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Phytochemistry 66 (2005) 1407–1416
PHYTOCHEMISTRY
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Molecular and biochemical characterisation of a serine acetyltransferase of onion, *Allium cepa* (L.)

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Received 29 November 2004; received in revised form 21 April 2005

Abstract

We have previously cloned a cDNA, designated *SAT1*, corresponding to a gene coding for a serine acetyltransferase (SAT) from onion (*Allium cepa* L.). The *SAT1* locus was mapped to chromosome 7 of onion using a single-stranded conformation polymorphism (SSCP) in the 3′ UTR of the gene. Northern analysis has demonstrated that expression of the *SAT1* gene is induced in leaf tissue in response to low S-supply. Phylogenetic analysis has placed SAT1 in a strongly supported group (100% bootstrap) that comprises sequences that have been characterised biochemically, including *Allium tuberosum*, *Spinacea oleracea*, *Glycine max*, *Citrullus vulgaris*, and SAT5 (AT5g56760) of *Arabidopsis thaliana*. This group can be divided further with the SAT1 of *A. cepa* sequence grouping strongly with the *A. tuberosum* sequence. Translation of *SAT1* from onion generates a protein of 289 amino acids with a calculated molecular mass of 30,573 Da and p*I* of 6.52. The conserved G_{277} and H_{282} residues that have been identified as critical for L-cysteine inhibition are observed at G_{272} and H_{277}. *SAT1* has been cloned into the pGEX plasmid, expressed in *E. coli* and SAT activity of the recombinant enzyme has been measured as acetyl-CoA hydrolysis detected at 232 nm. A K_m of 0.72 mM was determined for L-serine as substrate, a K_m of 92 μM was calculated with acetyl-CoA as substrate, and an inhibition curve for L-cysteine generated an IC_{50} value of 3.1 μM. Antibodies raised against the recombinant SAT1 protein recognised a protein of ca. 33 kDa in whole leaf onion extracts. These properties of the SAT1 enzyme from onion are compared with other SAT enzymes characterised from closely related species.
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Keywords: *Allium cepa (L.)*; Liliaceae; Onion; Recombinant enzyme; Serine acetyltransferase; Sulfur metabolism

1. Introduction

The amino acid, L-cysteine, is the first organic sulfur (S)-containing product of the reductive S-assimilation pathway in higher plants. This reductive sulfur-assimilation pathway in plants is now well characterised (Hawkesford and Wray, 2000; Leustek et al., 2000; Hell et al., 2002). The major form of S taken up by plants is highly oxidised sulfate that is first activated and then reduced by the sequential activities of ATP sulfurylase [EC 2.7.7.4], adenosine 5′-phosphosulfate (APS) reductase [E.C. 1.8.99.2] and sulfite reductase [EC 1.8.7.1] to produce sulfide. The final step in cysteine biosynthesis is the incorporation of the reduced sulfide into *O*-acetyl serine (OAS), to form cysteine.

This final step is catalysed by two enzymes, serine acetyltransferase (SAT; E.C. 2.3.1.30) that catalyses the formation of OAS from acetyl-CoA and L-serine, and *O*-acetylserine (thiol)lyase (OASTL; E.C. 4.2.99.8)

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doi:10.1016/j.phytochem.2005.04.034

that catalyses the formation of L-cysteine by the incorporation of the reduced sulfide into OAS. These two terminal enzymes co-operate to form a bi-enzyme complex, termed cysteine synthase (Bogdanova and Hell, 1997), and the structural domains that mediate the interaction between these two enzymes have now been characterised (Wirtz et al., 2001). This enzyme complex is of interest for two reasons: (i) it represents a key mechanism for cross-talk, via the generation of OAS, between S-assimilation and carbon and nitrogen metabolism in plants (Hesse et al., 2004), and (ii) the bi-enzyme complex acts as an exquisite control point for the formation of cysteine. In the SAT–OASTL complex, SAT is active and generates OAS, while the OASTL enzyme is inactive but serves to stabilise SAT. Because OASTL is inactive in the complex, the OAS is not channelled through the complex, but is utilised by excess OASTL to form cysteine (Droux et al., 1998). However, given the importance of S nutrition in crop productivity, and in the nutritional value of crops as both animal feeds and in human nutrition, there is still comparatively little detailed characterisation of the enzymes that comprise the reductive S-assimilation pathway in a wide range of higher plants.

For SAT, characterisation, either as partially or purified protein, or as a recombinant enzyme, has been reported for *Allium tuberosum* (Urano et al., 2000), *Arabidopsis thaliana* (Noji et al., 1998; Howarth et al., 2003), *Citrullus vulgaris* (Saito et al., 1995), *Glycine max* (Chronis and Krishnan, 2004), *Pisum sativum* (Droux, 2003), and *Spinacea oleracea* (Noji et al., 2001). In earlier protein purification studies with leaf extracts of *A. tuberosum*, the SAT enzyme co-purified with OASTL (Nakamura and Tamura, 1990), but has been characterised as a separate, active enzyme from chloroplast preparations of *S. oleracea* (Ruffet et al., 1994) and *P. sativum* (Droux, 2003). However, the expression of SAT genes in *E. coli* to generate recombinant protein has also provided a source of enzyme for characterisation studies. In a detailed study by Noji et al. (1998), three SAT genes of *A. thaliana* were isolated and each one shown to be targeted to either the chloroplast (SAT-p; SAT1 as designated in Hell et al., 2002), the mitochondrion (Sat-m; SAT3 as designated in Hell et al., 2002), or the cytosol (SAT-c; SAT 5 as designated in Hell et al., 2002). An important feature of this study was the differential feedback regulation of the three SAT genes by L-cysteine, in which SAT-c was feedback inhibited, while the activity of SAT-m and SAT-p was unaffected. Subsequent studies with recombinant enzymes have identified L-cysteine-sensitive SAT isoforms from *A. tuberosum* (Urano et al., 2000), *C. vulgaris* (Saito et al., 1995), *G. max* (Chronis and Krishnan, 2004), and *S. oleracea* (Noji et al., 2001), although only the isoform from spinach has been localised within the cell, and shown to be targeted to the chloroplast.

