession numbers, AB426587 (*SoCSaseA*), AB426588 (*SoCSaseB*), AB426589 (*SoCSaseC*) and AB426590 (*SoCSaseLP*).

2.4. Phylogenetic analysis

The full lengths of amino acid sequences deduced from the OASTL cDNAs were aligned using the GENETYX ver. 17 software package (GENETYX Co., Tokyo, Japan). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA ver6; http://www.megasoftware.net).

2.5. Northern blot analysis

Total RNA was prepared from leaves using TRIzol reagent (Thermo Fisher Scientific Inc., MA, USA) and 20 μg of total RNA was subjected to northern blot analysis as previously described [23]. The full-length cDNA fragment for *SoCSaseLP* was labelled with digoxigenin using the PCR DIG probe synthesis kit (Roche Diagnostics, Basel, Switzerland). Partial fragments of the tobacco plastid OASTL cDNA (nucleotide position 743-1242, GenBank acc. no. AM087457) and tobacco cytosol OASTL cDNA (nucleotide position 757-1256, GenBank acc. no. AM087458) were also used for preparation of digoxigenin-labelled DNA probes. Hybridization and detection of hybridized probes were carried out according to the manufacturer's protocol.

2.6. Determination of OASTL enzyme activity in the transgenic plants

Approximately 0.5 g of tobacco leaves were homogenized with 1 mL of 50 mM Tris–HCl, pH 8.0. The homogenate was centrifuged at 10,000 *g* for 15 min and the supernatant was supplemented with ammonium sulphate to 80% saturation. The resulting precipitant was dissolved in 50 mM Tris–HCl, pH 8.0, and desalted by dialysis against 50 mM Tris–HCl, pH 8.0. Protein content was determined as described by Bradford [24] with bovine serum albumin as the standard. Enzymatic activity of OASTL was determined according to Gaitonde [25]. One unit (U) corresponded to the amount of enzyme required to catalyse the formation of 1 μmol of cysteine per min at 25 °C.

2.7. Affinity chromatography

The plasmids pET-CSA, pGST-CSLP, and pGEX-2T were introduced into *Escherichia coli* BL21-CodonPlus(DE3)-RP cells (Agilent Technologies Inc., CA, USA). Production of recombinant proteins was induced by the addition of IPTG at a final concentration of 0.5 mM. After overnight culture, cells were collected by centrifugation. For isolation of GST and GST-fusion proteins, the *E. coli* cells were suspended in a binding buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3) and disrupted by sonication at 4 °C. The lysate was centrifuged at 10,000 *g* for 15 min and then applied to the glutathione affinity chromatography (GSTrap HP column; GE Healthcare). The GST and GST-fusion proteins were eluted in an elution buffer (called buffer-G; 50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione). For isolation of the recombinant His-CSA protein, proteins were released from *E. coli* cells into a binding buffer (20 mM sodium phosphate buffer containing 20 mM imidazole, 0.5 M NaCl, pH 7.4). The His-CSA protein was purified by immobilized metal affinity chromatography (HisTrap HP column, GE Healthcare). The trapped His-CSA molecules were eluted in an elution buffer (called buffer H; 20 mM sodium phosphate buffer containing 500 mM imidazole and 0.5 M NaCl, pH 7.4). To determine the potential interaction of SoCSaseLP with SoCSaseA, crude extracts of *E. coli* cells harbouring pGST-CSLP and pET-CSA were applied to the glutathione affinity chromatography. As a control, extracts of *E. coli* cells harbouring pGEX-2T and pET-CSA were also applied to the same chromatography.

2.8. Detection of recombinant proteins

For detection of recombinant proteins, the elution fractions were processed by SDS-PAGE and blotted onto PVDF membranes (FluoroTrans; Nihon Pall Ltd., Tokyo, Japan). Nonspecific binding of the antibodies was blocked with EZBlock reagent (ATTO Corp., Tokyo, Japan) overnight at 4 °C. The blots were washed three times with washing buffer (0.15 M PBS, pH 7.3, 0.1% Tween 20). The recombinant His-CSA proteins on the PVDF membranes were cross-reacted with a rabbit anti-spinach OASTL antibody [26] for 1 h. After washing three times for 10 min each, the blots were incubated for 60 min with a goat anti-rabbit IgG-HRP (Tanpaku Seisei Kogyo, Isezaki, Japan) at a 1:2000 dilution. The blots were washed as before and the colour was developed by adding EzWestBlue (ATTO Corp.). For detection of GST proteins, the blots were incubated with Anti-GST-HRP Conjugate (GE Healthcare) and the colour was developed as described above. When GST-CSLP proteins were detected, the gels were stained with silver nitrate using the 2D-Silver Stain Reagent II (Cosmobio, Tokyo, Japan).

