Peroxisomal alanine : glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*

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Summary

At least two glyoxylate aminotransferases are hypothesized to participate in the steps of photorespiration located in peroxisomes. Until recently, however, genes encoding these enzymes had not been identified. We describe the isolation and characterization of an alanine : glyoxylate aminotransferase (*AGT1*, formerly *AGT*) cDNA from *Arabidopsis thaliana*. Southern blot analysis confirmed that *Arabidopsis* AGT1 is encoded by a single gene. Homologs of this class IV aminotransferase are also known in other plants, animals, and methylotrophic bacteria, suggesting an ancient evolutionary origin of this enzyme. *AGT1* transcripts were present in all tissues of *Arabidopsis*, but were most abundant in green, leafy tissues. Purified, recombinant *Arabidopsis* AGT1 expressed in *Escherichia coli* catalyzed three transamination reactions using the following amino donor : acceptor combinations: alanine : glyoxylate, serine : glyoxylate, and serine : pyruvate. AGT1 had the highest specific activity with the serine : glyoxylate transamination, and apparent K_m measurements indicate that this is the preferred *in vivo* reaction. *In vitro* import experiments and subcellular fractionations localized AGT1 to peroxisomes. Sequence analysis of the photorespiratory *sat* mutants revealed a single nucleotide substitution in the *AGT1* gene from these plants. This transition mutation is predicted to result in a proline-to-leucine substitution at residue 251 of AGT1. When this mutation was engineered into the recombinant AGT1 protein, enzymatic activity using all three donor : acceptor pairs was abolished. We conclude that *Arabidopsis* AGT1 is a peroxisomal photorespiratory enzyme that catalyzes transamination reactions with multiple substrates.

Keywords: alanine : glyoxylate aminotransferase, serine : glyoxylate aminotransferase, peroxisome, photorespiration, aminotransferase, *Arabidopsis*.

Introduction

Photorespiration is a metabolite salvage pathway that is necessary because of the dual activities of ribulose-1,5-bisphosphate carboxylase/oxygenase. When ribulose-1,5-bisphosphate is oxygenated by ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoglycerate and phosphoglycolate are formed. The reactions within the photorespiratory pathway convert phosphoglycolate into phosphoglycerate, with the concomitant release of CO_2 and NH_3. This pathway is co-ordinated between three cellular compartments: chloroplasts, peroxisomes and mitochondria. At least two aminotransferases hypothesized to be involved in photorespiration are thought to reside within peroxisomes. These aminotransferases use the 2-oxoacid glyoxylate as an amino acceptor, with various amino acids serving as donors (Husic *et al.*, 1987; Leegood *et al.*, 1995; Somerville and Ogren, 1980).

Studies of plant glyoxylate aminotransferases have depended on protein preparations isolated from various plants, including spinach and cucumber (Hondred *et al.*, 1985; Ireland and Joy, 1983; Nakamura and Tolbert, 1983; Noguchi and Fujiwara, 1982; Noguchi and Hayashi, 1980; Noguchi and Hayashi, 1981; Rehfield and Tolbert, 1972; Smith, 1973; Yu *et al.*, 1984). Chromatographic purification analyses provide evidence for at least two distinct glyoxylate aminotransferases in plant extracts. These enzymes are active with a wide range of donor : acceptor

combinations. Although these aminotransferases have somewhat overlapping substrate ranges (i.e. both catalyze an alanine : glyoxylate aminotransferase (AGT) reaction), they are distinguishable by their unique activities: one has serine : glyoxylate aminotransferase (SGT) activity, the other exhibits glutamate : glyoxylate aminotransferase (GGT) activity (Nakamura and Tolbert, 1983; Rehfield and Tolbert, 1972). There has been controversy regarding the identity of these two aminotransferases, in part due to their broad and overlapping substrate specificities, and also due to the partial purity of some of these preparations.

The confusion about the number and identity of plant glyoxylate aminotransferases is complicated by the system of nomenclature. Because genes for these enzymes were not previously identified, the enzymes were assigned names based on activity rather than homology with known genes. For consistency with nomenclature from other organisms, the *Arabidopsis* enzyme catalyzing AGT, SGT and serine : pyruvate (SPT) transaminations will be referred to as AGT1 because it is homologous to human AGT1 (Liepman and Olsen, 1998); this enzyme has been called AGT, SGT or tryptophan aminotransferase (Hondred *et al.*, 1985; Nakamura and Tolbert, 1983; Noguchi and Hayashi, 1980; Smith, 1973). A second glyoxylate aminotransferase, characterized by GGT and AGT activities and a lack of SGT activity, has been called AGT, GGT or glutamate : 2-oxoglutarate aminotransferase in prior publications (Nakamura and Tolbert, 1983; Noguchi and Fujiwara, 1982; Noguchi and Hayashi, 1981; Rehfield and Tolbert, 1972). Genes encoding the aminotransferase(s) responsible for these activities in plants have not been isolated.

The hypothesis that SGT activity (AGT1) is important for photorespiration is supported by mutagenesis studies, although the mutated genes were not identified (McHale *et al.*, 1988; Murray *et al.*, 1987; Somerville and Ogren, 1980). Mutants lacking SGT activity were characterized by a conditional lethal phenotype when grown in conditions allowing photorespiration. Under normal atmospheric conditions these mutants grew poorly, had greatly reduced rates of photosynthesis, and accumulated serine and glycine. When photorespiration was suppressed by increasing the CO_2 concentration in the growth environment, the mutants displayed no apparent phenotype, indicating that SGT activity is not required outside the photorespiratory pathway. AGT and GGT activity were maintained at nearly wild-type levels in *sat* mutants (Somerville and Ogren, 1980), suggesting that the aminotransferases responsible for SGT and AGT/GGT activities *in vivo* are probably distinct. An additional link between AGT1 and photorespiration is the finding that SGT activity and a corresponding cross-reactive polypeptide are undetectable in etiolated cucumber seedlings that do not photorespire; SGT activity increases and the cross-reactive protein becomes more abundant as the cucumber seedlings become photoautotrophic and begin to photorespire (Hondred *et al.*, 1985; Noguchi and Fujiwara, 1982).

We have isolated the *AGT1* gene from *Arabidopsis* (Liepman and Olsen, 1998) and studied its expression pattern. The catalytic activity of recombinant AGT1, expressed and purified from *Escherichia coli*, was examined free of additional plant proteins. AGT1 was localized exclusively to peroxisomes in plants. We demonstrated that air-sensitive *sat* mutant *Arabidopsis* plants contain a mutated *AGT1* gene, and that when introduced into the recombinant protein this mutation abolishes all activity. These findings implicate AGT1 as a key peroxisomal enzyme in the photorespiratory pathway.

Results

Identification and sequence analysis of Arabidopsis AGT1

Sequence analysis of the *Arabidopsis* EST 35A11T7 revealed that it contains the entire coding sequence of *AGT1* plus 5′ and 3′ UTR regions (formerly *AGT*; Liepman and Olsen, 1998). The 1441 bp cDNA contains a 1203 bp ORF, predicted to encode a polypeptide of 401 amino acids with a calculated molecular mass of 44 206 Da. A conserved variant of the type I peroxisomal targeting signal, ser-arg-ile (highlighted in Figure 1), resides at the carboxyl terminus of AGT1 (Olsen, 1998). A recent database search using the full-length protein sequence identified an AGT1 homolog from the lily *Fritillaria agrestis* with 85% sequence identity. Based on its sequence, the putative function of this protein was suggested to be SGT (unpublished results; Accession No. AF039000). Homologs of AGT1 with known function were found in the methylotrophic bacteria *Hyphomicrobium methylovorum* (Hagashita *et al.*, 1996) and *Methylobacterium extorquens* (Chistoserdova and Lidstrom, 1994), as well as many mammals, including humans. The *H. methylovorum* homolog shares 51% sequence identity and encodes an aminotransferase specific for the SGT reaction (Hagashita *et al.*, 1996; Izumi *et al.*, 1990). Human AGT1 is 33% identical to *Arabidopsis* AGT1; in addition to an AGT reaction, human AGT1 also catalyzes a serine : pyruvate aminotransferase (SPT) reaction (Watts, 1992). Partially sequenced cDNAs from other plants, including tomato, rice, soybean, the common ice plant (*Mesembryanthemum crystallinum*), and the moss *Physcomitrella patens*, also contained regions with a high degree of similarity to *Arabidopsis AGT1*. It should be noted that AGT1 is distinct from alanine aminotransferase (EC 2.6.1.2) (Muench and Good, 1994; Son and Sugiyama, 1992), as demonstrated by a very low degree of sequence similarity.