While these studies with *A. thaliana*, *A. tuberosum*, *C. vulgaris*, *G. max*, *P. sativum*, and *S. oleracea* have provided significant insight into the regulation of SAT, only *A. tuberosum* is a significant accumulator of organosulfur compounds. Tissue disruption of *Allium* vegetables like *A. tuberosum* and onion triggers enzymatic hydrolysis of alkyl cysteine sulfoxides (ACSOs) to form a complex mixture of products with flavour and medicinal properties (Randle and Lancaster, 2002). Therefore, characterisation of the regulation of the S-assimilation and downstream pathways that determine the nature and extent of organosulfur compound accumulation is required to inform breeding and production programmes for this economically important crop (Lancaster et al., 2000; McCallum et al., 2001; McCallum et al., 2005). As part of our longer-term aim to dissect the regulation of S-assimilation in onion, we report here on the characterisation of a SAT enzyme from onion (*Allium cepa* SAT1) including its feedback regulation by L-cysteine.

2. Results

2.1. Genetic mapping of the SAT1 locus

The *SAT1* locus was mapped, using a single-stranded conformation polymorphism (SSCP) in the 3′ UTR of the gene that segregated in the 'W202A' × 'Texas Grano 438' (WxT) F_2 mapping population (Fig. 1). This polymorphism showed linkage to the SSCP marker API76 (McCallum et al., 2001) and two simple sequence repeat (SSR) markers previously mapped to chromosome 7 in the population 'BYG15-23' × 'AC43' used to develop

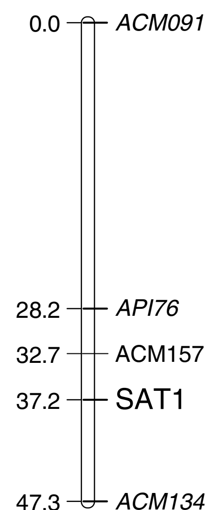

Fig. 1. Linkage mapping of the *SAT1* locus to chromosome 7 of onion in the 'W202A × Texas Grano 438' population. Loci in italics were previously mapped to chromosome 7 of the 'BYG15-23 × AC43' genetic map (Martin et al., 2005).

the onion linkage map (King et al., 1998; Martin et al., 2005).

2.2. Regulation of SAT1 expression by S-supply

RNA was isolated from leaf tissue of onion of the W202A cultivar at two developmental stages, designated pre-bulbing (ca. 10-week-old seedlings) and at bulbing (ca. 16-week-old seedlings). Plants were grown in either low or sufficient sulfur, and northern analysis used to determine the expression of the *SAT1* gene in response to S-supply (Fig. 2). In these experiments, there was no discernable difference in the level of *SAT1* expression, detected as an mRNA species of ca. 1.1 kb (expected size: 1094 bp), in response to S-supply in pre-bulbing leaf tissues, but in bulbing onions, the expression of *SAT1* was higher in leaves excised from plants growing in low S.

2.3. Phylogenetic analysis

Phylogenetic analysis was undertaken on 23 translated SAT sequences including the 5 genes of *A. thaliana* (designated SAT1–SAT5; Hell et al., 2002) (Fig. 3). The *A. cepa* SAT1 sequence is a member of a strongly supported group (100% bootstrap) that comprises sequences that have been characterised biochemically, including *A. tuberosum* (GI: 7384806), *S. oleracea* (GI: 7433702), *G. max* (GI: 18252508), *C. vulgaris* (GI: 1361979) and SAT5 (AT5g56760) of *A. thaliana*, which is equivalent to SAT-c, the cytosol-localised isoform as shown by Noji et al. (1998). This group can be divided further with the *A. cepa* SAT1 sequence grouping strongly with the *A. tuberosum* sequence, a second group comprising *S. oleracea*, *Beta vulgaris*, *G. max*, *A. thaliana* (SAT5), *Nicotiana tabacum* (SAT7) and *C. vulgaris*, and a third group comprising a sequence from *O. sativa*, and two from *Zea mays* (SATaseI and SATaseIII). None of the gene products in this third group have been characterised biochemically. The proposed or experimentally deduced sub-cellular localisation is also shown, but as yet very few sequences have been localised at the sub-cellular level.

The *A. cepa* SAT1 sequence is also quite distinct from the other *A. thaliana* sequences, with only 58% bootstrap support to group with SAT-m (SAT3; AT3g13110) and with SAT-p (SAT1; ATIg55920). A second gene from the TIGR *A. cepa* Gene Index (http://www.tigr.org; CF445069) was also used in the phylogenetic analysis, and was found to group with SAT1 and SAT3 of *A. thaliana*, rather than with the *A. cepa* SAT1 gene under analysis in this study.

2.4. Features of the deduced amino acid sequence

Translation of the *SAT1* gene from onion generates a protein of 289 amino acids with a calculated molecular mass of 30,573 Da and p*I* of 6.52. An alignment of the onion sequence with those more closely related sequences revealed by phylogenetic analysis, and for which some biochemical characterisation has been reported, is shown in Fig. 4. Comparison of the *A. cepa* sequence with *A. tuberosum* reveals an identity of 97% and a similarity of 100%, an identity of 66% and a similarity of 76% to *S. oleracea*, and an identity of 70% and a similarity of 85% with SAT5 of *Arabidopsis*. Further comparisons reveal an identity of 75% and a similarity of 87% with *G. max*, and an identity of 79% and a similarity of 90% with *C. vulgaris.* All of these sequences contain the conserved G_{277} and H_{282} residues that have been identified in *C. vulgaris* as critical for L-cysteine inhibition (Inoue et al., 1999), with

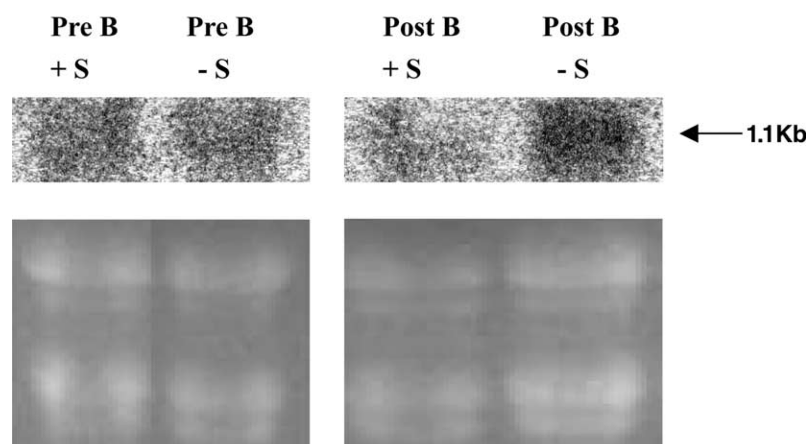

Fig. 2. Northern analysis of *SAT1* expression in leaf tissues of onion in response to S-supply at the developmental stage, as indicated. Thirty micrograms of total RNA was reduced in 2.2 M formaldehyde, separated through a 1% (w/v) agarose gel in 2.2 M formaldehyde, transferred to a nylon membrane and probed with the 3′-UTR region of the *SAT1* gene. Details of hybridisation and washing conditions are described in Section 4. Pre B = pre-bulbing; Post B = bulbing.

