**Direct targeting of Arabidopsis cysteine synthase complexes with synthetic polypeptides to selectively deregulate cysteine synthesis.**

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**ABSTRACT**

Biosynthesis of cysteine is one of the fundamental processes in plants providing the reduced sulfur for cell metabolism. It is accomplished by the sequential action of two enzymes, serine acetyltransferase (SAT) and O-acetylserine (thiol) lyase (OAS-TL). Together they constitute the hetero-oligomeric cysteine synthase (CS) complex through specific protein–protein interactions influencing the rate of cysteine production. The aim of our studies was to deregulate the CS complex formation in order to investigate its function in the control of sulfur homeostasis and optimize cysteine synthesis. Computational modeling was used to build a model of the *Arabidopsis thaliana* mitochondrial CS complex. Several polypeptides based on OAS-TL C amino-acid sequence found at SAT-OASTL interaction sites were designed as probable competitors for SAT3 binding. After verification of the binding in a yeast two-hybrid assay, the most strongly interacting polypeptide was introduced to different cellular compartments of Arabidopsis cell via genetic transformation. Moderate increase in total SAT and OAS-TL activities, but not thiols content, was observed dependent on the transgenic line and sulfur availability in the hydroponic medium. Though our studies demonstrate the proof of principle, they also suggest more complex interaction of both enzymes underlying the mechanism of their reciprocal regulation.

*Key words:* serine acetyltransferase; cysteine; cysteine synthase complex; synthetic polypeptide; protein-protein interaction

*Abbreviations:* AD, transcription activation domain of GAL4; BD, DNA-binding domain of GAL4; CS, cysteine synthase; Cys, cysteine; FW, fresh weight; GAL4, transcriptional activator of galactose regulon in yeast; OAS, *O*-acetylserine; OAS-TL, *O*-acetylserine

(thiol) lyase (EC 2.5.1.47); PEP, polypeptide; rbcS1, small subunit of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39); SAT, serine acetyltransferase (EC 2.3.1.30); Y2H, yeast two-hybrid.

**1. Introduction**

Cysteine is the first organic compound, biosynthesized in plant cells after sulfate assimilation and reduction. It is the main donor for all subsequent compounds with reduced sulfur. Together with methionine it is crucial for proper protein structure and function but it also constitutes the tripeptide glutathione, the first shield of defense against free radicals and the major storage compound for soluble reduced sulfur [[1](#_ENREF_1)]. Besides, it is the precursor of iron-sulfur clusters which are among the oldest and most versatile cofactors participating in many regulatory processes [[2](#_ENREF_2)].

Biosynthesis of cysteine in plants, but also in archea and eubacteria, is carried out by a two-step pathway [[3](#_ENREF_3)]. In the first step serine acetyltransferase (SAT; acetyl-CoA:L-serine *O*-acetyltransferase; EC 2.3.1.30) transfers an acetyl-moiety from acetyl coenzyme A to serine to form *O*-acetylserine (OAS). Subsequently, *O*-acetylserine (thiol) lyase (OAS-TL; *O3*-acetyl-L-serine:hydrogen-sulfide 2-amino-2-carboxyethyltransferase; EC 2.5.1.47) exchanges the activated acetyl group with sulfide by a β-replacement reaction to produce cysteine. It is well established, that OAS-TL and SAT physically interact to form multimeric complex known as cysteine synthase (CS) complex. Interestingly, the function of the complex formation is not metabolic channeling, but sensing the sulfur status of the cell to properly adjust the sulfur homeostasis. Whereas OAS-TL is only active outside the CS complex, SAT is strongly activated by association with OAS-TL [[3](#_ENREF_3)]. The stability of the CS complex is reciprocally regulated by free sulfide and OAS. When sulfur is not limiting, sulfide produced by assimilatory sulfate reduction pathway stabilizes the CS complex. However, when sulfide concentrations decrease due to sulfate deprivation, excess OAS promotes dissociation of CS complex, resulting in the formation of less active free SAT. When sulfide becomes available the CS complex associates again. In this way, the CS complex effectively senses both OAS and sulfur availability, and self-regulates further OAS production accordingly to supply and demand. The situation is additionally complicated by uneven distribution of both enzymes between cytosol, plastids and mitochondria. This subcellular compartmentalization is in contrast to provision of sulfide that takes place exclusively in plastids, therefore the formation of CS complex might serve different purposes in each compartment and likely provides an additional level of metabolic control. The catalog of the cysteine biosynthesis enzyme isoforms and their localizations is most complete in Arabidopsis thaliana. Five SAT and nine OAS-TL genes are found in the Arabidopsis genome [[4](#_ENREF_4), [5](#_ENREF_5)]. The predominant cytosolic isoforms are SAT5 and OAS-TL A, whereas SAT1 and OAS-TL B are targeted to the plastids and SAT3 and OAS-TL C localize to mitochondria [[3](#_ENREF_3), [4](#_ENREF_4)]. The existence of compartment-specific SAT and OAS-TL isoforms was long believed to be required by the protein biosynthesis machinery due to the membranes impermeability for cysteine [[6](#_ENREF_6)]. However, latest research with the analysis of insertion mutants, evidenced that OAS and cysteine are freely exchangeable between the compartments and synthesized at different rates by the organelles [[7-10](#_ENREF_7)]. Mitochondrial SAT3 contributes to approximately 80 % of total SAT activity in the Arabidopsis leaf cell, demonstrating the prominent role of mitochondria for total OAS production [[8](#_ENREF_8)]. Conversely, contribution of mitochondrial OAS-TL C to total OAS-TL activity in leaves is very low (<5 %). Cytosolic and plastidic isoforms of OAS-TL in Arabidopsis account both for more than 45 % of total OAS-TL activity; however only loss of a cytosolic isoform results in a decreased total cysteine production suggesting its predominant role in the synthesis of this amino acid [[7](#_ENREF_7)]. It also points out that in Arabidopsis the production of the pathway intermediates and the cysteine itself are spatially separated from each other: sulfide is generated in the plastids, the bulk of OAS in the mitochondria and both are finally combined to form majority of the cysteine in the cytosol.