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At    ---MDYMYGP G--------- -RHHLFVPGP VNIPEPVIRA MNRNNEDYRS PAIPALTKTL LEDVKKIFKT   57
Fa    ---MDYVYGP G--------- -RNHLFVPGP VNIPEPVIRA MNRNNEDYRA PAIPALTKTL LEDVKKLFKT   57
Hm    -----MTVTP ---------- ---HLFIPGP TNIPDAVRMA MNIPMEDMRS PEFPKFTLPL FEDLKKAFKM   52
Hs    MASHKLLVTP PKALLKPLSI PNQLLLGPGP SNLPPRIMAA GGLQMIGSMS KDMYQIMDEI KEGIQYVFQT   70
cons       .    *           .*..*** *.*. ... *   .. . .  . . . .   .  . . *.....*..   70

At    TSGTPFLFPT TGTGAWESAL TNTLSPGDRI VSFLIGQFSL LWIDQQKRLN FNVDVVESDW GQGANLQVLA  127
Fa    TTGTPFIIPT TGTGAWESAL TNTLSPGDTI VSFLIGQFSL LWIDQQQRLK FKVDVIESEW GQGANLDELA  127
Hm    KDGRVFIFPS SGTGAWESAV ENTLATGDKV LMSRFGQFSL LWVDMCERLG LKVEVCDEEW GTGVPVEKYA  122
Hs    RNPLTLVISG SGHCALEAAL VNVLEPGDSF LVGANGIWGQ RAVDIGERIG ARVHPMTKDP GGHYTLQEVE  140
cons   .  .. .. .*..*.*.*. *.* .** . .    *.... ...*   . .*... .. *  .   ..  140

At    SKLSQDENHT IKAICIVHNE TATGVTNDIS AVRTLLDHYK HPALLLVDGV SSICALDFRM DEWGVDVALT  197
Fa    SKLAADRTHT IKAVCIVHNE TATGVSSDVA AVRKALDAAK HPALLMVDGV SSICALDFRM DEWGIDVALT  197
Hm    DILAKDKNHE IKAVFVTHNE TATGVSSDVA GVRKALDAAK HPALLMVDGV SSVGSLDMRM GEWGVDCCVS  192
Hs    EGLAQ---HK PVLLFLTHGE SSTGVLQPLD GFGELCHRYK --CLLLVDSV ASLGGTPLYM DRQGIDILYS  205
cons   *. .  *  .... . *.* ..***..*  . ...  ..   . . .   ...**.**.* .*. ... .*   ...*.*. ...  210

At    GSQKALSLPT GLGIVCASPK ALEATKTSKS --LKVFFDWN DYLKFY---K LGTYWPYTPS IQLLYGLRAA  262
Fa    GSQKALSMPT GMGFICASPK ALEASKTAQS --ARVFFDWN DYLKFY---K IGTYWPYTPS IQMLYGLRAA  262
Hm    GSQKGFMLPT GLGILAVSQK ALDINKSKNG RMNRCFFSFE DMIKTN---D QG-FFPYTPA TQLLRGLRTS  258
Hs    GSQKALNAPP GTSLISFSDK AKKKMYSRKT KPFSFYLDIK WLANFWGCDD QPRMYHHTIP VISLYSLRES  275
cons   ****..  .*. *.... * * *..  ..   .  . .... . .....*. ...*..**  280

At    LDLIFEEGLE NIIARHARLG KATRLAVEAW GLKNCTQKEE WISNTVTAVM VPPHIDGSEI VRRAWQRYNL  332
Fa    LDLIFEEGLD NVIARHSRLG KATRLAVEAW GLKNCTQKEE WHSDTVTAVV VPPYIDSSEI VRRAWKRYNL  332
Hm    LDLLFAEGLD NVFARHTRLA SGVRAAVDAW GLKLCAKEPK WYSDTVSAIL VPEGIDSNAI TKTAYYRYNT  328
Hs    LALIAEQGLE NSWRQHREAA AYLHGRLQAL GLQLFVKDPA LRLPTVTTVA VPAGYDWRDI VSYVIDHFDI  345
cons   *.*....**. *.  ..*  ...     .....*. **..     .. .**....** ** .*   .*  .. . ...  ....  350

At    SLGLGLNKVA GKVFRIGHLG NVNELQLLGC LAGVEMILKD VGYPVVMGSG VAAASTYLQH HIPLIPSRI-  401
Fa    SLGLGLNKVA GKVFRIGHLG NLNELQLLGC LSGVEMVLKD VGYPVKLGSG VAAAATYLQN STPMIPSRI-  401
Hm    SFGLGLNKVA GKVFRIGHLG MLDEVMIGGA LFAAEMALKD NGVNLKLGSG TGAAAEYFSK NATKSATALT  398
Hs    EIMGGLGPST GKVLRIG--- ------LLGC NATRENVDR- -----VT-EA LRAA---LQH -CP--KKKL-  392
cons   ....**.... ***.***... . . .. ..*. . . *.....  .  . ..... ..** .... .  ....  420

At    ------- 401
Fa    ------- 401
Hm    PKQAKAA 405
Hs    ------- 392
cons           427
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**Figure 1.** Alignment of *Arabidopsis* AGT1 with other group IV aminotransferases.
Arabidopsis AGT1 (At, Accession No. AF063901) was aligned with a putative SGT from the lily *Fritillaria agrestis* (Fa, Accession No. AF039000); SGT from a methylotrophic bacterium *Hyphomicrobium methylovorum* (Hm, Accession No. D86125); and AGT1 from *Homo sapiens* (Hs, Accession no. 178273) to produce a consensus sequence (cons) using the CLUSTALW algorithm; minor adjustments were introduced manually. Asterisks denote residues identical in all four sequences; dots indicate residues conserved in at least three of the sequences. The four residues that best conform to the aminotransferase consensus sequence are shaded in grey. Putative type 1 peroxisomal targeting sequences are boxed in black at the carboxyl terminus of the *Arabidopsis*, lily and human proteins. The black triangle above proline residue 251 of *Arabidopsis* AGT1 marks the position of the proline-to-leucine amino acid substitution in the *sat* mutant CS51.

Southern blot analysis indicated that a single gene encodes AGT1 in *Arabidopsis* (Supplementary material, Figure S-1). A BAC clone (AC007209; Lin *et al.*, 1999), representing a region of *Arabidopsis* chromosome II and containing the *AGT1* genomic sequence, was also identified through database searching. Results from the Southern blot were confirmed by sequence analysis of the chromosome II region containing the *AGT1* gene; the size and number of hybridizing bands observed by Southern blot corresponded almost exactly to that predicted by a computer generated restriction map of that chromosomal region. Alignment of the cDNA and genomic sequences of *AGT1* demonstrated that this gene contains five exons and four introns, and the transcribed region is predicted to span ≈2000 bp.