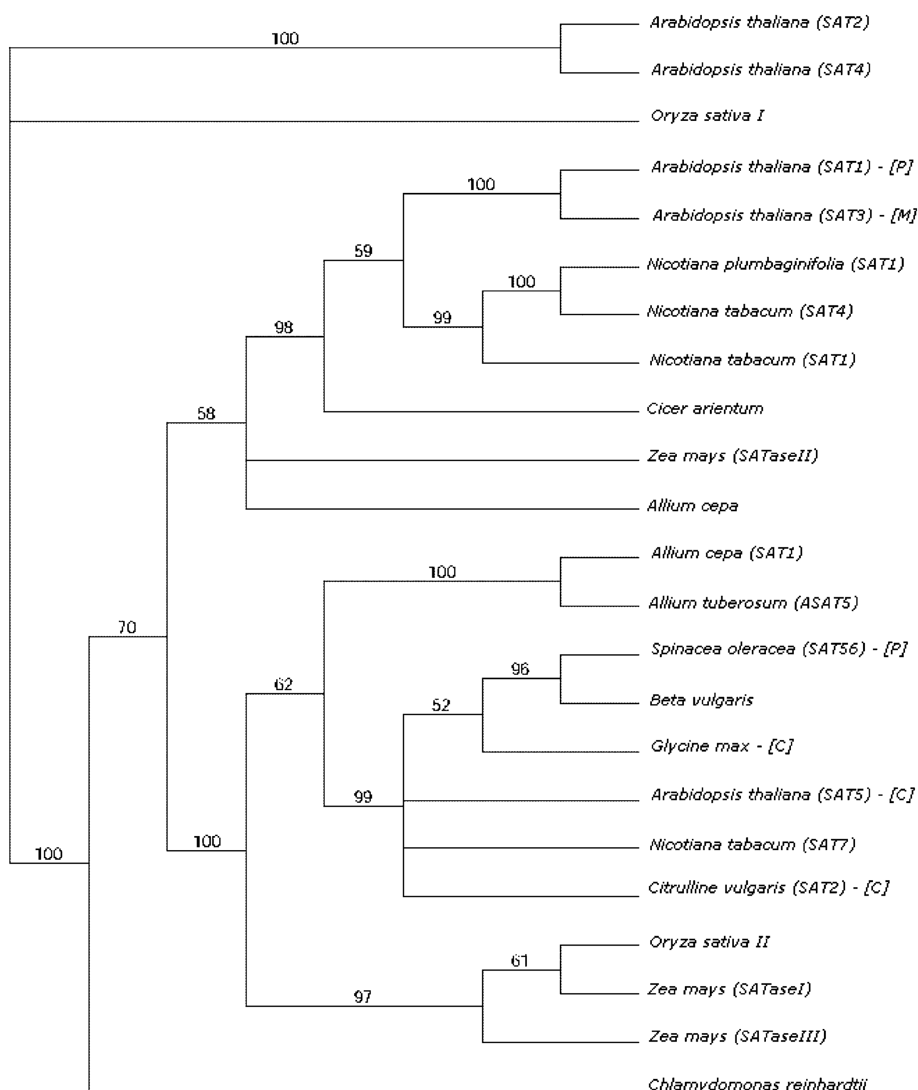

Fig. 3. Amino acid sequence based phylogeny of selected SAT sequences. The phylogenetic tree (generated using parsimony) was constructed from alignment of GenBank sequences with deduced amino acid sequences. Accession numbers for the sequences shown are: *Allium cepa (putative cytosolic)* – GI 6601494, CF 445069_1; *Allium tuberosum* – GI 7384806; *Arabidopsis thaliana*, *SAT1* – AT1g55920; *Arabidopsis thaliana*, *SAT2* – AT2g17640; *Arabidopsis thaliana*, *SAT3* – AT3g13110; *Arabidopsis thaliana*, *SAT4* – AT4g35640; *Arabidopsis thaliana*, *SAT5* – AT5g56760; *Beta vulgaris* – GI 28460066; *Chlamydomonas reinhardtii* – GI 20750301; *Cicer arientum* – GI 21615413; *Citrullus vulgaris* – GI 1361979; *Glycine max* – GI 18252508; *Nicotiana plumbaginifolia*, (*SAT1*) – GI 38350581; *Nicotiana tabacum*, *SAT1* – GI 23343581; *Nicotiana tabacum*, *SAT4* – GI 23343583; *Nicotiana tabacum*, *SAT7* – GI 23343585; *Oryza sativa* I – GI 22758278; *Oryza sativa* II – GI 34910686; *Spinacea oleracea* – GI 7433702; *Zea mays*, *SATaseI* – GI 25991547; *Zea mays*, *SATaseII* – GI 25991549; *Zea mays*, *SATaseIII* – GI 25991545. [C] = cytoplasm location; [M] = mitochondrial location; [P] = chloroplast location.

G_{272} and H_{277} the corresponding residues in the *A. cepa* SAT1 sequence.

2.5. Expression of SAT in E. coli and kinetic analysis of the recombinant enzyme

The *SAT1* gene of *A. cepa* was cloned into the pGEX expression vector and expressed in *E. coli* as a GST:SAT protein fusion. In response to IPTG induction, the fusion accumulated with a mass of ca. 58 kDa (cf. lane b with lane a, Fig. 5A). Affinity purification of the fusion protein was achieved using a glutathione–Sepharose matrix, with the SAT protein moiety released using a recombinant protease. This procedure yielded a protein of ca. 33 kDa, as determined by SDS–PAGE (lane c, Fig. 5A), and further purification of the protein was undertaken using ion-exchange column chromatography. The major protein of ca. 33,000 Da, the putative recombinant SAT protein, eluted at 210–240 mM NaCl. Activity assays confirmed that this fraction contained SAT activity (data not shown), and SDS–PAGE revealed this protein to be the major component in this fraction, as determined by coomassie blue staining (lane d, Fig. 5A).