No crystal structure of the CS complex from any organism is currently available mainly because of instability of SAT proteins. However, a lot of biochemical data help to understand the mechanisms of SAT and OAS-TL interaction [[3](#_ENREF_3)]. Data from the studies of the bacterial CS complex suggest that two OAS-TL dimers bind to the SAT dimer of trimers, the SAT hexamer likely forming the core of the complex [[11](#_ENREF_11)]. In plants, most probably the CS complex has similar composition as suggested by the molecular masses of CS complexes from spinach, tobacco, Arabidopsis and soybean [[12-14](#_ENREF_12)]. At the molecular level, interaction between the C-terminal decapeptide of SAT and active site of OAS-TL is crucial for formation of the plant CS complex [[15-18](#_ENREF_15)]. The flexible C-terminal tail of SAT binds to OAS-TL from the side of catalytic cavity thus inhibiting its activity. The CS complex stability is based on competition of OAS with SAT for the substrate binding site of OAS-TL. It was proved that interaction between 10 last amino acids of SAT protein and OAS-TL is sufficient to allow tight binding between the enzymes [[16](#_ENREF_16), [19](#_ENREF_19)]. However, it cannot be excluded that additional domains are involved in establishment and control of the interaction. Determination of these interaction sites is a prerequisite for understanding the control of sulfur homeostasis by CS complex. Due to the lack of structural information of plant CS complex, the structure based computational techniques may provide a way to predict the interaction interfaces of SAT and OAS-TL and their mode of recognition [[17](#_ENREF_17), [20](#_ENREF_20)].

The aim of our study was to enhance cysteine production in plants through selective deregulation of CS complex with the synthetic polypeptides. Therefore, based on structural and functional information, specific polypeptides that might interfere with the CS complex formation via binding to SAT were designed. Such polypeptides would compete with OAS-TL for SAT binding but at the same time stabilize the latter enzyme for effective catalysis and formation of OAS that can be subsequently used by free OAS-TL for cysteine production. In our model we decided to use Arabidopsis mitochondrial CS complex as being the best characterized [[13](#_ENREF_13), [17](#_ENREF_17), [20](#_ENREF_20), [21](#_ENREF_21)]. Additionally mitochondrial SAT3 constitutes the bulk of cellular SAT activity; therefore targeting this isoform with polypeptides should bring the biggest profit. Interestingly, though mitochondrial CS complex was used to determine the interacting interface, the synthetic polypeptides build from the short amino acid fragments of OAS-TL C, showed ability to interact in vivo with not only Arabidopsis SAT3, but also with SAT1, SAT5 as well as SATs from other species (*Nicotiana plumbaginifolia* and *Escherichia coli*). However, studies with transgenic Arabidopsis expressing the most strongly interacting polypeptide in different compartments, showed only minor changes in the total activities of SAT and OAS-TL. The possible explanations are discussed.

**2. Methods**

**2.1. Molecular biology techniques**

Plasmids used in this study are listed in Supplementary Table A.1 and were constructed by conventional techniques [[22](#_ENREF_22)]. Oligonucleotides for PCR, RT-PCR and DNA sequencing are listed in SupplementaryTable A.2**.** All restriction enzymes (MBI Fermentas), *Taq* polymerase (Thermo Scientific), *Pfu* polymerase (Promega) and T4 DNA ligase (Promega) were used under conditions recommended by the suppliers. All PCR products were verified by sequencing after cloning and other constructs by restriction digestion. DNA sequences encoding artificial polypeptides were synthesized by GenScript USA Inc. with additional ATG starting codon and provided as inserts in pUC59 plasmids. To create bait plasmids for the Y2H analysis PEP 1-5 were amplified from those templates with the primers PEPExF and PEPBamR (Supplementary Table A.2) and cloned into pGBKT7 (Clontech). cDNAs encoding Arabidopsis SAT isoforms were amplified from Arabidopsis cDNA using gene-specific primers (Supplementary Table A.2) and cloned into the prey vector pGADT7 (Clontech). cDNA encoding OAS-TL C was generated with RT-PCR from Arabidopsis leaves and cloned into the bait vector pGBKT7. The construction of the vectors carrying *N. plumbaginifolia* and *E. coli* SAT’s coding regions were described elsewhere [[23](#_ENREF_23)]. To fuse PEP4 or OAS-TL C with GST tag PCR-generated fragments were inserted into the pGEX4T-1 (Promega). The His-tagged SAT3 was obtained by cloning the PCR fragment into pET28a (Novagen). For the PEP4 expression in plants, the PCR fragment containing PEP4 coding sequence was first cloned into the series of ImpactVector plasmids (Plant Research International, Wageningen UR), enabling targeting of the recombinant protein to cytosol, chloroplast or mitochondria. Additionally, since in these constructs the expression is under the control of light regulated rbcS1 promoter, the coding fragments of PEP4 were PCR-amplified together with the sequences of the transit peptides and recloned into the pROK2 vector [[24](#_ENREF_24)] enabling constitutive expression from CaMV 35S promoter.