Currently, aminotransferases are divided into four subgroups based on sequence homology; *Arabidopsis* AGT1 most closely resembles members of group IV (Mehta *et al.*, 1993). Examination of a comprehensive alignment of more than 50 aminotransferase sequences, including members of each subgroup, led to the discovery of four invariant residues common to all aminotransferases: gly197; asp/glu222; lys258; arg386 (Mehta *et al.*, 1993). The absolute positions of these four residues vary among aminotransferases, but the relative spacing is conserved. The functions of these conserved residues have been defined by mapping their positions on the crystal structure of aspartate aminotransferase (Kirsch *et al.*, 1984; McPhalen *et al.*, 1992). In this enzyme, gly197 is located within a turn at a domain interface; asp/glu222 participates in a salt bridge to N1 of its cofactor pyridoxal 5-phosphate; lys258 forms a Schiff base with pyridoxal 5-phosphate; and arg386 is involved in a salt bridge with the α-carboxylic acid group of the substrate (Mehta *et al.*, 1993). An

alignment of *Arabidopsis* AGT1 with other group IV aminotransferases is shown in Figure 1. Residues gly151, asp175, lys201 and arg347 of *Arabidopsis* AGT1 (shaded in Figure 1) closely conform to the invariant residues characteristic of all aminotransferases. Alternatively, residues gly118, glu147, lys217 and arg347 also loosely fit the pattern, and may represent the conserved residues in *Arabidopsis* AGT1.
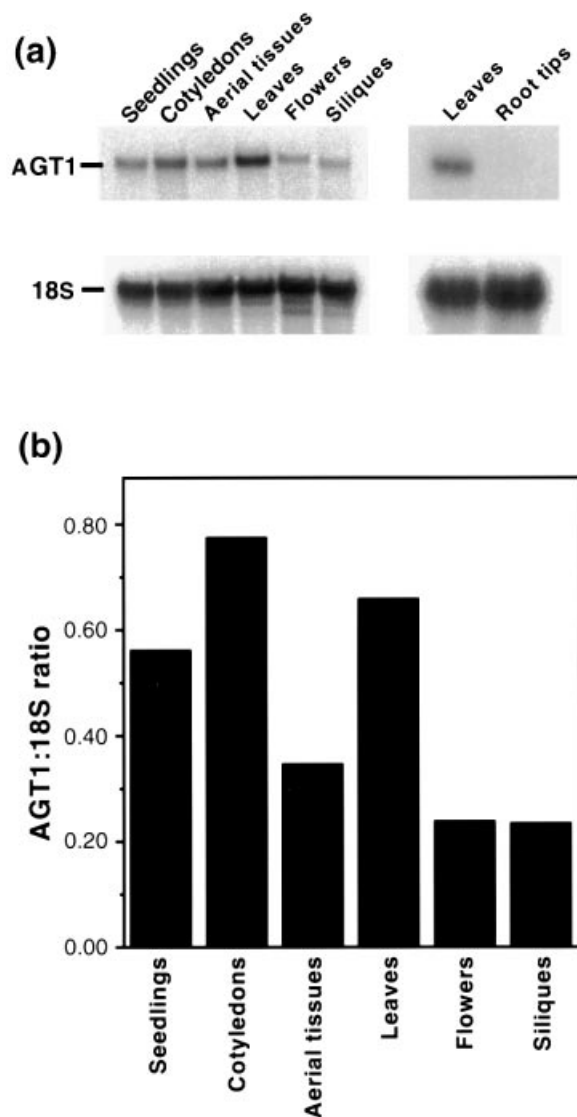
Figure 2. Analysis of *AGT1* mRNA accumulation in *Arabidopsis*.
Total RNA (≈5 μg) from the indicated *Arabidopsis* tissues was separated by electrophoresis and transferred to nylon membranes as described under Experimental procedures.
(a) Autoradiographs of membranes probed first with the ^{32}P-labeled *AGT1* cDNA fragment and subsequently with a fragment of the pea 18S rDNA, to control for potential loading differences.
(b) *AGT1* transcript accumulation was quantified using phosphorimaging analysis and normalized to 18S rRNA expression in above-ground tissues. A representative experiment is shown; this experiment was repeated twice.

Expression of AGT1

The mRNA expression pattern of *AGT1* was examined by RNA gel-blot analysis (Figure 2). *AGT1* cDNA, identical to that used for Southern analysis, was used to probe filters containing total RNA isolated from whole *Arabidopsis* plants, cotyledons of young seedlings, aerial tissues (stems, cauline leaves, meristems, flowers), leaves, whole flowers, siliques and root tips. A transcript of ≈1.5 kb was present in each tissue type (Figure 2a) except root tips, consistent with a predicted role in photorespiration. The length of the observed *AGT1* transcript is approximately equivalent to the length of the *AGT1* cDNA. To control for potential loading differences, filters were also probed with the 18S rDNA fragment, and approximately equal amounts of RNA were present in each lane (Figure 2a). Quantification of the *AGT1* mRNA level normalized to 18S rRNA expression in above-ground tissues of *Arabidopsis* is shown in Figure 2(b). *AGT1* expression was highest in green leafy tissue where the transcript was about twice as abundant as it was in flowers and siliques; expression in flowers and siliques may be limited to their green portions.

Purification and biochemical characterization of recombinant *Arabidopsis* AGT1

To study the activity of the enzyme encoded by *AGT1*, the protein-coding region of the cDNA was cloned into an *E. coli* expression vector. When protein extracts from bacterial cultures were separated using SDS–PAGE, a prominent band with the expected molecular weight of about 45 kDa was detected only in lysates of induced cells expressing recombinant *Arabidopsis* AGT1 (Figure 3a, lanes 2 vs. 4). Spectrophotometric assays were performed to determine whether recombinant AGT1 retained catalytic activity. The recombinant enzyme was active, and AGT activity (Figure 3b) was clearly elevated in bacterial lysates expressing the recombinant protein compared to bacteria transformed with the empty expression vector (Figure 3c).

Previously isolated plant glyoxylate aminotransferases have exhibited activity with multiple donor : acceptor combinations (Hondred *et al.*, 1985; Noguchi and Hayashi, 1980; Rehfield and Tolbert, 1972). To determine whether the *AGT1* cDNA encoded an aminotransferase active with other amino donors and acceptors, recombinant AGT1 was subjected to additional aminotransferase assays. Bacterial cell lysates with AGT activity also catalyzed SGT and SPT reactions (Figure 3b,c). Recombinant *Arabidopsis* AGT1 was most active with serine as amino donor and glyoxylate as amino acceptor; the SGT reaction was catalyzed at a rate of 6.96 × 10^{-5} μmol min^{-1} mg^{-1} (total *E. coli* protein) by the recombinant enzyme. AGT and SPT reactions were catalyzed at ≈14%

and 17% the rate of the SGT reaction, respectively (Figure 3c). In-gel aspartate aminotransferase staining assays were also carried out to ensure that the *AGT1* cDNA did not encode a non-specific aminotransferase. Lysates from *E. coli* expressing recombinant AGT1 and empty-vector controls both stained positive for the *E. coli* isozyme of aspartate aminotransferase, but lacked stained bands corresponding to those observed in whole extracts of *Arabidopsis* plants (data not shown). This result demon-

strated that AGT1 was not catalyzing non-specific transaminations.