2.9. Enzyme activity in the elution fraction of the affinity chromatography

The enzymatic activity of OASTL was affected by the composition of the elution buffer used in the affinity chromatography. His-CSA proteins in buffer G (an elution buffer for glutathione-affinity chromatography) showed a higher OASTL activity when compared with the activity determined in buffer H (an elution buffer for immobilized metal affinity chromatography). Equal amounts of purified His-CSA protein were mixed with buffer G and with buffer H, and the OASTL activity was determined in each solution. The mean enzyme activity in buffer G was 121 ± 5% (n = 3) of the corresponding activity in buffer H. The OASTL activity of the GST-CSLP and His-CSA complex that had been eluted in buffer G was normalized using the coefficient shown above. The His-CSA protein level was determined by quantification of immunologically detected bands marked with the anti-spinach OASTL antibody [26]. The OASTL activities of serial dilutions of purified His-CSA protein were determined. Then the His-CSA protein level in the diluted fraction was determined after immunological detection. The intensity of target bands was quantified using Image J software. Similarly, the OASTL activity of the GST-CSLP and His-CSA complex was determined. Then, the His-CSA protein in the GST-CSLP and His-CSA complex was immunologically detected. The level of His-CSA protein in the complex was estimated by comparison of the intensity of detected bands from serial dilutions of purified His-CSA. The specific activity of OASTL corresponded to the OASTL activity per the His-CSA band intensity. The specific activity in the GST-CSLP and His-CSA complex fractions were compared to the specific activity of the His-CSA purified from the crude extract of *E. coli* cells harbouring pET-CSA using immobilized metal affinity chromatography.

3. Results

3.1. Sequence analysis of spinach OASTL genes

We checked whether the *CSaseLP* gene and other spinach OASTL genes diverged from a common ancestral gene. Primer pairs were prepared from the 5′ and 3′ terminal sequences of spinach cDNAs and used for amplification of their genomic sequences. The *SoCSaseB* and *SoCSaseLP* gene

consisted of 10 exons separated by 9 introns. The number of exons was increased in the *SoCSaseA* because there was an additional intron insertion between exon 1 and exon 2 compared with the *SoCSaseLP* gene. Exon 3 of the *SoCSaseC* gene harboured the exonic sequences corresponding to exon 3 and exon 4 of the *SoCSaseLP* gene. The insertion position of intronic sequences were highly conserved among these four genes (Fig. 1). These results indicate that these four spinach OASTL genes are diverged from a common ancestral gene. Therefore, the *SoCSaseLP* gene is a homologue of the spinach OASTL gene family, although it does not show OASTL activity [20].

3.2. Phylogenetic analysis of SoCSaseLP

The genus *Spinacia* is placed in the *Chenopodioideae* subfamily of the *Amaranthaceae*/*Chenopodiaceae* alliance. Recently a reference genome sequence for *Beta vulgaris* was provided [27]. *B. vulgaris* (sugar beet) is placed in *Betoideae*, a subfamily closely related to *Chenopodiaceae* [28]. Therefore, we searched amino acid sequences of the *B. vulgaris* OASTL family to clarify the relatedness of SoCSaseLP to the other OASTL family members from spinach and sugar beets. Using the predicted amino acid sequences of spinach and *Arabidopsis* OASTL family genes in a BLASTp search, we identified five protein sequences from *B. vulgaris* and one novel spinach sequence that shared high similarities with the queried OASTL genes (E value $< e^{-100}$). The amino acid sequences of *B. vulgaris* accessions KMT13461, KMT15522, KMT20449 and KMT13462 showed high similarity to those of SoCSaseA, SoCSaseB, SoCSaseC and SoCSaseLP, respectively. In addition, a BLASTp search using the CS26 amino acid sequence revealed homologous proteins from *B. vulgaris* (KMS97610) and *S. oleracea* (KNA15399) [27]. A phylogenetic tree was constructed using these protein sequences (Fig. 2). The *Arabidopsis* OASTL family members excluding OAS-A2 were divided into five distinct clades. Clades I and II include major OASTL isoforms, namely OAS-A1, OAS-B and OAS-C. Clades III and IV include β-cyanoalanine synthase

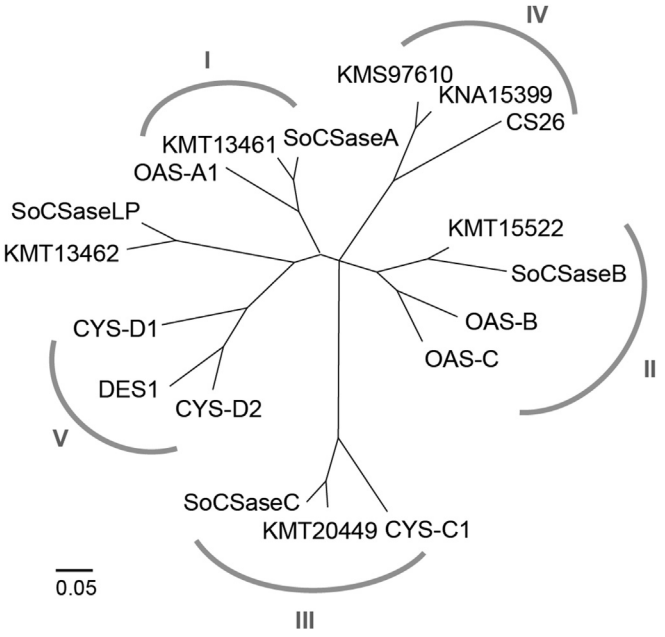

Fig. 2. Neighbour-joining phylogenetic tree of OASTL gene family members. The tree was built for the sequences from *Arabidopsis* (OAS-A1, OAS-B, OAS-C, CYS-C1, CYS-D1, CYS-D2, DES1 and CS26), sugar beet (KMT13461, KMT13462, KMT15522, KMT20449 and KMS97610) and spinach (SoCSaseA, SoCSaseB, SoCSaseC, KNA15399 and SoCSaseLP). The whole amino acid sequences were aligned and the tree was constructed with the MEGA tool. The scale bar indicates the distance corresponding to 5 changes per 100 amino acid positions.