**2.2. Yeast two-hybrid analysis**

The Yeast-2-hybrid system from Clontech Laboratories, Inc.was employed to test the protein-protein interaction between different polypeptides and SATs. The bait and prey constructs (Supplementary Table A.1) were introduced into the yeast strain AH109 by the lithium acetate method [[25](#_ENREF_25)]. The cotransformed clones were initially grown on plates with non-selective minimal medium lacking tryptophan and leucine (–Trp/Leu). These strains were then streaked onto selective minimal medium lacking tryptophan, leucine and histidine (–Trp/Leu/His). Interaction between the PEPs and SATs was monitored by assessing the growth of yeast co-transformants on selection media after 3–5 days at 30°C. The photos were taken at day 5. All yeast manipulations and media preparations were performed as described in the Clontech User manual.

### 2.3. Activity of β-galactosidase

The β-galactosidase activity was quantitatively assayed spectrophotomerically at 420 nm by using o-nitrophenyl-β-d-galactopyranoside (Sigma) as a substrate [[26](#_ENREF_26)] in yeast cells grown to OD600 ∼ 1. Three independent experiments using different yeast transformants were performed. The average activity was expressed in micromoles of o-nitrophenol/min/cell.

**2.4. Pull-down assay**

The expression of the His-tagged-SAT3 or GST-tagged-PEP4 was induced in *E. coli* strain BL21(DE3) with 1mM -D-1-thiogalactopyranoside (IPTG) at OD600 = 0,5. After 3h of induction, bacteria were pelleted, sonicated and the two protein extracts were combined. Following overnight incubation at 4°C with gentle rocking, the proteins were mixed in the native conditions with His-select HF Nickel Affinity Gel (Sigma-Aldrich). After that proteins were purified by 5-step wash under non-denaturing conditions and then released from the raisin with 250 mM imidazole. For the positive control reaction in pull-down analysis the GST-tagged OAS-TL C was used simultaneously. Next, SDS-PAGE electrophoresis and gel blots were performed with appropriate antibodies. As primary antibodies rabbit polyclonal anti-GST IgG (Santa Cruz Biotechnology, Inc.) or anti-His IgG (Santa Cruz Biotechnology Inc.) were used. Anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich) was next applied as secondary antibody and the signal was developed using NBT/BCIP substrate (Promega).

**2.5. Plant material and transformation**

*Arabidopsis thaliana* (ecotype Columbia-0) plants were grown in growth chamber (16/8 h light/dark cycle, 160 μmol m−2.s−1 photon flux density, 23°C). The binary plasmids described in Supplementary Table A.1 were introduced to *Agrobacterium tumefaciens* strainGV3101 and next the Arabidopsis plants were transformed using the flower dip method as described by [[27](#_ENREF_27)]. Transgenic plants were selected by growing on half-strength MS medium [[28](#_ENREF_28)] plus 0.8% agar and 50 μg.ml-1 kanamycin as selection marker, transplanted to soil and allowed to set seeds. For transgene segregation studies, seeds of transgenic Arabidopsis lines were surface-sterilized using a vapor-phase method [[29](#_ENREF_29)] and next germinated on agar-solidified (0.8%) half-strength MS medium with 50 μg.ml-1 kanamycin. After 10 days the segregation ratio was scored. The experiments described here were performed with T3 generation of the plants homozygous for the insert. For the biochemical studies the plants were grown hydroponically in the Araponics tanks (http://[www.araponics.com/](http://www.araponics.com/)) filled with 0.5x strength Hoagland medium [[30](#_ENREF_30)] and grown in the growth chamber with the weekly change of growth medium. For the sulfur starvation experiment MgSO4 present in the media was replaced with MgCl2 salt and the material was collected after 6 days of starvation. Rosette leaves of each 5-weeks old plant were pooled and used for molecular and biochemical analyses. The leaf samples were collected always at the same time of the day, i.e. 1 hour after light switch-on (to avoid variability of the rbcS1 promoter driven expression between samples [[31](#_ENREF_31)]).

**2.6. Semi-quantitative RT-PCR**

For semi-quantitative RT-PCR total RNA was extracted from frozen powdered leaf tissue using the TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Three micrograms of total RNA was next used as a template for cDNA synthesis with RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas). The obtained cDNA was diluted 10-fold and used as a template for PCR reaction. The amplification was performed with primers specific to PEP4 coding sequence and tubulin *TUA3* (TAIR: AT5G19770) listed in Supplementary Table A.2. Tubulin expression was used as an internal control for using equal cDNA quantities in the samples. 24 PCR cycles (30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C) were performed in a 10-μl volume.

**2.7. Enzyme assays and thiols content**

Total soluble proteins were isolated with 0.5 ml of 50 μm HEPES, pH 7.4, 1 μm EDTA, 30 μm dithiothreitol and Protease Inhibitor Coctail (Sigma-Aldrich) from 200 μg of the frozen leaf material. Cell debris was removed by centrifugation at 16,000g and 4°C for 10 min. The enzymatic activities of SAT and OAS-TL were assayed as described previously [[32](#_ENREF_32)]. Protein concentrations in the protein extracts were determined using Protein Assay Kit (BioRad) and bovine serum albumin as a standard. Total thiols content was measured in the same material using the method described previously [[32](#_ENREF_32)].