To test whether the multiple aminotransferase activities attributed to AGT1 were catalyzed by a single protein, AGT1 was chromatographically purified to apparent homogeneity (Figure 4). Purified AGT1 had the highest specific activity with the SGT reaction; it also catalyzed AGT and SPT reactions with much lower specific activity. This difference in reaction rates with various substrates can be at least partially explained by the widely variable apparent K_m values for alanine and serine (Table 1). The apparent K_m for alanine of AGT1 is well above predicted physiological levels, but the apparent K_m for serine is within measured physiological ranges (Riens *et al.*, 1991; Winter *et al.*, 1993). This suggests that AGT1 primarily catalyzes an SGT reaction *in vivo*.

Estimates of the molecular weight of putative plant AGTs range from 62 to 185 kDa (Hondred *et al.*, 1985; Ireland and Joy, 1983; Noguchi and Hayashi, 1980; Paszkowski and Niedzielska, 1990). The mass of recombinant *Arabidopsis* AGT1 was determined by gel filtration chromatography on a Superose-12 column. AGT1 eluted from the column between the β-amylase (200 kDa) and bovine serum albumin (66 kDa) standards (Supplementary material, Figure S-2). The molecular weight of AGT1 was calculated as 92 135 ± 1156 Da. This indicated that AGT1 is active as a homodimer, because the purified enzyme has an apparent subunit molecular weight of 45 kDa analyzed by standard denaturing gel electrophoresis.
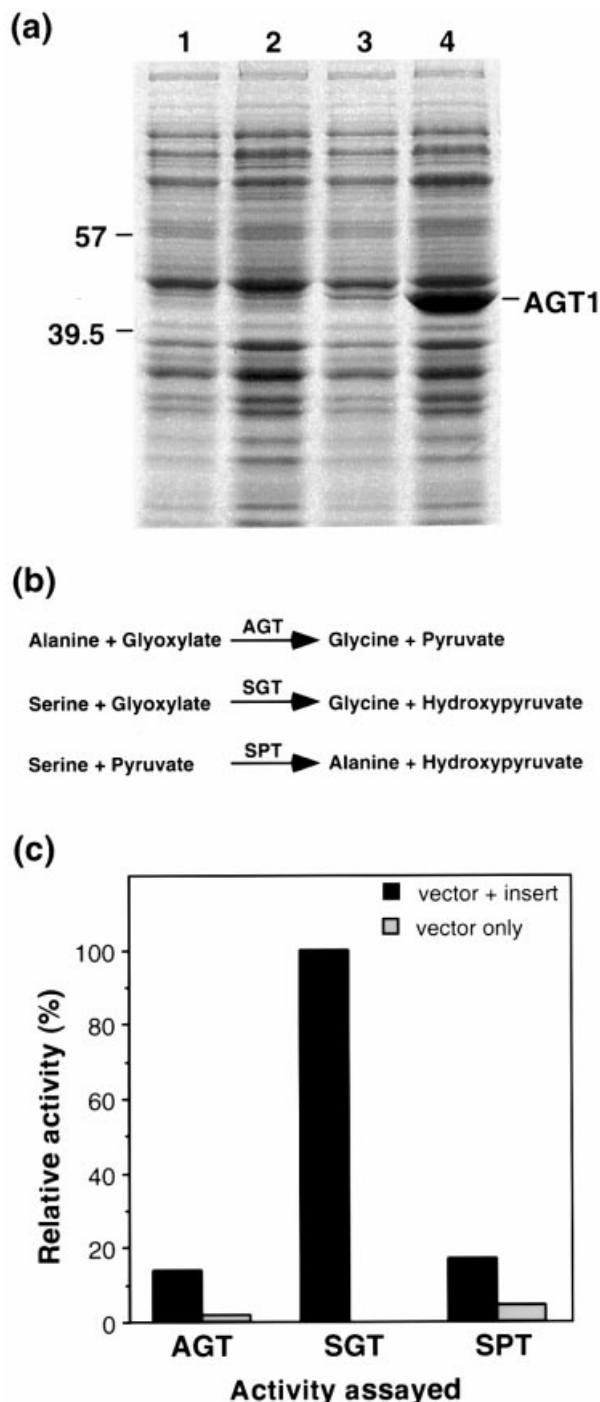
Subcellular localization of AGT1

The localization of mammalian AGT1 varies under the putative influence of dietary habit. In herbivores, AGT1 is localized to peroxisomes; in carnivores it is targeted to mitochondria; and in omnivores it is found in both organelles (Danpure, 1996). We hypothesized that AGT1 was localized to plant peroxisomes because it contains a

Figure 3. Expression and enzymatic characterization of recombinant *Arabidopsis* AGT1. The entire coding sequence of *AGT1* was subcloned into an *E. coli* expression vector (pET-28a).

(a) Lysates of bacteria expressing pET-AGT1 (lanes 3,4) or the empty pET-28 vector (lanes 1,2) were separated by SDS–PAGE and stained with Coomassie brilliant blue. Protein was collected prior to induction with IPTG (lanes 1,3), or 3 h after induction with IPTG (lanes 2,4). The size of known molecular weight markers (in kDa) is indicated.

(b) Three aminotransferase reactions catalyzed by AGT1: AGT, SGT and SPT. Only the forward reactions are indicated; the *in vivo* effects of the reverse reactions are probably minimal (see Discussion).

(c) Crude lysates of bacteria expressing AGT1 or the empty pET-28 vector shown in (a) were subjected to spectrophotometric enzyme assays to measure the three transaminations shown in (b). The catalytic activity of recombinant AGT1 for the SGT reaction (6.96×10^{-5} μmol min^{-1} mg^{-1}) was set as 100% relative activity.

peroxisomal targeting signal, and because its substrate glyoxylate is produced in peroxisomes.

An *in vitro* peroxisomal protein import assay (Brickner *et al.*, 1997) was used to determine whether AGT1 could be transported into isolated peroxisomes. *Arabidopsis* AGT1 was labeled with ^{35}S-met and incubated with isolated pumpkin glyoxysomes. Samples were next treated with proteinase K to digest those proteins free in solution or merely associated with the peroxisomal membrane;
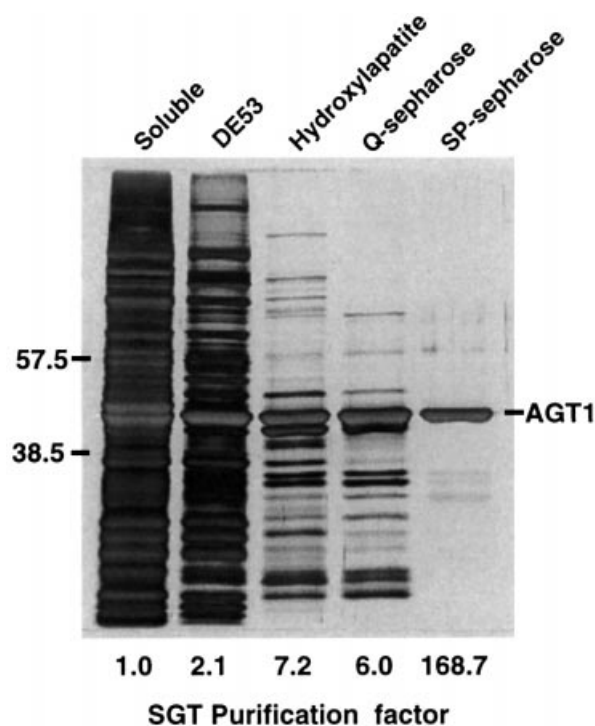
Figure 4. Chromatographic purification of recombinant *Arabidopsis* AGT1.

