Theor Appl Genet
DOI 10.1007/s00122-009-0988-3

ORIGINAL PAPER

Over-expression of aspartate aminotransferase genes in rice resulted in altered nitrogen metabolism and increased amino acid content in seeds

Ying Zhou · Hongmei Cai · Jinghua Xiao · Xianghua Li · Qifa Zhang · Xingming Lian

Received: 1 August 2008 / Accepted: 4 February 2009

Abstract Aspartate aminotransferase (AAT) is a key enzyme in the synthesis of amino acids. It plays an important role in regulating carbon and nitrogen metabolism in almost all organisms. In this study, we over-expressed in rice separately all three *AAT* genes from rice (*OsAAT1*∼*3*) and one *AAT* gene from *Escherichia coli* (*EcAAT*). Over-expression was driven by the *CaMV* 35S promoter and constructs were introduced into rice by *Agrobacterium tumefaciens*-mediated transformation. Compared with control plants, the transformants showed significantly increased leaf AAT activity and greater seed amino acid and protein contents. No other phenotypic changes were observed. The total leaf AAT activities in plants over-expressing *OsAAT1*, *OsAAT2*, and *EcAAT* were 26.6, 23.6, and 19.6 A min^{-1} mg^{-1} FW (A: units of activity, defined as increase of absorbency per min per mg; FW: fresh weight), which were significantly higher than that in the wild-type control (17.7 A min^{-1} mg^{-1} FW). The amino acid content in seeds of transgenic plants over-expressing *OsAAT1*, *OsAAT2*, and *EcAAT* was 119.36, 115.36, and 113.72 mg g^{-1}, respectively, which were 16.1, 12.0, and 5.4% higher, respectively, than that in the control plants. The transgenic plants over-expressing *OsAAT1*, *OsAAT2*, and *EcAAT* had significantly higher protein contents (increased 22.2, 21.1, and 11.1%, respectively) than wild-type plants. No significant changes were found in leaf AAT activity, seed amino acid content or protein content in *OsAAT3* over-expressed plants. The expression patterns of the three *OsAAT* genes and their different functions are also discussed.

Communicated by T. Tai.

Y. Zhou · H. Cai · J. Xiao · X. Li · Q. Zhang · X. Lian (✉)
National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, 430070 Wuhan, China
e-mail: xmlian@mail.hzau.edu.cn

Introduction

Nitrogen is an expensive and growth-limiting nutritional element in plants. Aspartate aminotransferase (EC 2.6.1.1; AAT) is an important enzyme involved in carbon and nitrogen metabolism. AAT catalyzes the reversible transfer of the amino group from aspartate to α-ketoglutarate, yielding oxaloacetate and glutamate (Givan 1980). In plants, AATs have been reported to play an important role in a number of physiological processes, such as recycling of carbon skeletons during ammonia assimilation in roots (Ryan and Fottrell 1974), providing amide precursors for biosynthesis of major nitrogen transport molecules such as asparagine and ureides (Rawstone et al. 1980), recruiting asparagine nitrogen during grain filling (Gordon et al. 1978), participating in intercellular carbon shuttles in C4 plants (Hatch and Osmond 1973), providing precursors for the biosynthesis of the aspartate-derived amino acids (Bryan 1980), and participating in the malate/aspartate shuttle, which moves reducing equivalents across the organelle membrane (Heber and Heldt 1981).

Unlike bacteria, which have only one AAT isoenzyme (Gelfand and Rudo 1977; Rudman and Meister 1953), plants have multiple AAT isozymes, which are located in different subcellular compartments such as cytosol, mitochondria, plastids, and glyoxysomes. For example, at least five genes are known to encode AATs in *Arabidopsis thaliana* (Coruzzi and Last 2000; Coruzzi 2003; Wilkie et al. 1995). Three *OsAAT* genes were identified in rice (*Oryza sativa*): *OsAAT1*, *OsAAT2*, and *OsAAT3*; these genes

Published online: 04 March 2009

encode chloroplastic, cytoplasmic, and mitochondrial AAT isoenzymes, respectively (Song et al. 1996).

Amino acid content determines the nutritional quality of crop plants used in the diet of humans and animals. An increase in the content of amino acids in animal feed sources, especially in rice grains, would eliminate the need for supplementation of amino acids. Using genetic engineering to increase amino acid content in plants is an attractive and practical technology. Shaul and Galili (1992) reported using such an approach to accumulate threonine in tobacco. Falco et al. (1995) increased lysine content in transgenic canola and soybean seeds. On the basis of AAT functions, a straightforward strategy for engineering nitrogen assimilation has been proposed to improve the amino acid content in plants (Murooka et al. 2002).

Rice is one of the most widely grown crops and a main staple food for about half the world's population. It also provides an important protein source for humans. The nutritional quality of rice grain is important to all rice consumers, especially where it is the population's main staple (Wang et al. 2005). Similar to other cereals, rice seed proteins are deficient in some amino acids (Chavan and Duggal 1978). Thus, increasing the amino acid content of the rice grain to develop high nutritional varieties is an important objective in breeding for grain quality. In the present study, we over-expressed in rice all three *AAT* genes from rice (*OsAAT1*∼*3*) and one *AAT* gene from *Escherichia coli* (*EcAAT*). The transgenic plants showed a significant increase in leaf AAT activity and grain amino acid content compared with that in the control plants.