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Arabidopsis thaliana (SAT5)     -------------------------MPPAGELRHQSPSKEKLSSVTQSDE 25
Citrullus vulgaris              -------------------------MP-VGELRFSSQS------------ 12
Glycine max                     -------------------------MP-TGLP------------------ 6
Spinacia oleracea               MLAVSYAIPSCCINRFYIREFSSSLQPALHHPWYKKRFGCCSTNQENCSL 50
Allium cepa                     -------------------------MPCSTLP------------------ 7
Allium tuberosum                -------------------------MPCSTVP------------------ 7
                                                          *

Arabidopsis thaliana (SAT5)     -----------AEAASAAISAAAADAEAAGLWTQIKAEARRDAEAEPALA 64
Citrullus vulgaris              ---------------STTVVESTTNNDETWLWGQIKAEARRDAESEPALA 47
Glycine max                     -----------------AANSLVAPDEEGWVWGQIKAEARRDAESEPALA 39
Spinacia oleracea               GSPTHLPRMPGGDLSVAPSVGHLTANNEAWLWDQIKGEARRDADSEPALA 100
Allium cepa                     ---------------IPTFPPPESESDESWVWNQIKAEAHRDAESEPALA 42
Allium tuberosum                ---------------FPTFPPPESESDESWVWNQIKAEARRDAESEPALA 42
                                                 .     :  :   :* ***.**:***::*****

Arabidopsis thaliana (SAT5)     SYLYSTILSHSSLERSISFHLGNKLCSSTLLSTLLYDLFLNTFSSDPSLR 114
Citrullus vulgaris              SYLYSTILSHSSLERSLSFHLGNKLCSSTLLSTLLYDLFLNAFSTDYCLR 97
Glycine max                     SYLYSTILSHSSLERSLSFHLGNKLCSSTLLSTLLYDLFLNAFSSDPSLR 89
Spinacia oleracea               SYLYSTILSHSSLERSLSFHLGNKLCSSTLLSTLLYDLFLNILSSDSSLL 150
Allium cepa                     SYLYSTIISHPSLARSLSFHLANKLCSSTLLSTSLYDLFLNTLSTFPTVL 92
Allium tuberosum                SYLYSTIISHPSLARSLSFHLANKLCSSTLLSTSLYDLFLNALSTFPTIL 92
                                *******:**.** **:****.*********** ******* :*:   :

Arabidopsis thaliana (SAT5)     NATVADLRAARVRDPACISFSHCLLNYKGFLAIQAHRVSHKLWTQSRKPL 164
Citrullus vulgaris              SAVVADLQAARERDPACVSFSHCLLNYKGFLACQAHRVAHKLWNQSRRPL 147
Glycine max                     SAAVADLRAARERDPACVSYSHCLLNYKGFLACQAHRVAHLLWRQSRRPL 139
Spinacia oleracea               DAVVADLRAARVRDPACVSFSHCLLNYKGFLACQSHRVAHKLWNQDRRPL 200
Allium cepa                     SASVADLIAARHRDPACVGFSHCLLNFKGFLAVQTQRIAHVLWSQSRRPL 142
Allium tuberosum                SATVADLIAARHRDPACIGFSHCLLNFKGFLAVQTQRIAHVLWSQSRRPL 142
                                .* **** *** *****:.:******:***** *::*::* ** *.*:**

Arabidopsis thaliana (SAT5)     ALALHSRISDVFAVDIHPAAKIGKGILLDHATGVVVGETAVIGNNVSILH 214
Citrullus vulgaris              ALALQSRIADVFAVDIHPAARIGKGILFDHATGVVVGETAVIGNNVSILH 197
Glycine max                     ALALHSRIANVFAVDIHPAARIGKGILFDHATGVVEGRHRNREQCVDPAP 189
Spinacia oleracea               ALALHSRISDVFAVDIHPAARIGKGILFDHATGVVIGETAIIGDNCSILH 250
Allium cepa                     ALALHSRVADVLSVDIHPAARIGKGILLDHATGVVIGETAVIGNNVSILH 192
Allium tuberosum                ALALHSRVADVLSVDIHPAARIGKGILLDHATGVVIGETAVIGNNVSILH 192
                                ****:**:::*::*******:******:******* *.     :  .

Arabidopsis thaliana (SAT5)     HVTLGGTGKACGDRHPKIGDGCLIGAGATILGNVKIGAGAKVGAGSVVLI 264
Citrullus vulgaris              HVTLGGTGKMCGDRHPKIGDGVLIGAGATILGNVKIGEGAKIGAGSVVLI 247
Glycine max                     -CSLGGTGKVGGDRHPKIGDGVLIGAGATILGNIKIGEGAKVGAGSVVLI 238
Spinacia oleracea               HVTLGGTGKAGGDRHPKVGDGVLIGAGATILGNVRIGDGAKIGAGSVVLI 300
Allium cepa                     HVTLGGTGKAGGDRHPKIGDGVLIGAGATILGNIRIGAGAKVGAGSVVLI 242
Allium tuberosum                HVTLGGTGKAGGDRHPKIGDGVLIGAGATILGNIRIGAGAKIGAGSVVLI 242
                                  :******  ******:*** ***********::** ***:********

Arabidopsis thaliana (SAT5)     DVPCRGTAVGNPARLVGGKEKPTIHDEECPGESMDHTSFISEWSDYII 312
Citrullus vulgaris              DVPPRTTAVGNPARLVGGKEKPS-QLEDIPGESMDHTSFISEWSDYII 294
Glycine max                     DVPPRTTAVGNPARLVGGKEKPS-KHEDVPGESMDHTSFISEWSDYII 285
Spinacia oleracea               DVPPRTTAVGNPARLIGGKEKPS-QNSDVPGESMDHTSFISEWSDYII 347
Allium cepa                     DVPPRTTAVGNPARLIGGKEKPS-VHEDVPGESMDHTSFISEWSDYII 289
Allium tuberosum                DVPPRTTAVGNPARLIGGKEKPS-MHEDVPGESMDHTSFISEWSDYII 289
                                *** * *********:******:   .: *******************
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Fig. 4. CLUSTAL-X alignment of the SAT sequences form *Arabidopsis thaliana SAT5* – AT5g56760, *Citrullus vulgaris* – GI 1361979, *Glycine max* – GI 18252508, *Spinacia oleracea* – GI 7433702, *Allium cepa* – GI 6601494; *Allium tuberosum* – GI 7384806. (*) represents identical residues, (:) represents similar residues, and (.) represents partial identity (4 out of 6 residues).

2.6. Western analysis of SAT expression

Whole leaf and chloroplast extracts were separated using gradient SDS–PAGE, and challenged with antibodies raised against the affinity purified SAT1 protein. The antibody strongly recognised a protein of ca. 33 kDa in the whole leaf extract (lane a, Fig. 5B), and weak recognition of two peptides of ca. 45 kDa and ca. 70 kDa was observed in the separated chloroplast extracts (lanes b, c, Fig. 5B).