Recombinant *Arabidopsis* AGT1 was chromatographically purified from the soluble fraction of *E. coli* lysates as described under Experimental procedures. Samples containing approximately equal levels of SGT activity were separated by SDS–PAGE and silver-stained. The sizes of known molecular weight markers (in kDa) are indicated. Each fraction was assayed for SGT, AGT and SPT activities. The purification factor after each purification step is indicated below the corresponding lane of the gel. The initial soluble fraction (specific activity 7.58×10^{-7} µmol min^{-1} mg^{-1} total *E. coli* protein) was given a relative purification factor of 1. The purification factor indicated after each additional step was calculated relative to this sample.

proteins shielded from protease by the peroxisomal membrane were not digested (Figure 5). As expected, AGT1 was not imported into peroxisomes at 4°C. When the peroxisomal membranes were lysed with the detergent Triton X-100 and sonication prior to protease treatment, nearly all of the radiolabeled protein was digested. The pattern of AGT1 import into peroxisomes was similar to that of the control peroxisomal protein glycolate oxidase.

Import experiments can determine if a protein is transported into peroxisomes, but they cannot rule out *in vivo* localization to additional subcellular compartments. Because AGT1 is found in both mitochondria and peroxisomes in some organisms (Danpure, 1996), subcellular fractionation experiments were used to further assess the localization of AGT1 *in vivo*. Previous attempts to localize plant glyoxylate aminotransferases indicated that there were peroxisomal isozymes of these proteins; however, because low levels of activity co-fractionated with other organellar peaks, it was not possible to exclude the possibility that AGTs reside in multiple subcellular compartments (Noguchi and Hayashi, 1980; Noguchi and Hayashi, 1981; Rehfield and Tolbert, 1972). Organelles from *Arabidopsis* seedling extracts were separated by sucrose density centrifugation. Gradient fractions were then assayed for the presence of marker enzymes for peroxisomes (catalase) and mitochondria (fumarase), as well as for chlorophyll (chloroplast marker) and sucrose concentration. SGT activity, the most easily assayed activity of AGT1, was also measured in each of the gradient fractions. Figure 6 shows the results from a representative subcellular fractionation experiment: the SGT activity peak co-localizes with the catalase peak at ≈53% sucrose, confirming the peroxisomal localization of AGT1. Very little SGT activity was detected in peak mitochondrial or chloroplast fractions. SGT activity was similarly localized to peroxisomes in pumpkin cotyledons (data not shown).

Molecular characterization of the sat mutation

About 20 years ago, Somerville and Ogren devised a genetic screen to isolate *Arabidopsis* plants with nuclear mutations in genes encoding photorespiratory enzymes (Somerville and Ogren, 1979; Somerville and Ogren, 1980; Somerville and Ogren, 1981). One mutant, called *sat*, is

Table 1. Kinetic measurements of AGT1 activity with various amino acid donors and 2-oxoacid acceptors

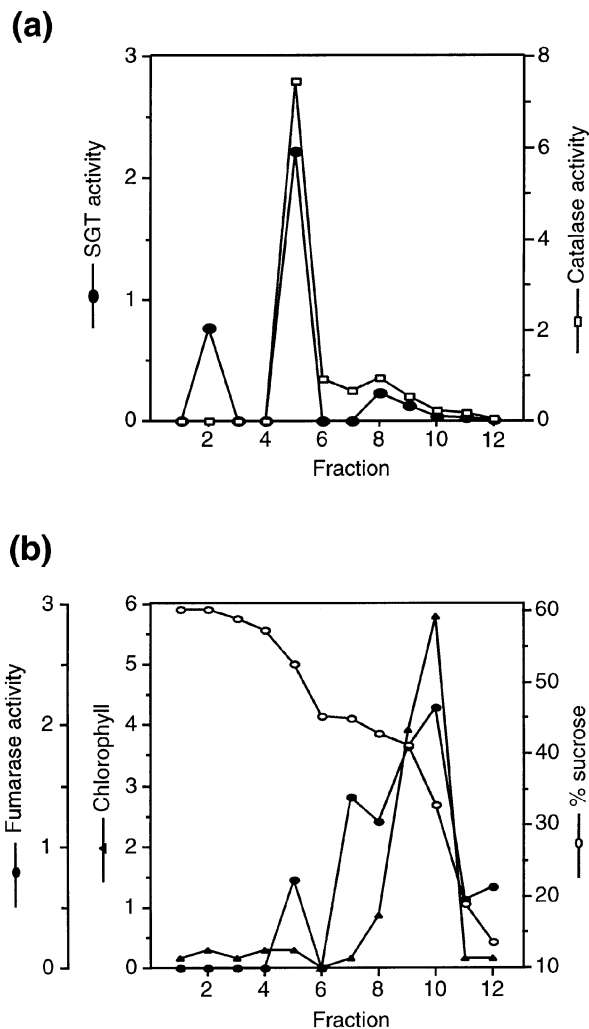| Reaction	Amino acid K_m	2-oxoacid concentration	2-oxoacid K_m	Amino acid concentration
SGT	1.52 mM serine	1 mM glyoxylate	0.11 mM glyoxylate	20 mM serine
AGT	101.2 mM alanine	1 mM glyoxylate	0.07 mM glyoxylate	200 mM alanine
SPT	0.39 mM serine	5 mM pyruvate	1.60 mM pyruvate	20 mM serine
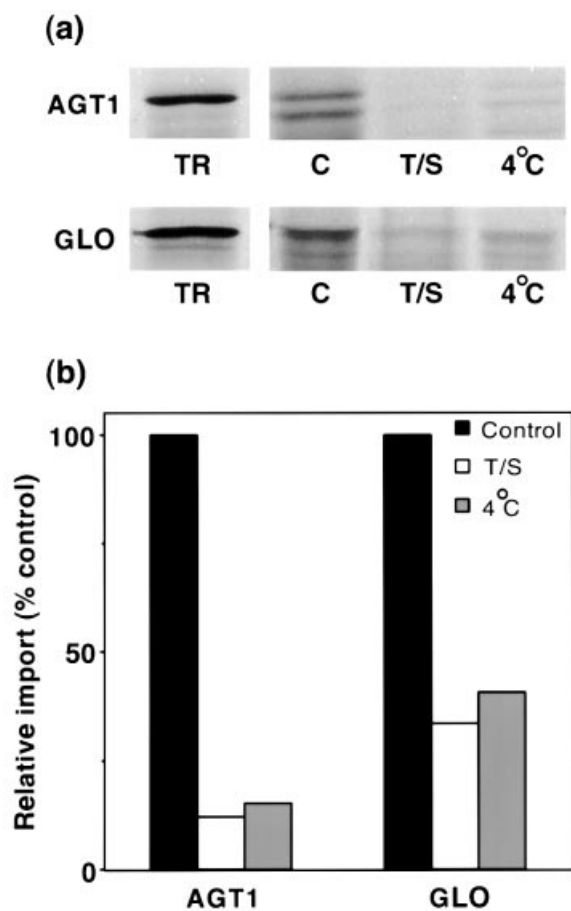
Figure 5. *In vitro* import of *Arabidopsis* AGT1 into isolated peroxisomes.

Standard *in vitro* peroxisomal protein import assays were performed as described under Experimental procedures. Radiolabeled translation products, either AGT1 or the peroxisomal protein glycolate oxidase (GLO), were incubated with isolated pumpkin glyoxysomes at 25°C. Following incubation, samples were treated with proteinase K to digest proteins not protected by the glyoxysomal membrane. For negative controls, import reactions were carried out at 4°C, or Triton X-100 and sonication treatments were administered prior to the addition of protease (T/S).

(a) Import samples were resolved by SDS–PAGE followed by fluorography and autoradiography.

(b) Quantification of the import samples shown in (a), using phosphorimaging analysis. The control for each protein was set as 100% relative import; this corresponds to 8% of the radiolabeled glycolate oxidase and 8.5% of radiolabeled AGT1 originally presented to the glyoxysomes. TR, translation products; C, protease-protected protein import controls. Results shown are from a representative experiment repeated four times.