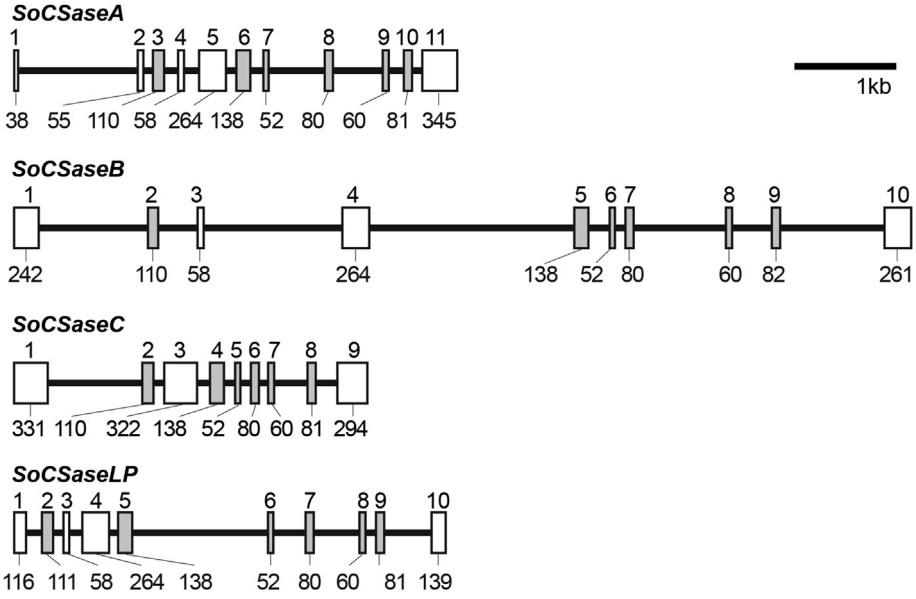

Fig. 1. The exon-intron structures of the spinach OASTL gene family members. Exons and introns are indicated by boxes and bars. The numbers above the boxes indicate the exon number. The numbers under the boxes are the nucleotide lengths of each exon. Grey boxes indicate the exons which show the same nucleotide length among these four spinach OASTL gene family members.

and *S*-sulfocysteine synthase, respectively. Clade V consists of DES-1 and two minor OASTL members (CYS-D1 and CYS-D2). The spinach four isoforms (SoCSaseA, SoCSaseB, SoCSaseC, KNA15399) and the corresponding four *B. vulgaris* isoforms (KMT13461, KMT15522, KMT20449, KMS97610) were assigned to Clades I, II, III and IV, respectively. However, we could not find spinach or *B. vulgaris* genes that were homologous to the *Arabidopsis* isoforms of Clade V using a BLASTp search. The SoCSaseLP and *B. vulgaris* KMT13462 protein formed a unique group that were distinct from any *Arabidopsis* OASTL family members (Fig. 2).

Fig. 3 shows the comparison of amino acid sequences of the OAS-A1, SoCSaseA, SoCSaseLP and the *B. vulgaris* KMT13462 protein. The OAS-A1 Ser-75 is highly conserved among the plant OASTL [29], SoCSaseLP and KMT13462 sequences. However, Gly replaces Ser-75 in *A. thaliana* DES1 [9], and Ala replaces Ser-75 in *Brassica napus* DES1 [29]. The substitutions of the *Glycine max* OASTL residues, T81M, S181M and T185S, caused a successful switch from OASTL reaction to β-cyanoalanine synthase reaction [30]. These three residues correspond to Thr-78, Ser-178 and Thr-182 in OAS-A1, and they are conserved in SoCSaseLP and KMT13462 sequences (Fig. 3). The 217–230 loop in OAS-A1 is crucial for the interaction with SAT [31] and conserved well in the SoCSaseLP and KMT13462 sequences. In contrast, the CS26 protein does not have the sequences corresponding to the 217–230 loop [11]. Therefore, the amino acid sequence of SoCSaseLP has similar characteristics to OASTL rather than the other family members, such as cysteine desulfhydrase, β-cyanoalanine synthase, and *S*-sulfocysteine synthase.

3.3. Ectopic expression of the SoCSaseLP gene in tobacco plants

To reveal the function of SoCSaseLP *in vivo*, an expression cassette for the *SoCSaseLP* gene was introduced into tobacco plants. In addition, we produced transgenic tobacco plants in which the *SoCSaseA* gene was expressed. Transgenic plants with a single T-DNA insertion locus were selected, and the OASTL activity in their leaves was determined (Fig. 4). The OASTL activity of the transgenic plants with the *SoCSaseA* gene (lines A3 and A9) showed approximately 5.5-fold and 1.8-fold increases, respectively, compared with that of the plants transformed with an EV. In contrast, OASTL activity in the tobacco plants (lines L6 and L7) transformed with the *SoCSaseLP* gene was reduced by 49% and 72%, respectively, in comparison with the activity in the EV plants. To determine whether the reduced OASTL activities resulted from transgene-induced RNA silencing, we determined the transcript levels of the *SoCSaseLP* transgene and the endogenous tobacco OASTL genes in the T-DNA-harbouring plants and null segregant plants of the L6 line. A reduced OASTL activity was consistently observed in the plants expressing the *SoCSaseLP* gene. These *SoCSaseLP* transgene-harbouring tobacco plants expressed endogenous tobacco OASTL genes encoding cytosolic and plastidial isoforms at the same level as the null segregant plants (Fig. 5). These results indicate that reduction of the OASTL activity was not caused by RNA silencing and strongly suggest that SoCSaseLP itself reduced the total OASTL activity in the transgenic tobacco plants.