**2.8. Computational analysis and in silico modeling**

Amino acids sequences of SATs as well as OAS-TLs of plant and bacterial origin were aligned using MAFT [[33](#_ENREF_33)] and Clustal W [[34](#_ENREF_34)] servers, and manually adjusted. Coordinates of the Protein Data Bank crystal structures are available for several bacterial isoforms of SAT (PDB: 1S80, 1SSM, 1SSQ, 1SST, 1T3D) as well as for several bacterial isoforms of OAS-TL (PDB: 2BHS, 2BHT, 1Y7L, 1OAS) and for OAS-TL A from Arabidopsis(PDB: 1Z7W, 1Z7Y, 2ISQ). Since sequences of both enzymes are strongly conserved a structural model of the Arabidopsis monomeric SAT3 and monomeric OAS-TL C was obtained using Sybyl 8.0 package (TRIPOS Inc., St. Louise, MO, USA) on the basis of 1T3D and 2ISQ, respectively. Structures of the proteins were subjected to energy minimization using the AmberFF99 forcefield as implemented in Sybyl 8.0. The computational model of the Arabidopsis mitochondrial CS complex was build using calculations of protein - protein docking using the own software [[35](#_ENREF_35)].

**3. RESULTS**

**3.1. Synthetic polypeptides design based on the computational model of Arabidopsis mitochondrial CS complex**

During our studies only crystal structures of SAT [[36-38](#_ENREF_36)] and OAS-TL [[39](#_ENREF_39), [40](#_ENREF_40)] from bacteria and of cytosolic OAS-TL A from Arabidopsis [[41](#_ENREF_41)] were known. Currently, there is also a crystal structure of Arabidopsis mitochondrial OAS-TL C available [[20](#_ENREF_20)]. Amino acid sequence similarities between enterobacterial and plant SAT are in the order of 34% to 44 %. They are sufficient for reliable protein modeling of the plant orthologues. The crystallized Arabidopsis OAS-TL A also share 30 to 40 % amino acid sequence identity with Salmonella typhimurium and *H*aemophilus influenza OAS-TLs and are indeed structurally very similar [[39-41](#_ENREF_39)]. Here, we used homology modeling of 3D structure of the enzymes and docking techniques to construct our own model of the mitochondrial CS complex from Arabidopsis. The C-termini of SAT3, missing in the template structures but crucial for CS formation, was structured de novo. The computational model of Arabidopsis mitochondrial CS complex was proposed in parallel by Feldman-Salit et al. [[17](#_ENREF_17)] and corroborated our findings. The mitochondrial CS complex was chosen for our studies as the interaction of SAT-OASTL in mitochondria seems to have biggest impact on the regulation of the sulfur flux in Arabidopsis [[21](#_ENREF_21)]. Although mitochondrial OAS-TL C accounts for less than 5 % of total OAS-TL activity, only OAS-TL C loss-of-function mutant shows a retarded growth phenotype, conversely to the loss-of-function mutants for OAS-TL A and OAS-TL B [[7](#_ENREF_7)]. This clearly demonstrates additional function of mitochondrial OAS-TL than only cysteine synthesis and points to its regulatory function when complexed with SAT3. Further support comes from the OAS-TL/SAT activity ratios in mitochondria. It was shown, that for efficient cysteine synthesis a 200-fold excess of OAS-TL over SAT activity is needed [[42](#_ENREF_42), [43](#_ENREF_43)]. While in the cytosol and plastids, the OAS-TL/SAT activity ratio is high (200:1 and 300:1, respectively), in mitochondria it is very low (4:1) allowing for efficient complex formation but not cysteine synthesis [[7](#_ENREF_7)].

Our computational model of the mitochondrial CS complex allowed us to predict the potential interacting sites of the enzymes. Four regions belonging to OAS-TL C, located not farther than 10 Å from SAT3 atoms, were selected as potential recognition surfaces for the construction of the SAT3-binding polypeptides. Based on the amino acid sequence of these regions, the specific five linear polypeptides were designed. Four of them are hybrids of three shorter polypeptides from three different regions of OAS-TL C linked together in different combinations and length (Fig. 1). In our model they compose the common interface for the possible interaction; however they are non-contiguous in sequence. The linkers consist of 3-4 small amino acids, namely glycine and serine, to ensure the appropriate distance and flexibility between the regions. In the fourth polypeptide (PEP4) the sequence of the C-terminal region of OAS-TL C, selected as potential recognition surface, was repeated twice. The amino acids building the fifth polypeptide (PEP5) come from the already defined “SAT-binding loop”, a region highly conserved among all OAS-TLs at the active site entrance. This region was proved to be crucial for the formation of CS complex by cytosolic isoforms in Arabidopsis and bacteria [[15](#_ENREF_15), [41](#_ENREF_41)], but also computational mutations of this sequence in the modeled mitochondrial CS complex resulted in increase of the electrostatic binding energy, thereby making complexation less favorable [[17](#_ENREF_17)].

After completion of our experiments, structure of Arabidopsis OAS-TL C has been deposited in Protein Data Bank (PDB: 4AEC) [[20](#_ENREF_20)]. Superposition of our modeled structure on crystallographic structure of OAS-TL C gives an average all-atom RMSD of about 0.5Å using backbone of the whole molecule and 0.9 Å for the designed peptides, being inside margin of measurement errors. This data validated the presented mitochondrial CS complex model.