2.7. Kinetic characterisation of the recombinant SAT enzyme

SAT activity was measured, using the recombinant enzyme obtained by affinity purification, by acetyl-CoA hydrolysis detected at 232 nm. Using L-serine as substrate, a K_m of 0.72 mM was determined while a K_m of 92 μM was calculated with acetyl-CoA as substrate. An inhibition curve for L-cysteine was generated using substrate concentrations of 5 mM L-serine and

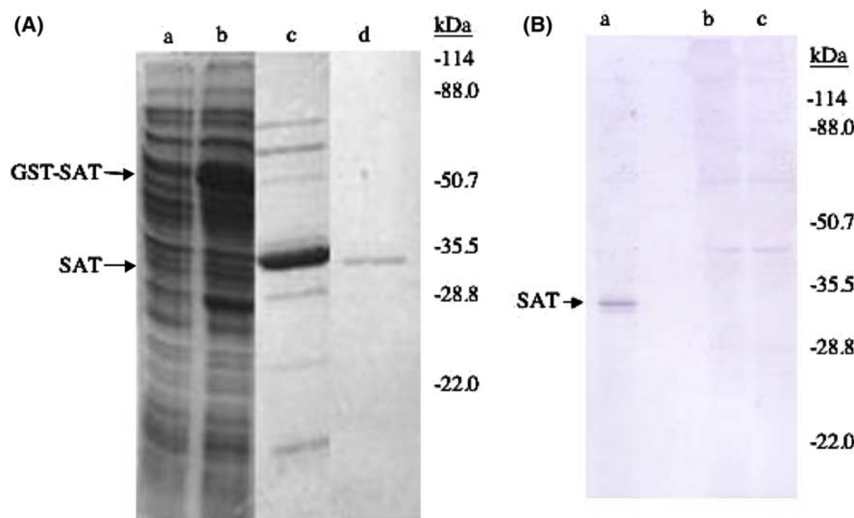

Fig. 5. (A) Expression of the *SAT1* gene of *A. cepa* in *E. coli* strain BL-21 and purification using affinity chromatography and ion-exchange chromatography. The proteins at each stage of the purification process were separated through a 12% SDS–PAGE gel and visualised using coomassie blue staining. Lane a = whole cell lysate of non-induced *E. coli* Bl-21 cells; lane b = whole cell lysate of *E. coli* Bl-21 cells induced with 0.6 mM IPTG; lane c = affinity purification of SAT1 using glutathione–Sepharose after cleavage of the GST–SAT1 fusion protein with protease; lane d = purification of SAT1 using ion-exchange chromatography from the preparation shown as lane c. (B) Western analysis of a whole leaf extract of *A. cepa* line W202A (lane a) and choloroplast preparations from *A. cepa* lines W202A (lane b) and Texas Grano W438 (lane c). Extracts were separated though a 10–20% gradient gel, blotted onto a PVDF membrane, and challenged with antibodies to SAT1 from *A. cepa.* Antibody recognition was determined using alkaline-phosphatase-linked secondary antibodies.

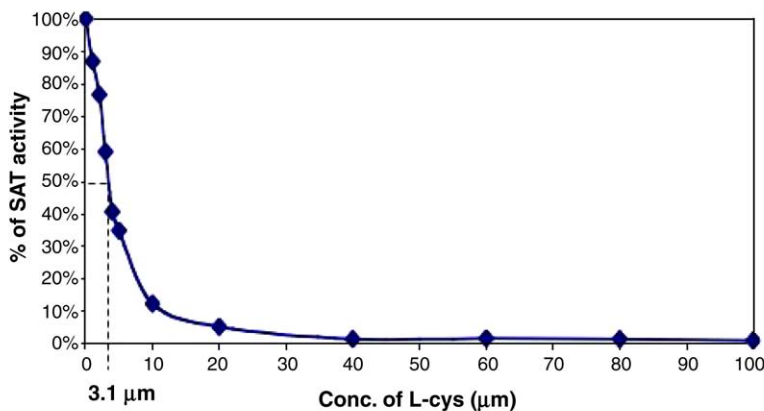

Fig. 6. Titration of recombinant SAT1 enzyme from *A. cepa* against increasing concentrations of L-cysteine using substrate concentrations of 5 mM L-serine and 1 mM acetyl-CoA. The IC_{50} value is indicated.

1 mM acetyl-CoA, from which an IC_{50} value of 3.1 μM was calculated (Fig. 6).

3. Discussion

Knowledge of the regulation of accumulation and allocation of S by the *Allium* group of vegetables represents an important avenue for the improvement of their flavour and medicinal benefits. However, there has been comparatively little characterisation of the mechanism of control of S uptake and assimilation in this important group of crop plants. In roots of onion, the expression of one member of the high-affinity-uptake gene family, and an APS reductase gene have been shown to be upregulated in response to S-deprivation in a pungent (high accumulator of ACSOs) and mild (lower accumulator or ACSOs) cultivar, while the transcription of an ATP sulfurylase gene decreased in response to S-deprivation (McCallum et al., 2001). Further, the activity of the APS reductase enzyme in leaf tissue of field-grown onions in response to nitrogen (N) and S inputs did not show the expected induction in response to low S inputs (McCallum et al., 2005). Collectively, these studies suggest that there are differences in the control of the reductive S-assimilation pathway in onion when compared to the model species, *A. thaliana*. Thus the characterisation of enzymes in the pathway in onion is

important to understand the accumulation of S in this important crop species, and this study focusses on the serine acetyltransferase enzyme, SAT1.

In terms of the biochemical data, a molecular mass of 30,573 Da for the onion enzyme compares well with determinations for other SAT enzymes that are deduced from SAT genes including 31,536 Da from *C. vulgaris* (Saito et al., 1995), 32,700 Da from SAT5 (SAT-p; Noji et al., 1998) and 30,300 Da from *G. max* (Chronis and Krishnan, 2004). The K_m value for L-serine as substrate of 0.72 mM for the onion enzyme compares well with a value of 0.59 mM for *C. vulgaris* when expressed as a recombinant enzyme (Saito et al., 1995), but is lower than other recombinant enzyme values including 2.71 mM for SAT5 of *A. thaliana* (SAT-c; Noji et al., 1998) and 2.27 mM for *G. max* (Chronis and Krishnan, 2004), and with the isoform purified from plastids (2.29 mM; Ruffet et al., 1994). For acetyl-CoA, a K_m value of 92 μM again compares well with *C. vulgaris* (0.13 mM), but is lower than values determined for SAT5 (SAT-c; 0.28 mM) and *G. max* (0.31 mM), and a determination of 0.35 mM for the purified plastid enzyme of *S. oleracea* (Ruffet et al., 1994). These data suggest that SAT1 of *A. cepa* is not significantly distinct, kinetically, from the SAT1 enzyme that has been characterised in both S-accumulating and non S-accumulating plants.