3.4. Interaction of SoCSaseLP with SoCSaseA

Because there is no N-terminal transit peptide in the predicted amino acid sequence, SoCSaseLP is considered to be a cytosolic isoform in the OASTL family (Fig. 3). One of the possible explanations for the reduction in OASTL activity caused by expression of the *SoCSaseLP* gene (Fig. 5) is that SoCSaseLP inactivates the tobacco cytosolic OASTL isoform after formation of a complex between them. We tried to detect the recombinant SoCSaseLP and SoCSaseA proteins in the tobacco protein extracts prepared from the corresponding transgenic plants, but we could not detect them with an anti-spinach OASTL antibody [26]. The failure to detect the recombinant proteins may be explained by their low level of accumulation in tobacco plants. We then investigated whether SoCSaseLP interacts with other spinach OASTL family member by using the *E. coli* expression system. We prepared a GST fusion protein with SoCSaseLP (GST-CSLP) and a His-tagged protein with SoCSaseA (His-CSA). When the GST protein was purified from *E. coli* cells carrying pGEX-2T and pET-CSA, the GST protein was detected by an anti-GST antibody, but there was no detectable His-CSA protein in the elution fraction (lane 2, Fig. 6). In contrast, the His-CSA protein was co-purified with the GST-CSLP from *E. coli* cells harbouring pGST-CSLP and pET-CSA by glutathione affinity chromatography (lane 3, Fig. 6). The immunological detection of the GST-CSLP with an anti-GST antibody was successful only when a large amount of GST-CSLP protein was subjected to the western analysis. Therefore, the detection of a recombinant GST-CSLP protein in the elution fraction was confirmed by silver staining (Fig. 6, lanes 1 and 3). These results indicate an interaction of SoCSaseLP with SoCSaseA.

We next determined the OASTL activity of the SoCSaseA–SoCSaseLP complex. The His-CSA protein was purified from *E. coli* cells carrying pET-CSA by immobilized metal affinity chromatography and used as a control. The complex consisting of the His-CSA and GST-CSLP proteins was prepared from *E. coli* cells carrying pET-CSA and pGST-CSLP by glutathione affinity chromatography. The His-CSA protein level in the elution fraction from glutathione affinity chromatography was determined by quantification of His-CSA protein band intensities in the SDS-PAGE electrophoretogram. Because the OASTL activity was affected by the composition of the elution buffer, the OASTL activity of His-CSA in the elution buffer used in glutathione affinity chromatography was normalized and then compared with the activity of His-CSA purified by immobilized metal chromatography (see Materials and methods). When the OASTL activity of the purified His-CSA protein was expressed as 100%, the OASTL activity of the His-CSA protein in the His-CSA and GST-CSLP complex was estimated as 68 ± 3% (mean ± SD,