**3.2. Synthetic polypeptides are able to interact with SAT**

Yeast Two-Hybrid (Y2H) assay was used to assess whether the synthetic polypeptides are able to interact with SAT3. The proposed amino acid sequence of the five polypeptides (named PEP 1-5) was backtranslated into nucleotide sequence using codons that meet the demands for efficient translation in *Sacharomyces cerevisie*, but also in *A. thaliana* (Supplementary Fig. A.1). The DNA coding sequence was next synthesized, cloned into yeast bait vector and introduced to yeast together with SAT3 in fusion with the GAL4 activation domain. The interactions were monitored with the ability of yeasts to grow on selective media lacking histidine and with quantitative -galactgosidase assay (Fig. 2A). All of the five polypeptides were able to interact with SAT3, with polypeptide PEP4 showing the strongest and PEP5 the weakest strength. Because all five polypeptides were designed based on the amino acid sequence from regions strongly conserved among OAS-TLs (Fig. 1B), the interactions of PEP1-5 with Arabidopsis isoforms SAT1, SAT5, but also SATs from other species: *N. plumbaginifolia* and *E. coli* were investigated. Judging from the ability of yeast transformants to grow on selective media, all PEPs bound strongly to SAT1 and SAT5 and with different strength to NpSAT and EcSAT (Fig. 2A). Since PEP4 was the strongest interactor it was chosen to follow this interaction in planta. Additionally, interactions between PEP4 and SAT3 were confirmed by the “pull-down” assay (Fig. 2B).

**3.3. Construction of transgenic Arabidopsis with PEP4 expression**

Six different constructs were used to obtain transgenic Arabidopsis plants with polypeptide PEP4 expression (Supplementary Table A.1). In the transgenic groups CB, HB, MB the expression of the transgene is under the control of the light-inducible rbcS1 promoter [[44](#_ENREF_44)], while in the groups CR, HR, MR the CaMV 35S constitutive promoter drives the PEP4 expression. The groups transformed with cPEPBIN and cPEPROK plasmids (Supplementary Table A.1), CB and CR respectively, express PEP4 in cytoplasm. The products of recombinant genes in the HB and HR group contain the chloroplast-targeting leader sequences thus PEP4 polypetide is expected in chloroplast. In MB and MR groups the PEP4 coding sequence was preceded with mitochondrial transit peptide thus PEP4 should be present in mitochondria. About 20 transformants from each group were obtained and screened for numbers of T-DNA insertion loci with the Quantitative Dual target PCR method [[45](#_ENREF_45)]. The findings were supported by segregation ratios of T2 generation after selection in the presence of kanamycin (data not shown). No phenotypic differences caused by the introduced genetic modifications were obvious in any of the transgenic line as compared with the wild type plants. The homozygous T3 progeny from the chosen lines (all with one insertion) was analyzed for the expression of the transgene at the transcription level (Fig. 3). The expression was only tested in leaf tissue of 5-weeks old plants. On average, the expression of the PEP4 was quite uniform among the plant lines, with the CR, HR, MR groups showing higher transcript level than CB, HB, MB groups (Fig. 3). Because the expression of the latest groups may vary depending on the light conditions due to the presence of light-regulated rbcS1 promoter, all the samples were collected 1 hour after the light switched-on. It is the time when rbcS1 promoter ensures the highest level of transgene expression [[31](#_ENREF_31)]. Interestingly, it was demonstrated that the rbcS1-driven expression is 8-fold higher at protein level in transgenic tobacco than for the commonly used CaMV 35S promoter [[44](#_ENREF_44)]. In our transgenic Arabidopsis lines the opposite level of expression was observed, at least at the level of transcription.

**3.4. Transgenic Arabidopsis lines with PEP4 expression show moderate changes in SAT and OAS-TL activities**

The effect of expression of synthetic polypeptide in different compartments of plant cell was determined. Young leaves of three individuals of 1-3 independent transgenic Arabidopsis lines were analyzed for SAT and OAS-TL total activities and compared to wild type grown under the same conditions (Fig. 4A). Determination of total SAT activity in protein extracts from wild type Arabidopsis leaves revealed a specific activity of 655 ± 123 pmol OAS min-1.mg-1 protein. In general, SAT activity in transgenic plants was slightly higher (up to 1.4-fold) than that of wild type control plant (Fig. 4A), with the CB lines (cytosolic PEP4 expression) being most affected. This increase cannot be correlated directly to the level of PEP4 transcript in the leaves of transgenic plants (Fig. 3). Although the PEP4 coding DNA is under the control of different promoters, either the light-inducible rbcS1 or the constitutive CaMV 35S promoter, only a slight variation was seen in the SAT activity among the transgenic groups. As expected, the activity of OAS-TL was much higher than that of SAT [[42](#_ENREF_42), [46](#_ENREF_46)] and was not significantly affected in any transgenic line as compared to wild type plants (Fig. 4A), where specific activity of 513 ±  68 nmol Cys min-1.mg-1 protein was detected. The slight changes of OAS-TL activity were also not correlated with the changes of SAT activity.