Titrations with L-cysteine determined that the SAT activity is inhibited by the presence of the amino acid, L-cysteine. An IC_{50} value of 3.1 μM L-cysteine compares well with values of 2.9 μM for *C. vulgaris*, 1.8 μM for SAT5 of *Arabidopsis*, and 7.6 μM for *S. oleracea* (Noji et al., 2001). In addition, a plastid isoform of SAT isolated from *S. oleracea* has also been shown to be inhibited by L-cysteine with an IC_{50} value of 12.2 μM (Droux, 2003). However, the IC_{50} value calculated for SAT1 is lower than a value of 48.7 μM calculated for the recombinant enzyme from *A. tuberosum* (Urano et al., 2000). In their study, Urano et al. (2000) argued that the cytosolic form of SAT they had characterised was feedback-insensitive to cysteine and that this, in part, accounted for the significant accumulation of S-containing in *A. tuberosum*. As onion also accumulates significant levels of S-containing compounds, one would expect that at least one of the SAT family may also be feedback-insensitive to cysteine. However, SAT1 is feedback regulated by cysteine suggesting that the function of this isoform is not as part of the regulatory mechanism that allows significant pools of S-containing compounds to accumulate. Indeed, northern analysis has shown that *SAT1* is induced in leaves of bulbing onions by low S-supply, indicating that this isoenzyme may be involved in the response to low S-stress. Further, it has been well established in *A. thaliana* that the SAT multi-gene family displays tissue-specific patterns of expression (Howarth et al., 2003), and so it is probable that the multi-gene family in onion also displays differential expression in response to developmental or environmental cues.

Phylogenetic analysis supports this biochemical similarity in that the *SAT1* gene of *A. cepa* is placed in a group of genes whose protein products have been characterised biochemically, and shown to be feedback inhibited by L-cysteine. Of those in which sub-cellular localisation data have been sought, the SAT5 protein of *A. thaliana* has been shown to be localised in the cytosol (Noji et al., 1998), while the isoform encoded for by the *SAT* gene of *C. vulgaris* has been shown not to occur in the chloroplast. The activity of the SAT-m (SAT3) and SAT-p (SAT1) enzymes of *A. thaliana* are not feedback inhibited by L-cysteine suggesting that this property of SAT may be restricted to the cytosolic enzyme. However, the *SAT1* gene of *A. cepa* characterised in this study, and the *SAT5* gene of *A. thaliana* also groups with the SAT gene from *S. oleracea*, which displays inhibition of activity by L-cysteine and has been localised to the chloroplast (Noji et al., 1998). In another study, using a plastid isolated SAT enzyme from *P. sativum*, Droux (2003) also demonstrated feedback inhibition by L-cysteine. Further, Droux and colleagues reported that the mitochondrial isoform was comparatively weakly inhibited (IC_{50} of 205.3 μM) and no evidence of L-cysteine inhibition could be detected in SAT activity from a cytosol preparation (with less than 5% of mitochondria or chloroplast contamination) (Droux, 2003).

Phylogenetic analysis has also determined that the *A. cepa SAT1* gene and the *A. tuberosum* sequence form a sub-group, suggesting a closer relationship between the two species of *Allium*.

In this study, western analysis has shown that antibodies raised against the recombinant gene product recognise a protein of the expected size in a leaf extract of onion, but not one of the expected size in the chloroplast preparation. We have raised antibodies to APS reductase, a plastid-localised enzyme and these antibodies do recognise a protein of the expected mass in the chloroplast (data not shown) suggesting that our plastid preparations are sufficiently pure. We cannot, however, exclude the possibility that the SAT enzyme under investigation in this study is localised in the mitochondria, but the phylogenetic analysis places the gene as distant from SAT-m and SAT-p from *A. thaliana* (Noji et al., 1998). Two proteins were recognised, albeit weakly, in the chloroplast preparation but the identity of these proteins is yet to be determined. However, we cannot exclude the possibility that the recognition is non-specific in nature, and these proteins are not related to SAT1.

A survey of the literature suggests that inhibition of SAT enzyme activity by L-cysteine is not restricted to cytosolic forms, but in *S. oleracea* and *P. sativum*, at least, the plastid-localised enzyme is inhibited (and for *P. sativum*, the cytosolic enzyme is not). For *A. cepa*, we have not characterised any plastid isoforms from

the protein family, and so cannot speculate further on this aspect of control of the SAT enzyme in different compartments of leaf cells of onion. However, the inhibition of activity by L-cysteine is common to other plant species that are not significant S-accumulators, e.g., water melon, suggesting that if there are different points of control of the pathway in S-accumulators such as onion, then these may not be focussed around this SAT gene.

4. Experimental

4.1. Plant material

An F_2 family of 82 plants was developed from the 'W202A' × 'Texas Grano 438' (W × T) population by self-pollinating a single F_1 plant derived from a bulk cross between 'W202A' (Goldman et al., 2000) and 'Texas Grano 438' (Asgrow Seed Co.).

4.2. Plant growth and regulation by S-supply

Germinated seeds of the W202A cultivar were transferred to 'Oasis' Horticultural foam blocks, and at the second leaf stage blocks were transferred to 18 L tubs containing Hoagland media (Hoagland and Arnon, 1950). Plants were maintained in the glasshouse as described in McCallum et al. (2002), and high S [4 Meq S] and low S [0.1 Meq] media, supplied by varying the concentration of $MgSO_4$, $Mg(NO_3)_2$ and $Ca(NO_3)_2$ in Hoagland media. Leaf material was excised from plants at a pre-bulbing stage (ca. 10-week-old seedlings), and at bulbing (ca. 16-week-old stage).

4.3. Genetic mapping

DNA isolation, PCR conditions and single-stranded conformation polymorphism analysis (SSCP) were performed as described previously (McCallum et al., 2001). The *SAT1* locus was amplified using the primers SAT 5′ AAGGTCGGAGCAGGGTCT and SAT3′ AACAACAAGACGATCACGTACAA and scanned for SSCPs. SSR markers were analysed according to Martin et al.(2005) and the SSR locus ACM157 was amplified using the primers ACM157L GCTAGTTGTACCTGCGCCTC and ACM157R TTGTTGTTGGTGTTTCCAGG. Linkage analysis was performed with Joinmap 3.0 software (Van Ooijen and Voorrips, 2001).