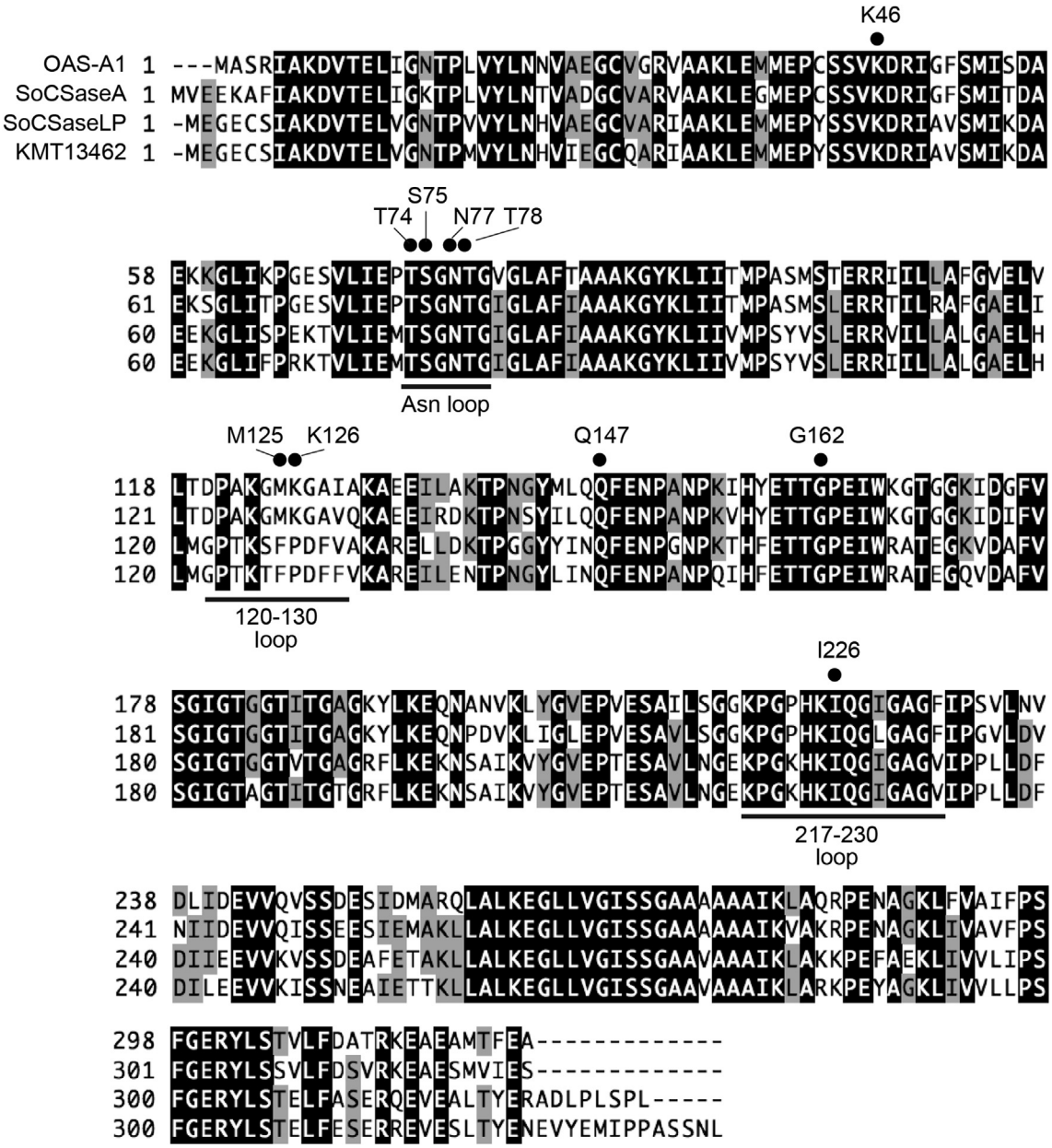

Fig. 3. Comparison of amino acid sequences of two cytosolic OASTL enzymes (OAS-A1 and SoCSaseA), SoCSaseLP and a *Beta vulgaris* homologue of SoCSaseLP, KMT13462. Identical amino acid residues are shown with black background. Grey shading indicates the 75% conservation among the aligned sequences. The amino acid residues indicated by closed circles are essential for the OASTL activity. Their position numbers were based on the *Arabidopsis* OAS-A1 amino acid sequences. The importance of residues K46, T74, S75, N77 and Q147 on the OASTL activity have been reported by Bonner et al. [31]. The residues T74, T78, M125, K126 and I226 have been suggested to interact with the C-terminal peptide of SAT [34]. Amino acid substitution mutation at G162 abolished OASTL activity [36]. The 120–130 loop and 217–230 loop are involved in the repartitioning of the surface cleft where the C-terminal peptide of SAT interacts [34]. The Asn loop is part of the OASTL catalytic centre [10].

n = 4). These results indicated that SoCSaseLP negatively regulates the OASTL activity of SoCSaseA.

4. Discussion

Here we showed that the *SoCSaseLP* gene is a member of the OASTL gene family and that it originated from a common ancestral gene (Fig. 1), but the *SoCSaseLP* is a dysfunctional OASTL enzyme. In fact, the OASTL activities of the SoCSaseLP and SoCSaseA were 0.013 U mg protein^{-1} and 56.7 U mg protein^{-1}, respectively [20]. SoCSaseLP and a *B. vulgaris* homologue, KMT13462, consist of a unique clade in the phylogenetic tree (Fig. 2). Cysteine biosynthesis from OAS and sulfide is catalysed by free OASTL homodimers but not by the OASTL subunits in the CSC [6,32]. There are 4 cytosolic OASTL isoforms in *Arabidopsis*, namely CYS-D1, CYS-D2, DES1 and OAS-A1. However, it is still unclear whether two distinct OASTL isoforms can form a heterodimer. Here, we show that SoCSaseLP negatively regulates cysteine biosynthesis in tobacco plants (Figs. 4 and 5). The cytosolic

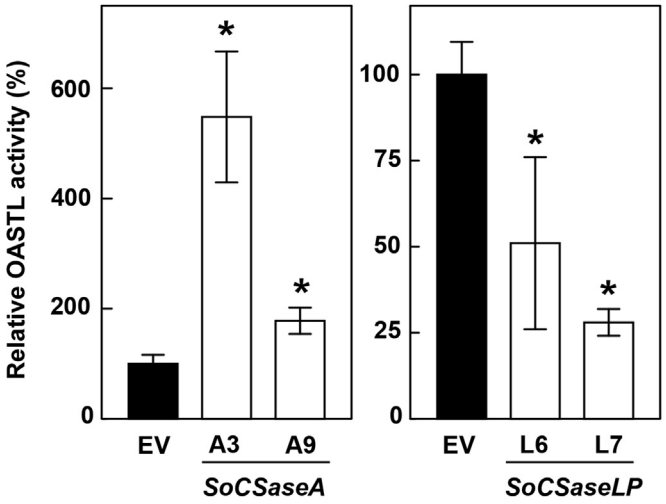

Fig. 4. The OASTL activity of the transgenic tobacco plants. Two independent transgenic lines, A3 and A9, expressed the *SoCSaseA* gene and other two lines, L6 and L7, expressed the *SoCSaseLP* gene. The relative values for the total OASTL activity of control transgenic EV line EV are shown. The data indicate the mean ± SD (n = 3). *indicates a statistically significant difference at $P < 0.05$ between the control EV plants and the plants transformed with the *SoCSaseA* or *SoCSaseLP* genes.