 The binding strength of the PEP4 to SAT3 was lower than OAS-TL C to SAT3 (Fig. 2) and in fact, in most cases, the affinity of a peptide for its target protein is much lower than that of the protein from which it is derived [[47](#_ENREF_47)]. However, this lower affinity can be compensated either by the very high expression of the PEP4 or by the specific conditions causing dissociation of CS complex. In most cases, peptides are competitive when added as the first component of the reaction and inactive when added after assembly [[47](#_ENREF_47)]. To ensure the high level of the peptide two different strong promoters were used to drive the expression of PEP4 in planta. Additionally, to induce the disconnection of OAS-TL from SAT protein, the plants were grown under sulfur deficit conditions for 6 days. It was shown, that upon 5 days of sulfur starvation sulfide concentration drops in cytosol to a concentration supporting the dissociation of the CS complex [[10](#_ENREF_10), [48](#_ENREF_48)]. Next, SAT and OAS-TL activities in the sulfur-starved Arabidopsis plants were measured (Fig. 4B). Similarly to the observations made by Krueger et al. [[48](#_ENREF_48)] upon sulfur starvation, no significant differences in SAT activity as well as OAS-TL activity were observed in leaves of wild type Arabidopsis compared to the non-starved control. In comparison to wild type, SAT activity was slightly elevated in some transformants (up to 1.6-fold), mostly in the transformants with cytosolic and mitochondrial PEP4 expression (Fig. 4B). Interestingly, the observed increase of SAT activity in particular transgenic lines was not correlated with the increase observed in non-stressed conditions (Fig. 4A). OAS-TL activity in the sulfur-starved transgenic lines was not significantly altered in comparison to the wild type (Fig. 4B).

**3.5. The level of thiols is not modified in plants with PEP4 expression**

In general, most sulfur in a plant tissue is bound in proteins [[49](#_ENREF_49)]; however the main, transient storage form of cysteine is glutathione [[1](#_ENREF_1)]. The expression of PEP4 able to interact with cytosolic, mitochondrial as well as plastidic isoform of Arabidopsis SATs might have an impact on the formation of CS complex in the respective cellular compartment and in turn result in changed level of cysteine and other sulfhydryl group-containing compounds of the downstream metabolism. In order to reveal potential differences in the level of reduced non-protein thiols, leaf material from 5-weeks old plants grown in either sulfur-sufficient medium or sulfur-starved for 6 days was analyzed. The method of thiols assay used in this work cannot distinguish between cysteine, glutathione, -glutamyl cysteine or other non-protein thiols. However, for the purpose of this study an assumption might be made that the majority of the detected sulfhydryl groups originates from glutathione [[1](#_ENREF_1)]. No significant changes in relation to the wild type Arabidopsis were noticed irrespective of the sulfur conditions (Fig. 5). The measured level of the thiols content in the leaves of the wild type Arabidopsis grown in sulfur-sufficient and sulfur-deficient conditions was 580 ±  62 and 161 ± 30 nmoles g-1 FW, respectively.

**4. Discussion**

One of the major challenges for the comprehension of physiological functions regulating the cellular processes is the elucidation of molecular mechanisms underlining protein–protein interactions. Protein-protein interactions play a central role in many cellular functions, including DNA replication, DNA transcription and translation, signal transduction, cell proliferation, and in metabolic pathways [[50](#_ENREF_50)]. Quantitative, functional and structural studies of protein-protein interactions enable understanding of the processes that are involved at the cellular and molecular levels. In animals, the defective protein-protein interactions leading to many pathological processes, are often therapeutic targets. Through application of small peptides, designed basing on the amino acid sequences found at protein-protein interfaces, it is possible to modulate those interactions. Indeed, a lot of peptide inhibitors have been already successfully designed and applied to affect protein-protein interactions involved in a number of diseases, including cancer and HIV [[51](#_ENREF_51), [52](#_ENREF_52)]. To our knowledge, such an approach has never been used in plants so far.

In our study, we wanted to interfere with the efficiency of CS complexes formation within the plant cell in order to modulate the intrinsic regulatory mechanisms maintaining the rate of cysteine synthesis on a balanced level. There were already a number of experimental trials producing transgenic plants with the aim to increase the rate of sulfate assimilation into cysteine, and subsequently into glutathione [[12](#_ENREF_12), [53](#_ENREF_53), [54](#_ENREF_54)]. The outcome of these studies is that increased SAT activity, but not OAS-TL activity is an efficient trigger for enhanced cysteine synthesis in planta. SAT activity is regulated by the formation of CS complex with SAT in the absence of OAS-TL being much less active [[55](#_ENREF_55)]. The mechanism of inactivation of SAT outside the complex is still unknown; however it’s possible that OAS-TL might act to ensure the proper folding of SAT protein. Such instability of SAT may be rescued by molecules that bind and stabilize the native protein, thereby enabling it to exert its function. The molecules of such function are termed ‘chemical chaperones’, since they act to retain the native folding of proteins [[56](#_ENREF_56)]. Therefore, the aim of this study was to create short polypeptides based on the regions on the surface of Arabidopsis OAS-TL C molecule potentially involved in its interaction with SAT3. The role of such polypeptide would be to bind, stabilize and activate SAT enzyme without the need of physical interaction with the native OAS-TL.

The five designed polypeptides showed ability to interact with three different isoforms of Arabidopsis SATs as well as with two SATs from other species. This shows that the regions of OAS-TL are conserved not only in sequence (Fig. 1B), but also in function. The weakest interactor was polypeptide PEP5 consisting of the OAS-TL C region already proved to be crucial for complex formation [[15](#_ENREF_15), [41](#_ENREF_41)]. PEPs 1-4 are modeled from the same three regions of OAS-TL molecule, however those regions differ between PEPs in order they are linked and amino acid length. From the interaction studies using the Y2H it seems that the sequence from C-terminus of OAS-TL C is very important since PEP2 and PEP4 showed the highest -galactosidase activity. In PEP2, the largest span of that region of twenty amino acids was used, while in PEP4 this region was shorter but it was used twice. However, the C-terminus of OAS-TL C is not the only region involved in the interaction, as deletion of 19 amino acid of bacterial OAS-TL had no significant influence on the OASTL–SAT interactions [[15](#_ENREF_15)], but resulted in the loss of OAS-TL activity. The N- and C- terminal domains flank the active site of the monomer [[57](#_ENREF_57)], therefore they may be important for both: proper structure and function of OAS-TL but also complex formation.

