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Isolation and characterization of o-acetylserine (thiol) lyase, an enzyme of the cysteine biosynthetic pathway of vetch (*Vicia sativa* L.)

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Abstract

A putative o-acetylserine (thiol) lyase (OAS-TL; EC 2.5.1.47) gene was successfully cloned and characterized from vetch, *Vicia sativa* L. cv. Blanche Fleur. The gene, named *Voas-tl5* (GenBank Accession No. DQ456491) had a cDNA length of 871 bp with its open reading frame coding for a polypeptide of 68 amino acid residues. In semi-quantitative RT-PCR, mRNA transcription patterns revealed that the *Voas-tl5* gene was highly transcribed in leaf, pod and seed tissues. Southern blot analysis indicated that *Voas-tl5* was a single-copy gene. Phylogenetic analysis revealed that the gene belonged to the Bsas 5 sub-group. Further, the function of the gene was indicated by over-expression in the *E. coli* NK3 mutant strain, lacking the ability to synthesize cysteine. The resultant transformed bacteria were able to grow on minimal media lacking cysteine. Understanding the molecular and biochemical properties of enzymes involved in the plant sulfate assimilation pathway, such as OAS-TL, can be applied to the future development of crops with greater agricultural productivity.

Keywords*:* Cysteine , vetch, amino acid biosynthesis, plant sulfur metabolism.

***Abbreviations*:** Bsas- β-substituted alanine synthase; OAS- o-acetylserine ;OAS-TL- o-acetylserine (thiol) lyase; PLP- pyridoxal phosphate; SAT- serine acetyl transferase

Introduction

Sulfur is a macronutrient essential for plant growth and is taken up from the plant environment in the form of sulfate. Unlike animals, plants can manufacture organic sulfur in the form of two of the most important amino acids, cysteine and methionine, together with other sulfur-containing vitamins like biotin and thiamine (Hell, 1997). The biological functions of sulfur are many but one of the most important is its direct involvement in the catalytic functions of molecules. In plants, sulfate is activated to adenosine 5'-phosphosulfate (APS), reduced to sulfite (SO_3^{2-}), and then to sulfide (S^{2-}). Cysteine is formed when sulfide is coupled with O-acetylserine (OAS), which is formed from serine and O-acetyl coenzyme A (Hesse et al., 2004). The two major enzymes involved in the production of cysteine from sulfate are serine acetyltransferase (SAT; EC 2.3.1.30) and OAS-TL. β-substituted alanines are

Table 1. Sequence similarity of *Voas-tl5* nucleotide sequence with other plant OAS-TL gene sequences

Species	GenBank Accession No.	Alignment with *Voas-tl5*		
		% Similarity	No. Similar Base Pairs	E-value
Arabidopsis	NM_111234.3	100	21	0.027
CS26	AB003041.1	100	21	0.027
mRNA for OAS-TL	AY099573.1	100	21	0.027
Atg03630 mRNA	BT002155.1	100	21	0.027

non-protein amino acids that are synthesized in plants as secondary metabolites (Ikegami and Murakoshi, 1994). The β-substituted alanine synthase (Bsas) gene family includes o-acetylserine (thiol) lyase (OAS-TL), also known as cysteine synthase. Together with SAT, OAS-TL is involved in the production of cysteine from sulfate.Members of the Bsas gene family encode the pyridoxal phosphate-dependent enzymes (Yamaguchi et al., 2000). Pyridoxal phosphate (PLP) is a cofactor for OAS-TL activity and binds with the OAS-TL lysine residue to form a Schiff base (Hayashi, 1995). The PLP enzymes are classified into α, β and γ subfamilies. Pyridoxal phosphate-dependent enzymes are vitamin B_6 derivatives that are versatile organic cofactors used by many enzymes in biological reactions. Over 600 amino acid sequences that encode for approximately 60 B_6 enzymes from various plant species are present in the published databases. The B_6 enzymes link carbon and nitrogen metabolism and almost all are known to participate in amino acid biochemical pathways (Mehta and Christen, 1998).