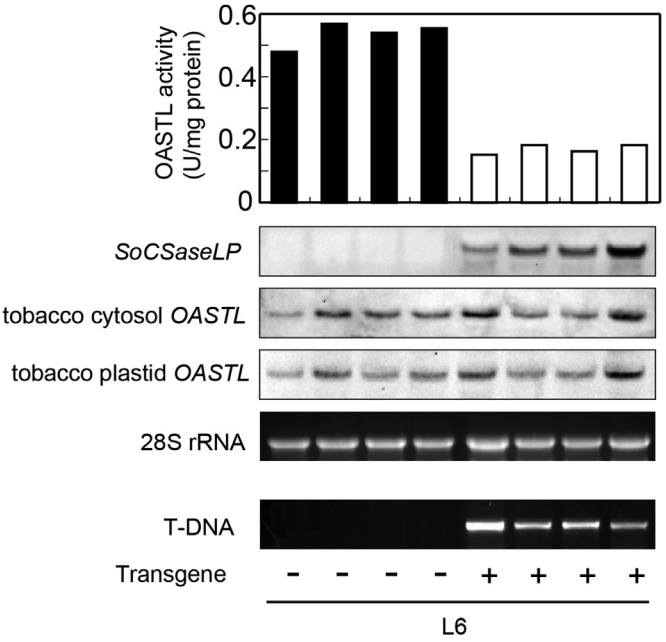

Fig. 5. Effects of overexpression of the *SoCSaseLP* gene on the expression of tobacco OASTL genes. Four null segregants and four segregants harbouring the *SoCSaseLP* transgenes of the L6 lines were provided. The total OASTL activities are shown with the amounts of the *SoCSaseLP* mRNA and tobacco mRNAs encoding the cytosolic and plastidial OASTLs. The 28S rRNA is shown to assess the equivalence of RNA loading. The entire SoCSaseLP transgene was amplified for the detection of the T-DNA.

OASTL isoform was a main contributor of OASTL activity in leaves (44% of total OASTL activity) and roots (80% of total OASTL activity) in *Arabidopsis* [10]. Therefore, the large reduction of total OASTL activity in tobacco was most likely caused by SoCSaseLP-mediated inhibition of tobacco

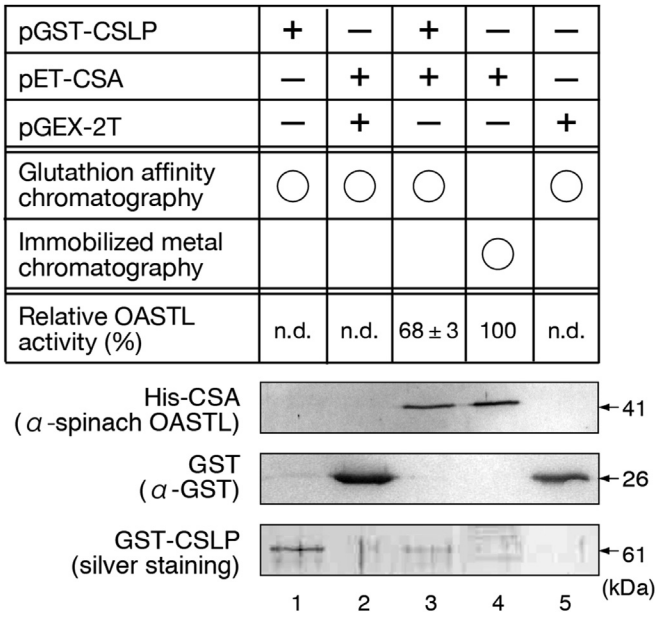

Fig. 6. Interaction of the SoCSaseLP with SoCSaseA in *E. coli* cells. The *E. coli* cells were transformed with the plasmids as indicated. The use of affinity chromatography for protein purification was also indicated. The SoCSaseLP and SoCSaseA proteins were fused with GST and histidine tags, respectively. In lanes 1, 2, 3 and 5, the crude extracts were used for purification with glutathione affinity chromatography. In lane 4, the crude protein fraction was subjected to immobilized metal affinity chromatography. The recombinant His-CSA was detected by western blotting with an anti-spinach OASTL antibody [26]. The fusion proteins with GST and GST by itself were detected by an anti-GST antibody. The production of a recombinant GST-CSLP protein was confirmed by silver staining. The OASTL activity of the GST-CSLP and His-CSA complex (lane 3) per the His-CSA protein level was determined as described in Materials and methods, and then normalized for the corresponding specific activity of the purified His-CSA fraction (lane 4). The data indicate mean ± SD (n = 4). n.d. means not determined.

cytosolic OASTL activity. Unfortunately, we failed to detect the recombinant SoCSaseLP and SoCSaseA proteins in the transgenic tobacco plants with an anti-spinach OASTL antibody. When the recombinant GST-CSLP and His-CSA proteins were co-expressed in *E. coli*, His-CSA was co-purified with GST-CSLP by glutathione affinity chromatography. Consistent with this interaction, the OASTL activity of co-purified His-CSA was reduced when compared with the His-CSA purified on its own from *E. coli* cells harbouring pET-CSA. These results indicate that SoCSaseLP interacts with cytosolic OASTL family members and negatively regulates their OASTL activity.

Two OASTL homodimers and a SAT homohexamer form a CSC, which results in a reduction of OASTL activity [6]. In the CSC, one of the two active sites in the OASTL homodimer (designated here as the first active site) is occupied by the last 10 C-terminal residues of one of the SAT subunits (called the C10 peptide) [33,34]. Upon binding of the C10 peptide to the first active site, another active site of the OASTL homodimer (designated as the second active site) loses affinity for the C10 peptide. Therefore, the second active site of OASTL homodimers in the CSC is free of the C10 peptide, and shows significantly reduced catalytic OASTL activity [5,6,35]. These