Figure 6. Localization of AGT1 to peroxisomes by subcellular fractionation.

Extracts of 2-week-old *Arabidopsis* seedlings were separated by sucrose density centrifugation, and each fraction assayed for SGT activity as described under Experimental procedures. Gradient fractions were also assayed for the organellar markers catalase, fumarase and chlorophyll, as well as sucrose content. The specific activity (in μmol min^{-1} mg^{-1} total protein) of each enzyme is shown (catalase $\times$ 10^4; fumarase $\times$ 10^8; SGT $\times$ 10^7); chlorophyll is expressed as mg chl mg^{-1} protein. Fraction 1 is from the bottom of the gradient; fraction 12 is the top.

characterized by a conditional lethal phenotype when grown under normal atmospheric conditions. The *sat* mutants lack SGT activity, but maintain nearly wild-type levels of AGT and GGT activities. The molecular basis of the *sat* mutation was unknown prior to this study of AGT1.

Because *sat* mutants lack SGT activity, we hypothesized that they contain a defective *AGT1* gene. To test this hypothesis we sequenced *AGT1* from the mutant plants.

When grown under normal atmospheric conditions, the mutant seedlings were clearly stunted at the two-cotyledon stage and appeared chlorotic compared to wild-type seedlings (Supplementary material, Figure S-3). Sequence analysis of *AGT1* from the *sat* mutant line revealed the presence of a single mutation that is predicted to result in a proline-to-leucine substitution at amino acid position 251 of AGT1. Analysis of the predicted secondary structure of AGT1 using computer programs [PROF v. 1.0; PSIPRED (Jones, 1999); PREDATOR (Frishman and Argos, 1996; Frishman and Argos, 1997)] indicated that proline 251 is situated adjacent to an α-helix.

It is possible that the proline-to-leucine substitution could alter the conformation of AGT1 near this α-helix, resulting in a grossly misfolded, or otherwise inactive protein. To determine whether a proline-to-leucine substitution at position 251 would inactivate AGT1, site-directed mutagenesis was used to introduce the *sat* mutation into the *AGT1* cDNA. The mutated recombinant protein, when expressed in *E. coli*, was the same size on SDS–PAGE as wild-type AGT1. Although the recombinant AGT1 mutant protein remained soluble, it lacked any detectable SGT, AGT or SPT activity (data not shown).

Discussion

In plants, two isozymes of AGT are thought to reside within peroxisomes (Nakamura and Tolbert, 1983; Rehfield and Tolbert, 1972), but until recently (Liepman and Olsen, 1998) genes encoding these enzymes had not been identified. These aminotransferases utilize a broad and overlapping range of amino donor : acceptor combinations and may act within various metabolic pathways, including the photorespiratory glycolate cycle and general amino acid metabolism (Husic *et al.*, 1987; Rehfield and Tolbert, 1972; Somerville and Ogren, 1982). There has been some confusion regarding the identity, substrate specificity and subcellular localization of these enzymes in plants – probably due to heterogeneity within the protein preparations and the variety of plants used for source material by previous researchers (Hondred *et al.*, 1985; Ireland and Joy, 1983; Nakamura and Tolbert, 1983; Noguchi and Fujiwara, 1982; Noguchi and Hayashi, 1980; Noguchi and Hayashi, 1981; Rehfield and Tolbert, 1972; Smith, 1973; Yu *et al.*, 1984).

We have isolated *AGT1* from *Arabidopsis*, and Southern analysis confirms that there is a single *AGT1* gene in *Arabidopsis* (Supplementary material, Figure S-1). *Arabidopsis* AGT1 encodes a 45 kDa class IV aminotransferase, consistent with the subunit size reported for the corresponding enzyme from spinach, cucumber and pea (Hondred *et al.*, 1985; Ireland and Joy, 1983; Noguchi and Hayashi, 1980). Using computer searches, *AGT1* homologs in many other plants including tomato, rice, soybean, the common ice plant, the lily *F. agrestis*, and the moss *P. patens* have been found, suggesting that *AGT1* is conserved among all plants.

Because AGT1 is thought to play an important photorespiratory role, high levels of gene expression were expected in green tissues. *AGT1* mRNA was found in all tissues, transcript accumulation being highest in green, above-ground tissues of *Arabidopsis* (Figure 2). A greatly reduced level of *AGT1* transcripts was observed in root tips, indicating that a low level of AGT1 may be sufficient in subterranean tissues.

Purified *Arabidopsis* AGT1 catalyzed at least three transamination reactions: AGT, SGT and SPT (Figures 3 and 4). AGT1 catalyzed the SGT reaction with the highest rate; AGT and SPT reactions were catalyzed at a much lower rate. Although most aminotransferases catalyze readily reversible reactions, proposed metabolic channeling in peroxisomes (Heupel and Heldt, 1994; Heupel *et al.*, 1991; Reumann, 2000), and the low reverse activities reported in the literature (Ireland and Joy, 1983; Nakamura and Tolbert, 1983), suggest that these AGTs function primarily in the forward direction. The observations that *sat* mutant plants maintain wild-type levels of AGT and GGT activity, and that purified recombinant AGT1 has highest specific activity with the SGT reaction, suggest that *in vivo* AGT1 functions mainly as an SGT. The apparent K_m measurements of AGT1 for serine versus alanine also support a primary role for AGT1 catalysis of an SGT reaction (Table 1). Thus the AGT and GGT activities observed in *sat* mutants are probably attributable to one or more additional aminotransferases.

Animals have two structurally distinct enzymes with AGT activity: AGT1 and AGT2. In vertebrates, AGT2 is consistently localized within mitochondria, while the subcellular localization of AGT1 varies according to the metabolic pathway in which it is involved (Danpure, 1996; Watts, 1992). In carnivores such as dogs and cats, AGT1 is involved primarily in gluconeogenesis and is localized to mitochondria. Herbivores such as rabbits and gorillas, and descendants of herbivores including humans, harbor AGT1 within peroxisomes where it detoxifies glyoxylate, a toxic plant metabolite. In most omnivores, such as rats, AGT1 is found in both mitochondria and peroxisomes where it participates in both gluconeogenesis and glyoxylate detoxification. In humans, the metabolic disorder primary hyperoxaluria type I, marked by build-up and excessive excretion of oxalate, is caused by mislocalization of AGT1 to mitochondria rather than to peroxisomes (Danpure, 1996). We hypothesized that AGT1 is a peroxisomal protein in plants because it contains a type I peroxisomal targeting signal and its substrate glyoxylate is abundant within peroxisomes. *In vitro* protein import studies demonstrated that AGT1 was imported into isolated peroxisomes (Figure 5); subcellular fractionation studies confirmed a strictly peroxisomal localization of *Arabidopsis* AGT1 *in vivo* (Figure 6).

The key role of AGT1 in the photorespiratory pathway is underscored by the air-sensitive phenotype of *sat* mutants (Somerville and Ogren, 1979; Somerville and Ogren, 1980; Somerville and Ogren, 1981), which contain a single amino acid substitution in AGT1. These mutants photosynthesize at greatly reduced rates and accumulate serine and glycine when grown under conditions promoting photorespiration. The *sat* mutants can be rescued and appear to photosynthesize normally when grown in environments

containing elevated CO_2, where photorespiration is reduced. This suggests that AGT1 is dispensable outside the photorespiratory pathway. The mechanism by which aminotransferase activity is abolished in the *sat* mutant is not clear. Since proline residues often introduce kinks into polypeptides, it may be that the proline-to-leucine substitution at residue 251 could result in an unstable or aggregated protein. Although recombinant mutant AGT1 was not detectably active when expressed in *E. coli*, the mutant protein remained soluble (data not shown), suggesting that the conformational defect is subtle. Studies designed to understand the molecular consequences of this amino acid substitution and to further define the substrate range of AGT1 are under way. In addition, it will be interesting to determine the function and identity of other plant peroxisomal glyoxylate aminotransferases. These aminotransferases would be expected to account for the AGT and GGT activities present in *sat* mutant plants.