4.4. Isolation of RNA and northern analysis

Total RNA was isolated from leaf tissue as described in McCallum et al. (2002). For northern analysis, 30 μg of total RNA was reduced in 2.2 M formaldehyde, and then separated through a 1% (w/v) agarose gel in the presence of 2.2 M formaldehyde. After transfer to a nylon membrane, the separated RNA was probed with the 3-UTR (284 bp) region of the *SAT1* gene. The probe was amplified using a forward primer, 5′- AAGGTCGGAGCAGGGTCT-3′, positioned at +740 to +758 bp in the *SAT1* sequence, and a reverse primer, 5′-AACAACAAGACGATCACGTACAA-3′, positioned at +1001 to +1024 bp in the *SAT1* gene sequence. The 284 bp probe was labelled with [α-^{32}P]dATP using the Megaprime™ DNA labelling kit (Amersham Biosciences, Piscataway, NJ, USA), and northern analysis performed essentially as described in Hunter et al. (1999). Hybridisation was recorded using a FUJI PLA-5000 Phospho-Imager.

4.5. Expression and purification of SAT as a recombinant protein in E. coli

The SAT cDNA, previously cloned (AF212156; McCallum et al., 2002) into the pBluescript vector, was used as template for the PCR amplification with a forward primer (SAT-F1) comprising 5′-CAGCAGCCGAATTCCATGCCATGCTCAACTCTT-3′, and a reverse primer (SAT-R1) comprising 5′-CAGCAGCTCGAGTCAGATGATATAATCCGACC-3′. The amplified product had a 5′-*Eco*R1 site and a 3′-*Xho*1 site to afford the directional cloning of the reading frame into the polylinker of the pGEX-GP-3 plasmid (Amersham Biosciences), to provide a fusion protein with glutathione-*S*-transferase (GST), and a N-terminal 8-amino acid residue extension in the translated protein product after protease digestion (Gly-Pro-Leu-Gly-Ser-Pro-Asn-Ser). The pGEX-GP-3 vector with the SAT insert was transformed into *E. coli* strain BL21, and translation of the SAT gene insert induced with the addition of 0.6 mM IPTG and incubation at 20 °C for 18 h. After this incubation period, cells were collected by centrifugation at 3000*g* for 10 min at 4 °C, the cells lysed by French Press at 15 kpsi, and the slurry centrifuged at 12,000*g* for 20 min at 4 °C. The fusion protein was isolated by incubation with glutathione–Sepharose (Amersham BioSciences), pre-equilibrated in phosphate-buffered saline, for 30 min at 25 °C. The Sepharose containing the bound GST–SAT fusion protein was then collected by centrifugation at 500*g* for 5 min at 25 °C, the resin then washed once with PBS, and then once with protease cleavage buffer (50 mM Tris–HCl, pH 7.0 containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT) before release of the SAT protein by incubation with Precission Protease (Amersham BioSciences) for 18 h at 25 °C. The Sepharose was collected by centrifugation at 500*g* for 5 min at 25 °C, and an aliquot of the supernatant containing the recombinant SAT protein was examined by SDS–PAGE. Staining revealed a protein of ca. 33,000 Da as the major protein in the fraction, the expected mass of the recombinant SAT protein with the

8-amino acid N-terminal extension, but other minor contaminants were clearly discernable.

To purify the recombinant SAT protein, the supernatant after protease digestion was exchanged into 50 mM Tris–HCl, pH 7.5 (buffer A), and loaded onto a Mono Q HR 5/5 ion-exchange column (Amersham BioSciences). Bound proteins were eluted within a gradient of 100% A:0% buffer B (buffer A containing 1.0 M NaCl) to 0% buffer A:100% buffer B. To localise the eluted SAT enzyme, activity was then measured in each of the FPLC fractions.

4.6. Measurement of SATase activity

SAT enzyme activity was determined from the rate of decrease in absorbance at A_{232} nm due to cleavage of the thioester bond in acetyl-CoA (Baecker and Wedding, 1980). For K_m determinations with L-serine as substrate, a concentration range of 0.1–20 mM was used, while for the determination of the K_m for acetyl-CoA, substrate concentrations of 0.5–10 mM were used. For the L-cysteine titrations, substrate concentrations of 5 mM L-serine and 1 mM acetyl-CoA were used. All assays were buffered with 50 mM Tris–HCl, pH 8.0, the enzyme reaction initiated with the addition of L-serine, and conducted at 25 °C. The molar extinction co-efficient of acetyl-CoA of $\varepsilon = 4500$ was used to calculate rates.

4.7. Isolation of chloroplasts from leaf tissue of A. cepa

Ice-cold leaf tissue of *A. cepa* lines W202A and Texas Grano 438 (5 g) were homogenised in 25 ml of ice-cold isolation medium (50 mM Tricine–KOH, pH 7.9, containing 2.0 mM EDTA, 1 mM $MgCl_2$ and 330 mM sorbitol) using an Ultra-Turrax blender for 10 s. The slurry was filtered through three layers of ice-cold miracloth into a 50-mL capacity plastic centrifuge tube, and the filtrate centrifuged at 1465*g* for 3 min at 4 °C. The supernatant was carefully decanted and the pellet resuspended with 5 mL of isolation medium. The slurry was then carefully layered onto a 40% (v/v):80% (v/v) (12 mL of each made up in isolation medium) discontinuous Percoll gradient (Sigma–Aldrich) made in a 30-mL capacity Corex glass centrifuge tube, and centrifuged at 3300*g* for 10 min at 4 °C. The upper 40% layer was removed down to the chloroplast fraction at the 40%–80% interface, fresh isolation medium added, the content mixed and then centrifuged as before. On the conclusion of centrifugation, the chloroplast fraction had migrated towards the bottom of the Corex tube, and so the upper layer was again removed, fresh ice-cold isolation medium added, the contents mixed and centrifuged as described. After centrifugation, the isolation medium supernatant was removed and the chloroplast pellet was resuspended in a small volume (ca. 500 μL) of residual isolation medium, before storage at −20 °C until use.

4.8. SDS–PAGE and western analysis

To make chloroplast extracts, whole chloroplasts were thawed, an equal volume of ice-cold water added and the slurry homogenised using a mortar and pestle. The slurry was then centrifuged at 13,000 *g* for 10 min at 4 °C, and the supernatant used directly for SDS–PAGE. For leaf extracts, whole leaf tissue from onion line W202A was extracted in a morter and pestle with 50 mM potassium phosphate buffer, pH 7.5 containing 1.0 mM DTT and 1.0 mM EDTA, using a buffer:fresh weight ratio of 3:1. The slurry was then centrifuged at 13,000 *g* for 10 min at 4 °C, and the supernatant used directly for SDS–PAGE. SDS–PAGE was performed either using a 12% continuous, or 10 % to 20% gradient gel and western transfer used the method essentially as described in Lancaster et al. (2000). Determination of molecular masses of proteins was as described by Hames and Rickwood (1981).