findings indicate that a conformational change in the first active site allosterically induces a conformational change in the second active site. In fact, the binding of the C10 peptide at the first active site induces allosteric closure of the second active site and hinders substrate binding [35]. In *Arabidopsis* OAS-A1, several amino acid residues have been identified as important for the regulation of OASTL catalytic activity. These residues, Thr-74, Ser-75 and Gln-147, lock the C10 peptide in the active site [34]. Two residues, Thr-74 and Ser-75, are located on the Asn-loop (Thr-74 to Gly-79) and function, together with Gln-147, as a gate that determines substrate accessibility [31,35]. The Gly-162 residue has been shown to be critical to OASTL activity [36]. The binding site of the C10 peptide extends from the Schiff base formed by Lys-46 and a pyridoxal 5′-phosphate through a surface cleft formed by the 217–230 loop (Lys-217 to Phe-230) on one side and the 120–130 loop (Asp-120 to Ile-130) on the opposite side [34]. The binding of the C10 peptide to OAS-A1 is accompanied by an interaction with Met-125/Lys-126 of the 120–130 loop and Ile-226 in the 217–230 loop [34]. Therefore, the C10 peptide-induced conformational changes should include the conformational alignment of 120–130 and 217–230 loops, and such conformational changes eventually induce allosteric closure of the second active site of the OASTL homodimer [35]. Most of the amino acid residues relevant to the active site responsible for OASTL activity are highly conserved among the cytosolic isoforms OAS-A1, SoCSaseA, SoCSaseLP and a *B. vulgaris* homolog, KMT13462 (Fig. 3). However, the amino acid residues in the C120-130 loop are quite different between the OAS-A1/SoCSaseA and SoCSaseLP/KMT13462, which should cause conformational differences of the cleft structure and affect substrate accessibility to the active site. If the SoCSaseLP and SoCSaseA form a heterodimer, it is possible that the differences in the residues of the 120–130 loop of SoCSaseLP down-regulate the OASTL activity of SoCSaseA. In addition, the variation in amino acid residues located on the 120–130 loop in the SoCSaseLP should alter the formation of a complex with the SAT homohexamer. Here we showed the interaction between the SoCSaseLP and SoCSaseA, but it still remains to be clarified whether these two proteins exist as a heterodimer or a highly polymerised form. Although additional comprehensive studies are required to fully explain the function of SoCSaseLP, our results suggest that an isoform of the OASTL family binds to another member of the OASTL family and regulates its activity in a manner that differs relative to the corresponding homodimer.

5. Conclusion

In general, a free OASTL homodimer catalyses cysteine synthesis and OASTL activity is negatively regulated by the formation of a CSC with a SAT homohexamer. Here, we showed another mode of regulation of OASTL activity through the formation of a hetero-complex with a different OASTL isoform. A spinach OASTL isoform, SoCSaseLP, showed no apparent OASTL activity. In addition, ectopic expression of the *SoCSaseLP* gene in tobacco plants reduced total OASTL activity. The interaction of SoCSaseLP with the spinach cytosolic isoform of OASTL, SoCSaseA, also negatively regulated the OASTL activity of the SoCSaseA protein. These results suggest the formation of a hetero-complex between the OASTL isoforms that modulates OASTL activity.

Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biopen.2016.01.002.

References

[1]. M.S. Khan, F.H. Haas, A.A. Samami, A.M. Gholami, A. Bauer, K. Fallenberg, M. Reichelt, R. Hänsch, R.R. Mendel, A.J. Meyer, M. Wrtz, R. Hell, Sulfite reductase defines a newly discovered bottleneck for assimilatory sulfate reduction and is essential for growth and development in *Arabidopsis thaliana*, Plant Cell 22 (2010) 1216–1231.

[2]. I. García, L.C. Romero, C. Gotor, Cysteine homeostasis, in: J.P.F. D'Mello (Ed.), Amino Acids in Higher Plants, CABI Publishing, Wallingford, 2015, pp. 219–233.

[3]. J.M. Jez, S. Dey, The cysteine regulatory complex from plants and microbes: what was old is new again, Curr. Opin. Struct. Biol. 23 (2013) 302–310.

[4]. M. Wirtz, R. Hell, Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties, J. Plant Physiol. 163 (2006) 273–286.

[5]. A. Feldman-Salit, M. Wirtz, R. Hell, R.C. Wade, A mechanistic model of the cysteine synthase complex, J. Mol. Biol. 386 (2009) 37–59.

[6]. M. Droux, M.L. Ruffet, R. Douce, D. Job, Interactions between serine acetyltransferase and *O*-acetylserine(thiol)lyase in higher plants, Eur. J. Biochem. 255 (1998) 235–245.

[7]. C. Barroso, J.M. Vega, C. Gotor, A new member of the cytosolic *O*-acetylserine(thiol)lyase gene family in *Arabidopsis thaliana*, FEBS Lett. 363 (1995) 1–5.

[8]. R. Jost, O. Berkowitz, M. Wirtz, L. Hopkins, M.J. Hawkesford, R. Hell, 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 (2000) 237–247.

[9]. C. Álvarez, L. Calo, L.C. Romero, I. García, C. Gotor, An *O*-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis, Plant Physiol. 152 (2009) 656–669.

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

[11]. M.A. Bermúdez, M.A. Páez-Ochoa, C. Gotor, L.C. Romero, *Arabidopsis S*-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control, Plant Cell 22 (2010) 403–416.

[12]. I. García, J.M. Castellano, B. Vioque, R. Solano, C. Gotor, L.C. Romero, Mitochondrial β-cyanoalanine synthase is essential for root hair formation in *Arabidopsis thaliana*, Plant Cell 22 (2010) 3268–3279.

[13]. C. Álvarez, I. García, L.C. Romero, C. Gotor, Mitochondrial sulfide detoxification requires a functional isoform O-acetylserine(thiol)lyase C in *Arabidopsis thaliana*, Mol. Plant 5 (2012) 1217–1226.