Experimental procedures

Primer sequences

AGT1*Nco*I: 5′-GGAAACCATGGACTATATGTATG-3′
AGT1*Sal*I: 5′-GAAGAGCTCGAAAGAGAAGGAC-3′
AGT1*Gen*1: 5′-CGAAGGAGTCCAAAACCTCCCTC-3′
AGT1*Gen*2: 5′-CCCTCATACCCATATAAGTTGAAG-3′
AGT1P3: 5′-GCTTGCTCTGGATTGACCAGCAG-3′
AGT1P4: 5′-CCAGCCACTTTGTTGAGACCAAG-3′
AGT1P6: 5′-GTCCACTCCCCACTCATCCATTC-3′
AGT1P9: 5′-CAATGACATCTCTGCTGTCCGCAC-3′
AGT1Mut1: 5′-CCATACACACTTTCCATTCAACTTC-3′
AGT1Mut2: 5′-GAATGGAAAGTGTGTATGGCCAATAGG-3′

Constructs

pZL-AGT1 contains a full-length *Arabidopsis AGT1* cDNA in the pZL1 vector (BRL); it was obtained from the *Arabidopsis* Biological Resource Center (EST 35A11T7; Newman *et al.*, 1994). PCR with Taq polymerase (Perkin Elmer, Boston, MA, USA) was used to amplify the coding region of pZL-AGT1. The mutagenic primers AGT1NcoI and AGT1SacI were used to introduce an *Nco*I site at the 5′ end of the ORF and a *Sac*I site within the 3′ UTR to facilitate further manipulations. The PCR product was ligated into pCRII vector (Invitrogen, Carlsbad, CA, USA), producing pCR-AGT1. For expression in *E. coli*, the 1242 bp *Nco*I/*Sac*I fragment from pCR-AGT1 was subcloned into the corresponding sites of the expression vector pET-28a (Novagen, Madison, WI, USA), resulting in pET-AGT1. Site-directed mutagenesis was performed on pZL-AGT1 to introduce a proline-to-leucine substitution at residue 251 of the predicted polypeptide. First, two PCR products were generated using the AGT1NcoI/AGT1Mut2 and AGT1SacI/AGT1Mut1 primer combinations and Vent DNA polymerase (NEB, Beverly, MA, USA). Next, aliquots from these reactions were used as template for a second PCR reaction with Vent polymerase and the AGT1NcoI/AGT1SacI primer pair. This PCR product was gel purified, ligated into the PCRII vector (yielding pCR-AGT1P251L)

and subcloned into pET-28a in the same manner as the wild-type construct. Sequence analysis of the resulting construct (pET-AGT1P251L) indicated that only the intended mutation was introduced by these procedures. pP055 contains a fragment of the 18S rDNA from pea; it was generated in the laboratory of Dr Xing-Wang Deng at Yale University (McNellis *et al.*, 1996) and donated by Dr Keiko Torii (University of Washington).

Nucleic acid hybridization

DNA fragments to be used as probes were labeled with ^{32}P-dCTP (3000 Ci mmol^{-1}, Amersham, Piscataway, NJ, USA) using a random-hexamer labeling kit (Promega, Madison, WI, USA). Unincorporated nucleotides were removed by size-exclusion chromatography through a column packed with fine Sephadex G-50, equilibrated with 10 mM Tris pH 8, 50 mM EDTA. The *AGT1* probe corresponds to the 1242 bp *Nco*I/*Sac*I fragment of pCR-AGT1 containing the full coding sequence of *AGT1*. A 1 kb *Eco*RI/*Bam*HI fragment of pP055 was labeled to produce the 18S rDNA probe.

Genomic DNA was extracted from 1 g *Arabidopsis* (ecotype C24) leaf tissue (Dellaporta, 1994), and 10 μg genomic DNA subjected to standard Southern blot protocols (Sambrook *et al.*, 1989). Blots were exposed to film for 10 days at −70°C.

RNA was extracted from 150 mg of various *Arabidopsis* tissues using a plant RNA collection system (Qiagen, Valencia, CA, USA). Root-tip RNA was generously provided by Dr Myeong-Min Lee (University of Michigan). Approximately 5 μg total RNA from each sample was subjected to standard Northern blot techniques (Sambrook *et al.*, 1989). Blots probed with *AGT1* were exposed to film for 2 days. Blots probed with 18S rDNA were exposed to film for 25 min. Band intensities were quantified by phosphorimaging analysis using a GS-363 Molecular Imager (Bio-Rad, Hercules, CA, USA).

Cloning and sequencing of AGT1 *genomic fragments from wild-type and* sat *mutant* Arabidopsis

DNA was collected from the cotyledons of *sat* mutant seedlings using a DNA micro-extraction technique (Li and Chory, 1998). Genomic fragments of the *AGT1* gene from *sat* mutant seedlings and wild-type plants (ecotype Col-0) were amplified by PCR with Taq polymerase and various primer combinations (AGT1Gen1/AGT1P4, AGT1Gen1/AGT1P6, AGT1Gen2/AGT1P3, AGT1Gen2/AGT1P9). After recovering PCR products from low-melt agarose gels, fragments were cloned into the pCR-II vector (pCR-II TOPO, Invitrogen). Plasmids from clones carrying genomic fragments were isolated using standard methods (Sambrook *et al.*, 1989), and the inserts were fully sequenced with an automated sequencer (ABI Prism 310, Perkin Elmer). To account for potential mutations introduced during amplification and cloning of genomic fragments, each region was amplified, cloned and sequenced at least twice. A single nucleotide mutation at position 751 (relative to the translation initiation site) was observed in multiple clones.

AGT1 expression in E. coli

For aminotransferase assays of bacterial lysates, *E. coli* BL-21 cells were transformed with pET AGT1 and grown at 37°C in 50 ml Luria Bertani (LB) super broth (32 g tryptone, 20 g yeast extract, 5 g NaCl l^{-1}) with kanamycin selection to an OD_{600} ≈0.5 before

induction with 450 μM isopropyl-beta-D-thiogalactopyranoside (IPTG). Following the addition of IPTG, cultures were shaken at 25°C. Three hours after induction, 2.5 ml aliquots were removed from each of the 50 ml cultures and the cells in these aliquots were pelleted by centrifugation at 4°C for 10 min at 10 000 *g* in a microcentrifuge. Bacterial cell pellets were resuspended in 200 μl lysis buffer consisting of 50 mM Tris pH 8, 10 mM EDTA, 10 mM NaCl, 10% glycerol, 0.1% β-mercaptoethanol, 1 mM phenyl methyl sulfonyl fluoride (PMSF). Cells were then mechanically lysed by repeated freeze–thaw cycles using a dry ice/ethanol bath. Bacterial lysates were quick frozen in dry ice/ethanol and stored at –70°C. Inductions of *E. coli* BL-21 cells expressing pET-AGT1P251L were carried out the same way, except that cells were resuspended at 10 OD_{600} per ml lysis buffer prior to freeze–thaw lysis.