The expression of the PEP4 peptide showing the strongest ability to interact with SATs in Y2H assay caused minor effect on the total SAT activity in the plant cells (Fig. 4). This may be caused by the short lifetime of the polypeptide, which is the main shortcoming of this approach [[58](#_ENREF_58)]. There are methods to improve the stability of peptides and to capture their bioactive conformations, e.g. acetylation or amidation, peptide cyclization and hydrocarbon stapling [[58](#_ENREF_58)]. However, as the PEP4 was produced in plant cells *de novo* and not introduced as a molecule, there were no modifications used. Only the level of transcription of PEP4 in transgenic Arabidopsis lines was monitored (Fig. 3), however the level of polypeptide after translation, its proper localization within the cell and its stability are unknown. Additionally, it should be noted that the interfacial surface area necessary for specific recognition by proteins of different complexes is typically large (approximately 750–1500 Å2), suggesting that large ligands may be required to compete effectively with the natural protein partner [[50](#_ENREF_50)]. Because PEP4 might be therefore ousted by the native OAS-TL molecule from the interaction with SAT during sufficient sulfur supply, where the CS complex is further stabilized by high sulfide concentration, the SAT activity in the plants stressed for 6 days with sulfur deficiency was followed. This should result in the diminished sulfide concentration and CS complex dissociation making SAT available for interaction with PEP4 [[10](#_ENREF_10), [48](#_ENREF_48)]. In our assumption such interaction should stabilize SAT in conformation needed for efficient enzymatic activity. A statistically significant difference in the total SAT activity of wild type Arabidopsis was not observed in sulfur-starved plants as compared to plants grown in sulfur-sufficient conditions. However, sulfur deficiency stress had a big impact on plants metabolism, as evidenced by the significant drop in non-protein thiol level. The same phenomenon was already observed in other studies [[48](#_ENREF_48)]. Nonetheless, some of the transgenic lines, especially those with PEP4 expression in cytosol or mitochondria, showed statistically significant increase of SAT activity, proving that PEP4 may actually stabilize SAT. SAT produces *O*-acetylserine, which is limiting for cysteine synthesis, but only under normal growth conditions. It was shown that OAS still accumulates to the same extent under sulfur starvation conditions as sulfide content decreases and cysteine synthesis is limited [[48](#_ENREF_48)]. Paradoxically, then, the approach of using polypeptides to stabilize SAT cannot be used for biotechnological purposes, because one cannot expect enhanced synthesis of sulfur containing compounds in the conditions of sulfur deficiency.

The transgenic lines with PEP4 expression did not show any significant changes in the content of non-protein thiols, despite minor alterations of SAT activity. Concentration of thiols decreases upon 6 days of sulfur starvation to nearly equal levels and no significant difference could be observed between wild type and transgenic plants. Glutathione is an integral component of redox homeostasis [[59](#_ENREF_59)]. It is conceivable that the maintenance of thiol at certain levels is a prerequisite for the proper function of cell metabolism. Studies of the Arabidopsis knock-out mutants as well as transgenic plants seem to support this hypothesis. The lack of the particular SAT or OAS-TL activity or elevated level of the total activity did not have much influence on the level of thiols pool [[7-9](#_ENREF_7), [12](#_ENREF_12), [53](#_ENREF_53)] proving the existence of compensating and controlling mechanisms.

The formation of the CS complex is also important because of the inhibition of the free SAT by cysteine. In the light of the newest results [[20](#_ENREF_20)] another, next to activation of the SAT activity, important feature for the regulatory function of the plant CS complex is the modification of SAT feedback sensitivity to cysteine in the complex compared to free SAT. The cytosolic soybean CS complex reported by Kumaran et al. [[18](#_ENREF_18)] exhibits a 2-fold increase in SAT activity and decreased feedback sensitivity by cysteine compared to free SAT. SAT activity remains unchanged in the Arabidopsis cytosolic and mitochondrial CS complexes but feedback sensitivity to cysteine is significantly reduced in both complexes [[13](#_ENREF_13), [21](#_ENREF_21)]. The loss of feedback inhibition in the CS complex appears to be caused by sequestration of the SAT C-terminus within the OAS-TL active site, which prevents exposure of cysteine binding site in SAT [[60](#_ENREF_60)]. It is therefore important to remember that, not only OAS and sulfide, as primary readouts for the sulfur supply of the cell, regulate the rate of the cysteine synthesis. Cysteine itself serves as a signal for the sulfur demand of the cell [[3](#_ENREF_3)] hence the formation of CS complex plays also the sensory function [[21](#_ENREF_21)]. In that context, the interaction of SAT with the short polypeptides seems to be not a good strategy to enhance SAT activity, since the whole OAS-TL molecule is needed to properly shape SAT structure and make it insensitive to cysteine. However, the polypeptides designed in this work still offer new insights into the mode of molecular recognition of SAT and OAS-TL enzymes in plants.

Extensive studies are yielding insight into the molecular bases of the CS complex formation and its regulatory function in plants. Interaction of SAT–OAS-TL in the CS complex provides a variety of means to modulate SAT activity by monitoring the level of products, allosteric regulation, protection against feedback inhibition, and maintenance of physical activity. Our results demonstrate that rather than a short polypeptide the usage of bigger synthetic molecule with stronger affinity to SAT would be more applicable to completely destroy CS complex formation in planta and provide answers to questions regarding multi-faceted regulation of sulfur homeostasis. To avoid misinterpretation and provide more detailed structural, quantitative and biophysical characterization of the biomolecular interactions additional analyses could be employed, such as NMR spectroscopy, ITC, fluorescence anisotropy and surface plasmon resonance.