A gene encoding an OAS-TL enzyme was the first gene cloned from the plant sulfur assimilation pathway (Romer et al., 1992; Saito et al., 1992). Since the formation of cysteine is the first occurrence of sulfur in a reduced, organic form in the cell, all compartments involved in protein biosynthesis are known to contain the OAS-TL and SAT genes. The cytosol, plastids and mithochondria of spinach and cauliflower had been found to contain these two enzymes (Lunn et al., 1990; Rolland et al., 1992). In spinach, CAS activity was found to be predominant in the mitochondria but was also detected in the chloroplast and cytosol. The protein isoforms from the cytosol and plastids of spinach have subsequently been well characterized (Droux et al., 1992; Rolland et al., 1992; Warrilow and Hawkesford, 1998).

There are at least three isoforms of OAS-TL in higher plants and *Arabidopsis*, the most investigated plant, has about nine OAS-TL-like genes (Wirtz et al., 2004). Lunn et al (1990) suggested that the presence of multiple isoforms in different compartments may be due to the inability of the plant to transport the enzyme among the compartments. In general, the compartmentalization of isoforms and their varying substrate specificities has made the progress towards elucidating the exact functions of plant OAS-TL enzymes slow (Wirtz et al., 2004). To date, the exact number of OAS-TL isoforms and their functions has not been determined fully for any plant species.

This study describes the isolation, characterization and expression of an OAS-TL gene from vetch. Elucidating the characteristics of enzymes of the cysteine biosynthetic pathway at the molecular level is vital in uncovering the numerous unanswered questions pertaining to sulfur metabolism in plants.

Materials and Methods

RNA isolation and cDNA synthesis

Total RNA was extracted from leaf tissues of six-week-old *V. sativa* cv. Blanche Fleur plants. The plants were grown in a glasshouse at 22^{o}C with 16h light/8h dark photoperiod. The TRIzol reagent (Invitrogen, USA) was used as the extraction agent following the manufacturer's protocol. The RNA concentration was assessed using a spectrophotometer at 260 nm and the quality was assessed by running 5 µL of the re-suspended pellet on a 1.4 % RNAse-free agarose gel.

Table 2. O-acetylserine (thiol) lyase enzyme activities in the *E. coli* NK3 mutant strain after transformation with the Voas-tl and pGEM-T plasmids

Treatment	Enzyme Activity (µmol/min) ± S.E.
NK3	not detected
NK3/pGEM-T	not detected
NK3/pVoas-tl5	0.022 ± 0.001
NK3/pVoas-tl6	0.022 ± 0.001

The RNA samples were treated with DNAse I (Invitrogen, USA) to eliminate contaminating DNA. Synthesis of cDNA was performed using the Monsterscript 1st-Strand cDNA synthesis kit (Epicentre, USA). First-strand cDNA was primed using V3-Oligo(dT)21 primers (Epicentre, USA). The primers were annealed to the RNA samples using the manufacturer's protocol. Second-strand cDNA was synthesized using the Access-RT PCR System (Promega, USA) following the manufacturer's protocol with modifications mentioned below.

The cDNA was primed with gene-specific primers (Forward: 5'-TCCTCTACCGGAACGAAATC-3' Reverse: 5'- AACTAAACAGAGCCGTTGTG-3') designed from the conserved regions of OAS-TL amino acid sequences sourced from the databases. The reaction mixture was prepared by combining the following reagents in a 0.2 mL PCR tube, to a final volume of 25 µL: 12 µL nuclease-free water, 5 µL AMV/*Tfl* 5 x reaction buffer, 0.5 µL 0.2 mM dNTP mix, 1.7 µL 1 µM forward primer, 1.8 µL 1 µM reverse primer and 2.5 µL of 25 mM $MgSO_4$. The above reagents were mixed by pipetting before adding the following: 1 µL AMV/RT (0.1 U), 1 µL *Tfl* DNA polymerase (0.1 U) and 1 µL of the RNA template (10 ng/µL). The PCR cycling program was as follows: 1 cycle of reverse transcription at 48°C for 45 min, followed by 1 cycle of AMV/RT inactivation and RNA/cDNA/primer denaturation, 40 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 1 min and extension at 68°C for 2 min. A final extension was performed at 68°C for 7 min.