4.9. Phylogenetic analysis

A majority rule consensus tree (neighbour joining/observed distances/100 bootstraps) was built using the computer programme 'Phylogenetic Analysis Using Parsimony *' (PAUP* version 4.0 od64, Sinaur Associates, Inc. Publishers, Sunderland, MA, USA). Sequences used to construct the tree are identified by accession number in the figure legend.

Acknowledgements

The authors thank Dr. Peter Lockhart, Institute of Molecular BioSciences, Massey University for assistance with the phylogenetic analysis.

References

Baecker, P., Wedding, R.T., 1980. Purification of serine acetyltransferase, a component of a multienzyme complex, by immunoadsorption and selective dissociation of the complex. Anal. Biochem. 102, 16–21.

Bogdanova, N., Hell, R., 1997. Cysteine synthesis in plants: protein–protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J. 11, 251–262.

Chronis, D., Krishnan, H.B., 2004. Sulfur assimilation in soybean (*Glycine max* [L.] Merr.): molecular cloning and characterization of a cytosolic isoform of serine acetyltransferase. Planta 218, 417–426.

Droux, M., 2003. Plant serine acetyltransferase: new insights for regulation of sulphur metabolism in plant cells. Plant Physiol. Biochem. 41, 619–627.

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

Goldman, I.L., Schroek, G., Havey, M.J., 2000. History of public onion breeding programs in the United States. Plant Breeding Reviews 20, 68–103.

Hames, B.D., Rickwood, D., 1981. Gel Electrophoresis of Proteins: a Practical Approach. IRL Press, Oxford, Washington, DC.

Hawkesford, M.J., Wray, J.L., 2000. Molecular genetics of sulphate assimilation. Adv. Bot. Res. 33, 159–223.

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

Hesse, H., Nikiforova, V., Gakière, B., Hoefgen, R., 2004. Molecular analysis and control of cysteine biosynthesis: integration of nitrogen and sulphur metabolism. J. Exp. Botany 55, 1283–1292.

Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ., No.347.

Howarth, J.R., Dominguez-Solis, J.R., Gutierrez-Alcala, G., Wray, J.L., Romero, L.C., Gotor, C., 2003. The serine acetyltransferase gene family in *Arabidopsis thaliana* and the regulation of its expression by cadmium. Plant Mol. Biol. 51, 589–598.

Hunter, D.A., Yoo, S.D., Butcher, S.M., McManus, M.T., 1999. Expression of 1-aminocyclopropane-1-carboxylate oxidase during leaf ontogeny in white clover. Plant Physiol. 120, 131–141.

Inoue, K., Noji, M., Saito, K., 1999. Determination of the sites required for the allosteric inhibition of serine acetyltransferase by L-cysteine in plants. Eur. J. Biochem. 266, 220–227.

King, J.J., Bradeen, J.M., Bark, O., McCallum, J.A., Havey, M.J., 1998. A low-density genetic map of onion reveals a role for tandem duplication in the evolution of an extremely large diploid genome. Theor. Appl. Genet. 96, 52–62.

Lancaster, J.E., Shaw, M.L., Pither-Joyce, M.D., McCallum, J., McManus, M.T., 2000. A novel alliinase from onion (*Allium cepa* L.) roots: biochemical characterization and cDNA cloning. Plant Physiol. 122, 1269–1279.

Leustek, T., Martin, M.N., Bick, J.A., Davies, J.P., 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 141–165.

Martin, W., McCallum, J., Shigyo, M., Jakse, J., Kuhl, J.C., Yamane, N., Sink, K.C., Town, C.D., Havey, M.J., 2005. Genetic mapping of expressed sequences in onion and in silico comparisons with rice show scant colinearity. Mol. Gen. Genet. (in press).

McCallum, J.A., Leite, D., Pither-Joyce, M., Havey, M.J., 2001. Expressed sequence markers for genetic analysis of bulb onion (*Allium cepa.* L.). Theor. Appl. Genet. 103, 979–991.

McCallum, J., Pither-Joyce, M., Shaw, M., 2002. Sulfur deprivation and genotype affect gene expression and metabolism in onion roots. J. Am. Soc. Hort. Sci. 127, 583–589.

McCallum, J., Porter, N., Searle, B., Shaw, M., Bettjeman, B., McManus, M.T., 2005. Sulfur and nitrogen fertility affects flavour of field-grown onions. Plant and Soil (in press).

Nakamura, K., Tamura, G., 1990. Isolation of serine acetyltransferase complexed with cysteine synthase from *Allium tuberosum*. Agric. Biol. Chem. 54, 649–656.

Noji, M., Inoue, K., Kimura, N., Gouda, A., Saito, K., 1998. Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from *Arabidopsis thaliana*. J. Biol. Chem. 273, 32739–32745.

Noji, M., Takagi, Y., Kimura, N., Inoue, K., Saito, M., Horikoshi, M., Saito, F., Takahashi, H., Saito, K., 2001. Serine acetyltransferase involved in cysteine biosynthesis from spinach: molecular cloning, characterization and expression analysis of cDNA encoding a plastidic isoform. Plant Cell Physiol. 42, 627–634.

Van Ooijen, J.W., Voorrips, R.E., 2001. JoinMap (r) 3.0, Software for the Calculation of Genetic Linkage Maps. Plant Research International BV, Wageningen, The Netherlands.

Randle, W.M., Lancaster, J.E., 2002. Sulfur compounds in *Alliums* in relation to flavour quality. In: Rabinowitch, H., Currah, L. (Eds.), *Allium* Crop Science – Recent Advances. CABI Publishing, Oxford, pp. 1–62.

Ruffet, M.-L., Droux, M., Douce, R., 1994. Purification and kinetic properties of serine acetyltransferase free of *O*-acetylserine(thiol)lyase from spinach chloroplasts. Plant Physiol. 104, 597–604.

Saito, K., Yokoyama, H., Noji, M., Murakoshi, I., 1995. Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon. J. Biol. Chem. 270, 16321–16326.

Urano, Y., Manabe, T., Noji, M., Saito, K., 2000. Molecular cloning and functional characterization of cDNAs encoding cysteine synthase and serine acetyltransferase tht may be responsible for high cellular cysteine content in *Allium tuberosum*. Gene 257, 269–277.

Wirtz, M., Berkowitz, O., Droux, M., Hell, R., 2001. The cysteine synthase complex from plants. Eur. J. Biochem. 268, 686–693.