Enzyme assays

AGT, SGT and SPT activities were all measured spectrophotometrically with a Shimadzu UV160U spectrophotometer, as described (Rehfield and Tolbert, 1972). AGT activity was measured at 340 nm by coupling the reduction of pyruvate produced by AGT1 to the oxidation of NADH, catalyzed by excess lactate dehydrogenase. To assay for AGT activity, 10 μl sample was added to a reaction mixture containing 70 mM Hepes pH 7, 0.17 mM NADH, 0.1 mM pyridoxal 5-phosphate, 1 mM glyoxylate, and 0.03 units lactate dehydrogenase in a final volume of 1 ml. SGT activity was measured at 340 nm by linking the reduction of hydroxypyruvate produced by AGT1 to the oxidation of NADH, catalyzed by excess hydroxypyruvate reductase. Reaction conditions were identical to the AGT assay except that 20 mM serine was substituted for alanine, and 0.05 units of hydroxypyruvate reductase was substituted for lactate dehydrogenase. SPT activity was measured at 340 nm by linking the reduction of hydroxypyruvate produced by AGT1 to the oxidation of NADH, catalyzed by excess hydroxypyruvate reductase. Reaction conditions were identical to the SGT assay, except that 3 mM pyruvate was substituted for glyoxylate, and 0.05 units of hydroxypyruvate reductase was substituted for lactate dehydrogenase. For each assay, the reaction was started by adding amino acid to a final concentration of 20 mM; water was added instead of amino acid to the reference cuvette. For kinetics measurements, substrate concentrations were varied over a range of 0.1–200 mM for amino acids and 0.025–10 mM for 2-oxoacids. Calculations of K_m values were performed using non-linear regression software (PRISM program from GraphPad).

Protein concentration was measured using the BCA protein assay system (Pierce, Rockford, IL, USA). Specific activity of AGT1 for each aminotransferase reaction is expressed in μmol $min^{-1} mg^{-1}$ and was calculated using the extinction coefficient for NADH of 6200 $cm^{-1} M^{-1}$.

Aspartate aminotransferase activity was assayed using an in-gel staining technique (Gebhardt *et al.*, 1998). Samples were resolved on native polyacrylamide gels that were stained by incubation in staining solution [0.1 M sodium phosphate pH 7.3, 13 mM aspartic acid, 13 mM α-ketoglutaric acid, 5 mM fast blue BB salt (catalog no. F-0250, Sigma-Aldrich Co., St. Louis, MO, USA), and 40 mM pyridoxal 5-phosphate] at 37°C for 15 min in the dark, then rinsed with water and dried.

Chromatographic purification of AGT1

Escherichia coli BL-21 cells containing the pET-AGT1 plasmid were grown at 37°C in 1.5 l LB super broth as described above.

Following cell harvest, bacterial pellets were combined and resuspended in 25 ml lysis buffer, then lysed by passing them twice through a French pressure cell. Cell lysates were centrifuged at 4°C twice for 15 min at ≈50 000 *g* (in a Sorvall F28/50 rotor) to remove insoluble debris. Aliquots of the resulting supernatant, representing the crude soluble fraction (specific activity 7.58 × 10^{-7} μmol $min^{-1} mg^{-1}$ total *E. coli* protein) were frozen in liquid nitrogen and stored at –70°C.

The crude soluble lysate (4 ml) was applied to a drip column packed with 10 ml DEAE cellulose resin (DE53, Whatman, Clifton, NJ, USA). AGT1 eluted with buffer A (50 mM Tris pH 8, 10% glycerol). Fractions containing AGT1 were pooled and applied to a hydroxylapatite (Bio-Rad) column connected to a fast-performance liquid chromatography (FPLC) system (Pharmacia, Piscataway, NJ, USA). AGT1 was eluted from the hydroxylapatite column by running a linear gradient of buffer A to buffer B (50 mM Tris pH 8, 400 mM sodium phosphate pH 8, 10% glycerol). Fractions containing AGT1 were again pooled and dialyzed against buffer A overnight at 4°C. AGT1 was next applied to a Q-sepharose Fast-flow (FFQ, Pharmacia) FPLC column; it was eluted from the FFQ column by running a linear gradient of buffer A to buffer C (50 mM Tris pH 8, 1 M KCl, 10% glycerol). Pooled fractions containing AGT1 were dialyzed against buffer D (50 mM MES pH 6, 10% glycerol) overnight at 4°C. The dialyzed preparation was applied to an SP-sepharose FF (Pharmacia) column, and AGT1 was eluted from this column by running a linear gradient of buffer D to buffer E (50 mM MES pH 6, 1 M NaCl, 10% glycerol). Following each chromatographic step, fractions were analyzed by SDS–PAGE and/or enzyme activity assays.

Gel filtration of AGT1

To determine the molecular weight of AGT1, ≈200 μg AGT1 (>90% pure, approximated by protein staining) in buffer A was mixed with 250 μg each of the following molecular weight standards: β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa). This mixture was applied to an FPLC column containing Superose 12 (Pharmacia) resin. Proteins were eluted with buffer A. To locate protein peaks, 15 μl aliquots from each fraction were resolved by SDS–PAGE, and gels were silver-stained. A standard curve was constructed by plotting the logMW of the molecular weight standards against the ratio of their elution volume : void volume. This line was used to estimate the molecular weight of AGT1.

In vitro *peroxisomal protein import assay*

pZL-AGT1 was linearized with *Not*I prior to *in vitro* transcription with T7 RNA polymerase (Promega). mRNA synthesized *in vitro* was added to a cell-free wheatgerm lysate translation system containing [^{35}S]methionine (43.5 Tbq $mmol^{-1}$; Amersham). To measure translation efficiency, translation products were acid-precipitated onto glass fiber filters, washed with ethanol, and quantified with a liquid scintillation counter (Model LS-6800, Beckman, Fullerton, CA, USA).

The import of *Arabidopsis* AGT1 and spinach glycolate oxidase was assayed as described previously (Brickner *et al.*, 1997) using 50 μg ml^{-1} proteinase K for AGT1 import reactions and 10 μg ml^{-1} proteinase K for glycolate oxidase import reactions. Approximately 8% of the radiolabeled glycolate oxidase and 8.5% of radiolabeled AGT1 was protected by the glyoxysomal membrane (imported) in the control import reactions.

Subcellular fractionation

Arabidopsis seedlings (ecotype C24) were grown on germination media in continuous light for 2 weeks (Olsen *et al.*, 1993). Green seedlings were harvested and ground in a cold Waring blender with 1.5 ml grinding buffer [150 mM Hepes–KOH pH 7.5; 10 mM KCl; 1 mM MgCl$_2$; 1 mM EDTA; 20% (w/v) sucrose; 1 mg ml^{-1} BSA; 5 mM DTT] g^{-1} FW. All subsequent steps were performed at 4°C. The resulting homogenate was centrifuged in a Sorvall HB6 (swinging bucket) rotor at 2600 *g* for 15 min. The supernatant was then layered onto a sucrose gradient containing 1 ml 30% sucrose, 2 ml 42% sucrose, 3 ml 45% sucrose, 2 ml 57% sucrose, and 3 ml 60% sucrose. Each sucrose solution (w/v) was prepared in 45 mM Hepes/KOH, 1 mM EDTA pH 7.5. The gradient was centrifuged in a Beckman SW40Ti rotor at 166 500 *g* for 4.5 h without braking. 1 ml fractions were collected from the bottom of the gradient and stored at –20°C.

Gradient fractions were analyzed for sucrose content using a Milton Roy refractometer, and for protein content using the BCA assay (Pierce). Catalase, fumarase and chlorophyll were each measured as described (Aebi, 1984; Arnon, 1949; Hatch, 1978). SGT was assayed as described above except that 0.1% Triton X-100 was included for maximal access to the enzyme (Reumann, 2000). Gradient samples were prepared with the assistance of Tanya Johnson (Olsen Laboratory, Department of Biology, University of Michigan).

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GenBank accession number AF063901.

Supplementary material

Three additional figures (Figures S-1, S-2 and S-3) can be found in the online version of this paper at http://www.blackwell-synergy.com/tpj