DNA hybridization analysis.

Genomic DNA (100 ng) was digested for 1 h with 1U of *HaeIII* and *HindIII* restriction enzymes. Digested DNA was separated on a 1% (w/v) agarose gel at 70 V for about 2 h. Separated DNA was transferred to a Hybond N^+ membrane using the standard protocol outlined by Sambrook and Russel (2001). The probe was prepared by labeling an 871 bp cDNA fragment previously identified, with the DIG High Prime Labeling Mix (Roche, Germany) following the manufacturer's instructions. Pre-hybridization was performed for 30 min at 50°C in a hybridization buffer containing 5 X SSC, 0.1% lauryl sarcosine, 0.02% SDS and 10 X blocking reagent (Roche, Germany). Hybridization of probe with membrane-bound DNA was performed overnight at 50°C using the same buffer with the addition of 1 mL DIG Easy Hyb solution (Roche, Germany). Four subsequent stringency washes were done at 65°C. The first and second washes were carried out with 2 X SSC and 0.1% SDS for 5 min. The third and fourth washes were performed using 0.1 X SSC and 0.1% SDS for 15 min. The membrane was incubated for 30 min in an antibody solution containing 1 µL of the Anti-Digoxigenin-AP conjugate (Roche, Germany). Hybridization products were detected using the CSPD ready-to-use chemilluminescent substrate (Roche, Germany) followed by exposure to a Biomax light film (Kodak, France).

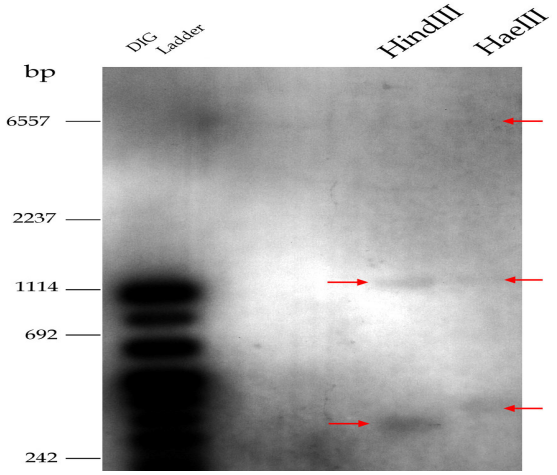

Fig 1. DNA hybridization analysis of *V. sativa* genomic DNA with *Voas-tl5* probe; arrows indicate restriction fragments