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

[15]. Y. Hatzfeld, A. Maruyama, A. Schmidt, M. Noji, K. Ishizawa, K. Saito, β-cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis, Plant Physiol. 123 (2000) 1163–1171.

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

[17]. K. Saito, N. Miura, M. Yamazaki, H. Hirano, I. Murakoshi, Molecular cloning and bacterial expression of cDNA encoding a plant cysteine synthase, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 8078–8082.

[18]. K. Saito, K. Tatsuguchi, I. Murakoshi, H. Hirano, cDNA cloning and expression of cysteine synthase B localized in chloroplasts of *Spinacia oleracea*, FEBS Lett. 324 (1993) 247–252.

[19]. K. Saito, K. Tatsuguchi, Y. Takagi, I. Murakoshi, Isolation and characterization of cDNA that encodes a putative mitochondrion-localizing isoform of cysteine synthase (*O*-acetylserine(thiol)-lyase) from *Spinacia oleracea*, J. Biol. Chem. 269 (1994) 28187–28192.

[20]. R. Asai, J. Nakata, K. Takahashi, H. Kodama, M. Masada, Molecular cloning of a cDNA that encodes a spinach cysteine synthase-like protein, Plant Biotechnol. 21 (2004) 73–77.

[21]. H. Kodama, T. Hamada, G. Horiguchi, M. Nishimura, K. Iba, Genetic enhancement of cold tolerance by expression of a gene for chloroplast ω-3 fatty acid desaturase in transgenic tobacco, Plant Physiol. 105 (1994) 601–605.

[22]. V. Walbot, C. Warren, Regulation of *Mu* element copy number in maize lines with an active or inactive mutator transposable element system, Mol. Genet. Genom. 211 (1988) 27–34.

[23]. H. Kodama, M. Ito, N. Ohnishi, I. Suzuka, A. Komamine, Molecular cloning of the gene for plant proliferating-cell nuclear antigen and expression of this gene during the cell cycle in synchronized cultures of *Catharanthus roseus* cells, Eur. J. Biochem. 197 (1991) 495–503.

[24]. M.M. Bradford, A rapid and sensitive methods for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[25]. M.K. Gaitonde, A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids, Biochem. J. 104 (1967) 627–633.

[26]. S. Kitamura, T. Yamaguchi, X. Zhu, M. Masada, Physiological study of cysteine synthase isozymes in *Spinacia oleracea* seedlings, Biosci. Biotechnol. Biochem. 60 (1996) 1608–1611.

[27]. J.C. Dohm, A.E. Minoche, D. Holtgräwe, S. Capella-Gutiérrez, F. Zakrzewski, H. Tafer, O. Rupp, T.R. Sörensen, R. Stracke, R. Reinhardt, A. Goesmann, T. Kraft, B. Schulz, P.F. Stadler, T. Schmidt, T. Gabaldón, H. Lehrach, B. Weisshaar, H. Himmelbauer, The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*), Nature 505 (2014) 546–549.

[28]. G. Kadereit, T. Borsch, K. Weising, H. Freitag, Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of C_4 photosynthesis, Int. J. Plant Sci. 164 (2003) 959–986.

[29]. Y. Xie, D. Lai, Y. Mao, W. Zhang, W. Shen, R. Guan, Molecular coning, characterization, and expression analysis of a novel gene encoding L-cysteine desulfhydrase from *Brassica napus*, Mol. Biotechnol. 54 (2013) 737–746.

[30]. H. Yi, M. Juergens, J.M. Jez, Structure of soybean β-cyanoalanine synthase and the molecular basis for cyanide detoxification in plants, Plant Cell 24 (2012) 2696–2706.

[31]. E.R. Bonner, R.E. Cahoon, S.M. Knapke, J.M. Jez, Molecular basis of cysteine biosynthesis in plants: structural and functional analysis of *O*-acetylserine sulfhydrylase from *Arabidopsis thaliana*, J. Biol. Chem. 280 (2005) 38803–38813.

[32]. M. Wirtz, H. Birke, C. Heeg, C. Müller, F. Hosp, C. Throm, S. König, A. Feldman-Salit, K. Rippe, G. Petersen, R.C. Wade, V. Rybin, K. Scheffzek, R. Hell, Structure and function of the hetero-oligomeric cysteine synthase complex in plants, J. Biol. Chem. 285 (2010) 32810–32817.

[33]. B. Huang, M.W. Vetting, S.L. Roderick, The active site of *O*-acetylserine sulfhydrylase is the anchor point for bienzyme complex formation with serine acetyltransferase, J. Bacteriol. 187 (2005) 3201–3205.

[34]. J.A. Francois, S. Kumaran, J.M. Jez, Structural basis for interaction of *O*-acetylserine sulfhydrylase and serine acetyltransferase in the *Arabidopsis* cysteine synthase complex, Plant Cell 18 (2006) 3647–3655.

[35]. A. Feldman-Salit, M. Wirtz, E.D. Lenherr, C. Throm, M. Hothorn, K. Scheffek, R. Hell, R.C. Wade, Allosterically gated enzyme dynamics in the cysteine synthase complex regulate cysteine biosynthesis in *Arabidopsis thaliana*, Structure 20 (2012) 292–302.

[36]. R. Shirzadian-Khorramabad, H.C. Jing, G.E. Everts, J.H.M. Schippers, J. Hille, P.P. Dijkwel, A mutation in the cytosolic O-acetylserine(thiol) lyase induces a genome-dependent early leaf death phenotype in *Arabidopsis*, BMC Plant Biol. 10 (2010) 80.