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**Appendix A. Supplementary data**

 Supplementary data associated with this article can be found in the online version at (web address to be inserted later)

**Figure CaPTIONS**

**Figure 1.** **Synthetic polypeptides used in this study.**

(A) Amino-acid sequence of five polypeptides PEP1-5. The number of amino acids of each polypeptide is given in brackets; in grey are linkers joining different regions of OAS-TL C used for construction. (B) Sequence alignment of Arabidopsis cytosolic (OAS-TL A; ref: NP\_193224.1), plastid (OAS-TL B; ref: NP\_181903.1), mitochondrial (OAS-TL C; ref: NP\_191535.2), *N. plumbaginifolia* (NpOAS-TL; gb: AAR18402.1) and *E. coli* (EcOAS-TL; gb: AAA23654.1) proteins were created with Clustal W. Below the alignment the high consensus is marked with asterisks. A black rectangles mark regions selected as possibly involved in interaction with SAT used to compose polypeptides in (A). The amino acids sequence of PEP5 comes from the already defined “SAT-binding loop” [[15](#_ENREF_15), [41](#_ENREF_41)].

**Figure 2. Interactions of different polypeptides and SAT proteins.**

(A) Yeast-Two-Hybrid screen of the interactions. DNA sequences encoding PEP1-5 or cDNA of Arabidopsis OAS-TL C (NP 191535.2) were fused with DNA binding domain (BD) of the transcription factor GAL4 while cDNA encoding Arabidopsis cytosolic (SAT5; ref: NP\_200487.1), plastid (SAT1; ref: NP\_175988.1), mitochondrial (SAT3; ref: NP\_187918.1), *N. plumbaginifolia* (NpSAT; gb: AAR18403.1) and *E. coli* (EcSAT; gb: P0A9D4.1) SATs were fused with activation domain (AD) of the transcription factor GAL4. The yeast strains co-transformed with indicated BD- and AD- plasmids were grown on selective media lacking histidine. The strength of the interactions between SAT3 and all PEPs were additionally assayed with β-galactosidase activity of three independent yeast transformants for each combination. (B) The protein gel blot with anti-GST (α-GST) and anti-His (α-HIS) antibodies showing the results of the “pull-down” experiment using the mixture of crude extracts prepared from the bacteria producing His-SAT3 or GST-PEP4. After affinity purification through the Ni column, both proteins are detected (elution lane) due to SAT3-PEP4 interaction. The positive control of the assay was run in parallel and confirmed the SAT3-OAS-TL C interaction. The size (kDa) of the recombinant protein is indicated on the left.

**Figure 3. Expression of the transgenes in the leaves of the 5-weeks old Arabidopsis.**

Semi-quantitative RT-PCR (24 cycles) for detection of PEP4 transcript was performed after RNA isolation from the pooled leaf material of one individual from each line. Amplification of tubulin *TUA3* (TAIR: AT5G19770) from the same cDNA preparations was used as quantity control. The abbreviations above photo stand for independent lines transformed with: CB – cytosol localized PEP4 with expression driven by rbcS1 promoter; HB – chloroplast localized PEP4 with expression driven by rbcS1 promoter; MB - mitochondria localized PEP4 with expression driven by rbcS1 promoter; CR – cytosol localized PEP4 with expression driven by CaMV 35S promoter; HR – chloroplast localized PEP4 with expression driven by CaMV 35S promoter; MR - mitochondria localized PEP4 with expression driven by CaMV 35S promoter.

**Figure 4. Enzymatic activity of SAT and OAS-TL in leaves of 5-weeks old Arabidopsis plants grown hydroponically in either sulfur-sufficient conditions (A) or in sulfur-deficient conditions for 6 days (B).** Relative activities of independent T3 generation lines (abbreviations as in Fig. 3). The activity of a wild type Arabidopsis is set as one on the Y axis and marked with dashed line. Light grey bars: SAT activity; dark grey bars: OAS-TL activity. Shown are mean values from 2 determinations of three individuals of each line (n=3 with 2 technical repetitions). Error bars indicate SD, and asterisks mark significant differences between wild type and transgenic line determined using the unpaired t test (\* P<0.05; \*\* P<0.01; \*\*\* P<0.001).

**Figure 5. Non-protein thiols contents in leaves of 5-weeks old wild type and transgenic Arabidopsis plants**.

Total content of thiols in leaves of the plants grown in hydroponic culture and transferred for 6 days to media with (light grey bars) or without (dark grey bars) sulfate. Data are presented in relation to the wild type as mean values ± SD of three biologically independent replicates collected from each indicated transgenic line (abbreviations as in Fig. 3). The level of thiols of a wild type Arabidopsis is set as one on the Y axis and marked with dashed line.

**Supplementary Figure A.1.** **Backtranslation of the designed polypeptides.**

The designed amino acid sequence was translated into DNA sequence using Reverse Translate program from The Sequence Manipulation Suite Ver. 2.0 using a codon usage database manually adjusted to fit *S. cerevisiae* and *A. thaliana* translation needs.

**Supplementary Table A.1.** **Plasmids used in this study.**

**Supplementary Table A.2.** **Oligonucleotides used in this study.**

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