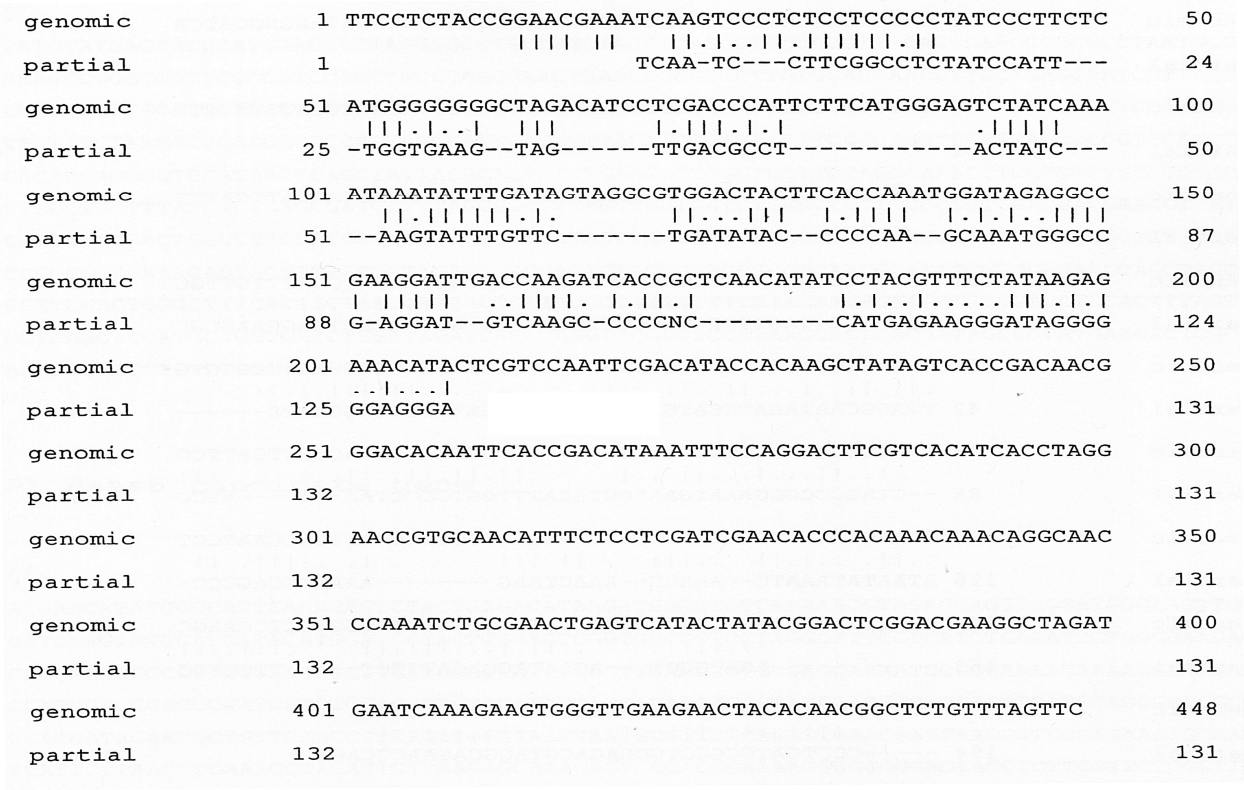

Fig 2. Probable location of the putative *V. sativa* OAS-TL partial cDNA within the genomic. Gene sequences were aligned using the Pair-wise Alignment tool from the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk)

Phylogenetic analysis

Deduced amino acid sequence of *Voas-tl5* was obtained using the Translate GCG software package (Accelerys, Inc. and Biomanager by ANGIS (http://www.angis.org.au). Amino acid sequences were aligned using ClustalW (Thompson et al., 1994). Identical and similar residues among sequences were highlighted using the Boxshade program (Hoffman, K. and Baron, M.D and Biomanager by ANGIS). Distance matrices between protein sequences were computed using Protdist, which computed the distances using maximum likelihood estimates based on the Dayhoff PAM matrix (Dayhoff, 1979) or Kimura's approximation (Kimura, 1983). The phylogenetic tree was constructed using the Neighbour-joining method (Saitou and Nei, 1987) sourced from the PHYLIP package version 3.2 (Felsenstein, 1989) and Biomanager by ANGIS, and drawn using Treeview version 1.4 (Page, 1996).

Over-expression of Voas-tl5 into the cys⁻ E.coli NK3 mutant strain

The coding regions of isolated cDNA clones were amplified by PCR to introduce the *AeII* recognition site in the 5' region adjacent to the first methionine using oligonucleotide primers 5'-ATGACGTCCATGCAGCCTAGGGG-3' and 5'-GTCTCGGCAAAACTTTCGCTCAGA-3'. The amplified fragments were cloned into the *AatII* site of the pGEM-T vector (Promega). The resultant plasmid (pVoas-tl5) was introduced into the *E.coli* cysteine auxotroph NK3 by heat-shock transformation. For genetic complementation of the cysteine requirement, *E. coli* was cultured on M9 minimal salts with or without 0.5 mM cysteine at 37°C overnight.

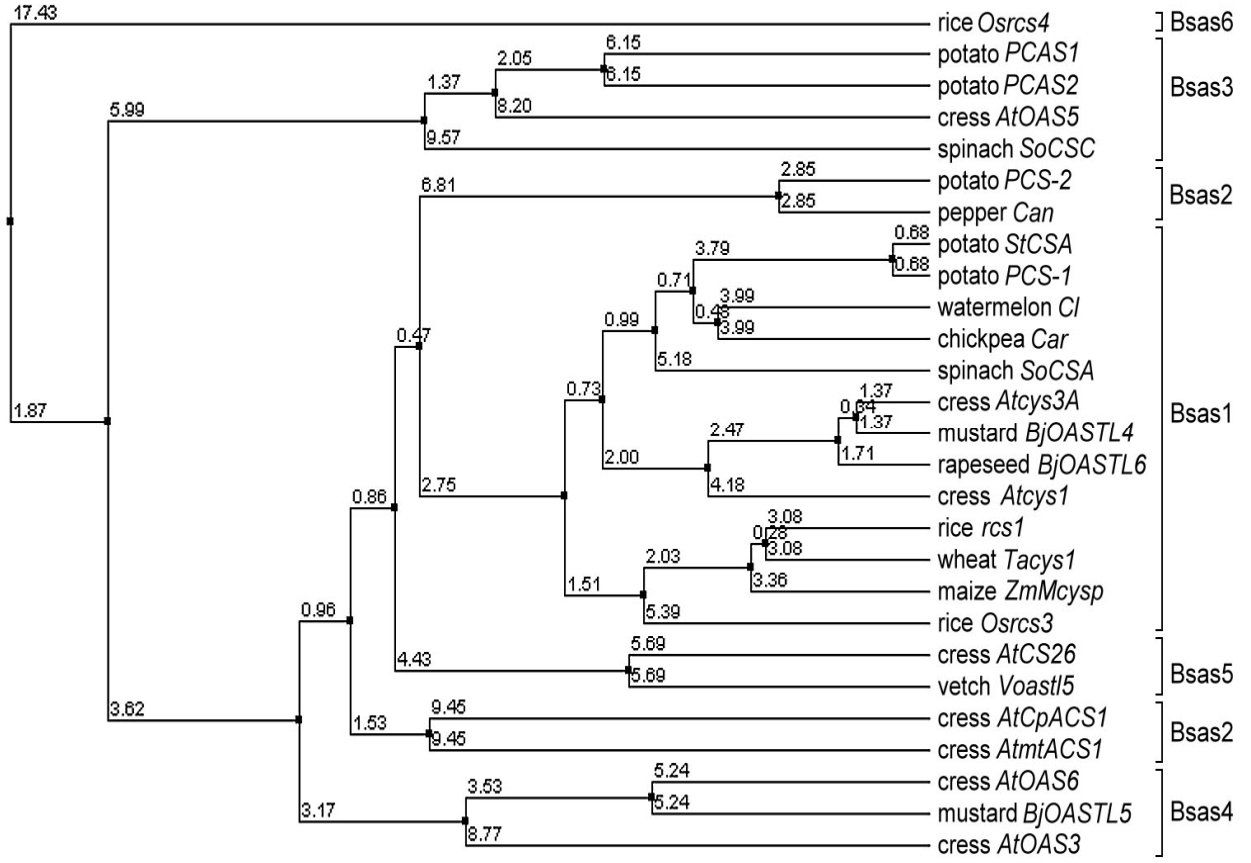

Fig 3. Phylogenetic relationships among members of the plant Bsas family including the deduced amino acid sequences of the putative *Voas-tl5* gene isolated from *V. sativa* cv. Blanche Fleur; Osrcs1 (*Oryza sativa*, GenBank Accession No. AF073695), Tacys1 (*Triticum aestivum*, GenBank Accession No. D13153), ZmMcysp (*Zea mays*, GenBank Accession No. X85803), Osrcs3 (*O. sativa*, GenBank Accession No. AF073697), Cl (*Citrulus vulgaris* var. *lanatus*, GenBank Accession No. D28777), Car (*Cicer arietinum*, GenBank Accession No. AJ006024), SoCSA (*Spinacea oleracea*, GenBank Accession No. D10476), Atcys3A (*A. thaliana*, GenBank Accession No. X84097), Atcys1 (*A.thaliana*, GenBank Accession No. X81697), BjOASTL4 (*Brassica juncea*, GenBank Accession No. Y10845), BjOASTL6 (*B.juncea*, GenBank Accession No. Y10847), AtcpACS1 (*A.thaliana*, GenBank Accession No. X81698), AtmtACS1 (*A.thaliana*, GenBank Accession No. X81973), PCS-1 (*Solanum tuberosum*, GenBank Accession No. AB029511, PCS-2 (*S. tuberosum*, GenBank Accession No. AB029512), Can (*Capsicum anuum*, GenBank Accession No. CAA46086), AtOAS6 (*A.thaliana*, GenBank Accession No. AB024283), BjOASTL5 (*B. juncea*, GenBank Accession No. Y10846), AtOAS3 (*A.thaliana*, GenBank Accession No. AJ011603), AtCS26 (*A.thaliana*, GenBank Accession No. NM_111234.3) and Osrcs4 (*O.sativa*, GenBank Accession No. AF073698). Similarities between sequences are shown by the distance matrix values indicated on the branches. The branch lengths of the phylogram were considered proportional to the inferred evolutionary change. Amino acid sequences were aligned using ClustalW. The tree was constructed using the Neighbor-joining method from the Phylip package version 3.2

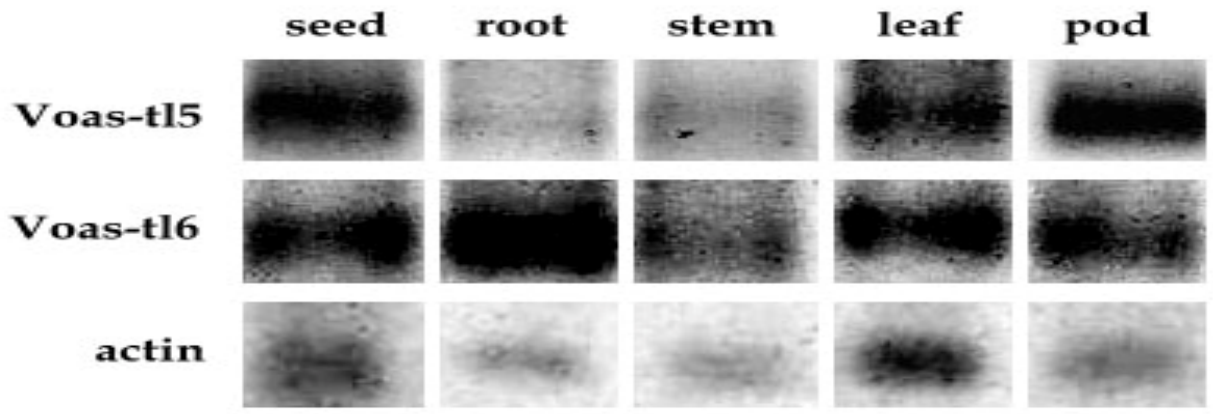

Fig 4. Messenger RNA transcription profiles of *Voas-tl5* among five plant tissues of *V. sativa* cv. Blanche Fleur

M9 minimal salts with or without 0.5 mM cysteine at 37^{o}C overnight.

OAS-TL enzyme assay of the transformed E. coli NK3 mutant strain

For enzyme assays, transformed *E. coli* NK3 was grown in liquid LB medium (Sambrook and Russel, 2001) containing 100 mg/L ampicillin at 37°C overnight following the method of Hatzfeld *et al*. (2000). This involved culturing 100 µL of the *E. coli* solution in 10 mL of fresh LB medium with agitation for 3 h at 37°C. One hundred microliters of 1 M IPTG.

Results and Discussion

A cDNA clone from *V. sativa*, designated as *Voas-tl5*, was isolated by PCR with oligonucleotide primers designed from the conserved amino acid regions of plant OAS-TL sequences deposited in the databases. The nucleotide sequence of *Voas-tl5* was 871 bp long. The coding region on the direct strand was predicted to extend from base 28 to 165, coding for 45 amino acids. Molecular copy number of *Voas-tl5* was determined by Southern hybridization of genomic DNA with cDNA encoding the OAS-TL genes. DNA-DNA hybridization of *Voas-tl5* at the calculated temperature (50°C) indicated two bands as a result of the *HindIII* restriction and three from the *HaeIII* restriction, suggesting a single copy (Fig. 1). Partial cDNA sequence of 131 bp for Bsas 5 candidate gene was obtained from the RT-PCR experiments using the same primer sets employed in the isolation of the Bsas 5 genomic sequence. Figure 2 shows the probable location of the partial cDNA sequences within the genomic sequence.

Nucleotide sequence homology search of the databases revealed that the *Voas-tl5* cDNA fragment was homologous to *A. thaliana* cysteine synthase CS26 (GenBank Accession No. NM_111234.3) with an e-value of 0.027 (Table 1). Results of the multiple sequence alignment showed that the putative *Voas-tl5* gene shared many conserved amino acid residues with plant OAS-TLs (data not shown). Phylogenetic analysis (Fig. 3) revealed that the putative *Voas-tl5* gene was most closely related to the *A. thaliana* cysteine synthase CS26 gene (GenBank Accession No. NM_111234.3) which belongs to the Bsas 5 sub-family as classified by Maruyama *et al* (2001).

In RT-PCR, the *Voas-tl5* gene was shown to be highly transcribed in leaf, pod and seed tissues. Figure 4 shows mRNA transcription patterns of *Voas-tl5.* The high amounts of transcription in leaf and seed tissues were consistent with the transcription of the actin housekeeping gene in the same tissues. When the coding regions of *Voas-tl5* and *Voas-tl6* were independently cloned into the *AatII* site of the pGEM-T vector, the plasmid pVoas-tl5 was produced. After transformation into

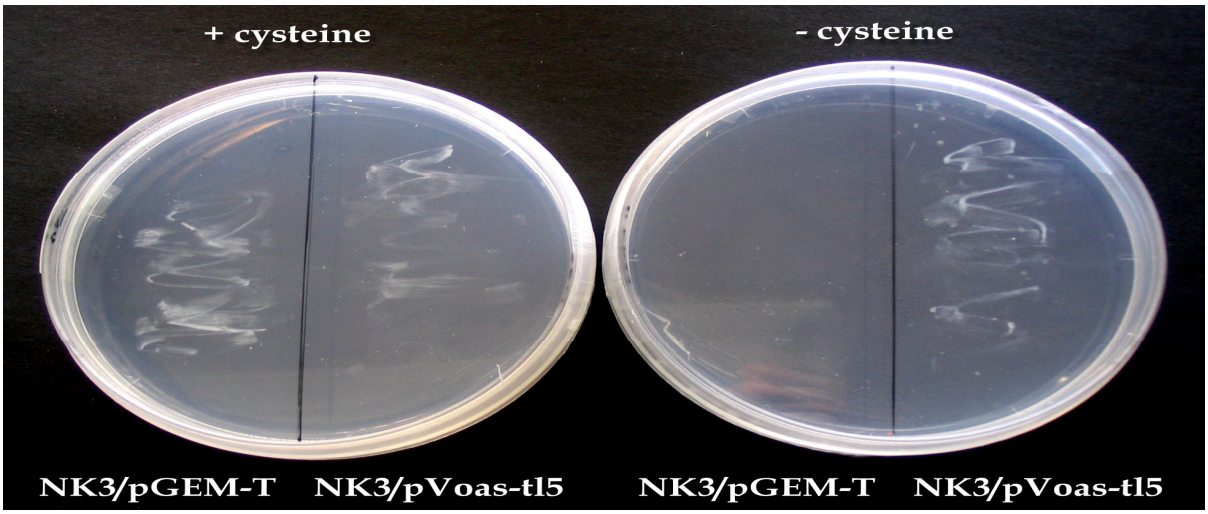

Fig 5. Functional complementation of the Cys$^-$ *E.coli* NK3 mutant by transformation with the expression vector (pVoas-tl5) carrying the *Voas-tl5* cDNA clone. The transformed NK3 was streaked onto the M9 minimal media containing 0.5 mM cysteine (left plate) or without cysteine (right plate). The empty vector pGEM-T was used as the control

the *E. coli* NK3 mutant strain, they were able to grow on M9 minimal medium without cysteine (Fig. 5). The NK3 mutant strain carrying the empty pGEM-T cloning vector however, did not grow in the absence of cysteine. No enzyme activity was detected in the *E. coli* NK3 mutant strain containing the empty pGEM-T cloning vector. The enzyme activity in the *E. coli* NK3 mutant transformed with the pVoas-tl5 construct was detectable (Table 2).

Characterization of cloned DNAs has been used to uncover important chemical and biological features. The ability to synthesize a cDNA from a mRNA fragment is proof that a gene is active in the cell from which the mRNA was isolated. An 871-bp OAS-TL cDNA fragment was cloned and sequenced from *V. sativa.* The presence of introns in the cDNA sequence was not surprising because the preparation of high quality RNA is a major hurdle in cDNA cloning. In plants, nuclear RNA represented 25% of the complexity observed in unprocessed introns of primary transcripts (Goldberg, 2001). Many studies involving intron-containing cDNAs were not published. Southern hybridization revealed a single gene copy gene. This was consistent with results obtained for OAS-TL isoforms A, B and C encoding cDNAs At.OAS.5-8 (GenBank Accession No. X80376), At.OAS.7-4 (GenBank Accession No. X80377) and EST181H17T7 (GenBank Accession No. AJ271727) from *Arabidopsis* and cys1 (GenBank accession No. D13153) from wheat. Protein sequence alignment showed many conserved domains that conformed to other plant OAS-TL genes. However, the degree of homology was small compared to most OAS-TL genes. This was not unexpected since four of the *Arabidopsis* OAS-TL ESTs studied by Hatzfeld et al. (2000) did not match any previously reported OAS-TL sequences but were ultimately proven to possess OAS-TL function. This demonstrated that predicting gene function on the basis of nucleotide or protein sequence homology should not be the foremost criterion in assigning a gene's function.

The *Voas-tl5* cDNA gene fragment isolated and cloned from *V. sativa* cv. Blanche Fleur was able to survive and grow in the presence of exogenous cysteine and may be involved in the cysteine biosynthetic pathway. The high transcription levels of the gene observed in the leaf tissues over the other tissues assessed were not surprising. Most of the reactions of the sulfate assimilation pathway are known to take place in leaf chloroplasts, but OAS-TL activities were also detected in both leaf and root tissues (Saito et al., 1993; Schneider et al., 1997; Nakamura et al., 1999). In spinach leaves under normal conditions, the *cysB* gene was more highly expressed in the leaves than the cysA and cysC genes although small amounts of transcripts were found to

have also accumulated in the roots (Saito et al., 1993).

The functional complementation of the bacterial mutant *E.coli* NK3 strain was previously employed by many researchers as the method of choice in helping to validate plant cDNAs as encoding OAS-TL-like genes. The success of this approach indicated that OAS-TL was most likely to be evolutionally conserved in function. Also, the ability of bacterial OAS-TL and SAT to sustain activity in plants was an indication that the mechanism of cysteine synthesis is similar for both. In this study, *Voas-tl5* was over-expressed within the *E. coli* NK3 mutant strain. OAS-TL enzyme activity was detected from the lysed bacterial cells, a clear indication that the *Voas-tl5* gene is actively involved in the sulfate assimilation pathway of *V. sativa.*

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