Materials and methods

Constructs and transformation

The coding sequence of the bacterial *AAT* gene was amplified using *E. coli* DNA as a template with primers EcAATsF/EcAATsR according to the sequence reported by Joachim et al. (1994) (GenBank accession number X03629). The PCR product was cleaved with *Kpn*I and *Xba*I, and ligated into the *pCAMBIA1301S* vector. The hptII selectable marker and *AAT* coding regions were cloned in opposite orientations, both driven by *CaMV* 35S promoter.

The coding sequences of *OsAAT1* and *OsAAT2* were amplified using rice genomic DNA as the template with the primers OsAAT1sF/OsAAT1sR and OsAAT2sF/OsAAT2sR (Table 1), respectively. The primers were designed according to the rice sequence, with *Sal*I/*Sal*I and *Sal*I/*Sac*II sites added to the primers for gene reconstruction. For *OsAAT*3, a full-length cDNA clone (EI$^{\#}$76-O14) was found in the Minghui 63 normalized cDNA library of our laboratory (http://www.redb.ncpgr.cn) (Chu et al. 2003). The clone was digested with *Bam*HI and *Kpn*I. The three rice *AAT* gene fragments (named *OsAAT1*, *OsAAT2*, and *OsAAT3*) were then ligated into the *pCAMBIA 1301S* vector. Standard molecular techniques (Sambrook and Russell 2001) were used for DNA manipulation. The constructs were transformed into the *japonica* rice cultivar Zhonghua 11 by an *Agrobacterium tumefaciens*-mediated transformation method (Hiei et al. 1994).

Plant growth and treatment

For hydroponic culture, both *AAT* transgenic plant seeds and wild-type Zhonghua 11 plant seeds were geminated and sown in sand. Young seedlings at the two-leaf stage were transferred to Yoshida culture solution (Yoshida et al. 1976), refreshed once in 7 days.

For field experiments in 2005 and 2006, germinated seeds of the wild-type Zhonghua 11 and T_1 and T_2 transgenic lines (containing positive and corresponding negative lines) were sown in a seedbed and transferred to the experimental field in Huazhong Agricultural University, Wuhan, China. Field management including irrigation, weeding and pesticide spraying followed the normal agricultural practices. At maturity, all the plants were harvested, dried, and stored in a refrigerator for further use.

For further analysis, three independent T_1 transgenic lines over-expressing *OsAAT1*, *OsAAT2*, *OsAAT3*, and *EcAAT* with a single copy transgene (named *OsAAT1*-OX, *OsAAT2*-OX, *OsAAT3*-OX, and *EcAAT*-OX, respectively) were planted in the field, following a randomized complete block design with three replications. Each block included five genotypes (four transgenic lines and one wild type) and was divided into five plots accordingly. Germinated seeds of wild-type Zhonghua 11 and the four transgenic lines were sown in a seedbed and transferred to the field, with 30 plants per genotype planted in a plot containing three rows of 10 plants each with a spacing of 25 cm between rows and 20 cm between plants within a row. At maturity, eight plants from the middle of each subplot were harvested (above-ground plant parts) and dried. Then amino acid content of the seeds was determined.

To confirm the measurements of amino acid contents of the T_1 families, T_2 lines were field-planted and harvested again in 2006 in Wuhan. For this experiment, three positive transgenic lines and three corresponding negative transgenic lines were planted for comparison.

Northern and Southern blot analysis

Total RNA was extracted from the leaves of 20-day-old seedlings using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Then 15 μg of total RNA was used for northern blot analysis.

Table 1 Primers used in PCR or qRT-PCR

Gene name	Primer pair	Primer sequences (5′–3′)	Product size (bp)	Function
OsAAT1	OsAAT1sF	ATTAAACCACGTGATGGGTGT	4,227	To obtain full-length genomic DNA of *OsAAT1*
	OsAAT1sR	GATTAACCACAATAGCAGTGACCT		
	OsAAT1tF	ACACCTGAAGTCGCAAAC	95	For qRT-PCR
	OsAAT1tR	GCAACTATCCTAGCACCAT		
OsAAT2	OsAAT2sF	GCGAGAAACAGCTATCCACGTCA	4,588	To obtain full-length genomic DNA of *OsAAT2*
	OsAAT2sR	AATCCTCAGAACACTGCTGCGGA		
	OsAAT2tF	AGGAGTTACCGCTACTATGA	167	For qRT-PCR
	OsAAT2tR	AACTGCCTGATCTGTTCC		
OsAAT3	OsAAT3tF	TGCGAACAGCACTCAAAG	208	For qRT-PCR
	OsAAT3tR	TTAGCCAGATAGGCAACAT		
EcAAT	EcAATsF	CTTCCAGAGCAATCTCACGT	1,285	To obtain full-length genomic DNA of *EcAAT*
	EcAATsR	GGAGCCATGTTATCTGGTGT		
	EcAATtF	TATCGACCCTACGCTGGAAC	503	For qRT-PCR
	EcAATtR	TCAGGCCACTGAAGGAGAAC		
GUS	GUSF	TGATTGATGAAACTGCTGCTG	555	Positive or negative check
	GUSR	ACATATCCAGCCATGCACACT		
Actin	ACTINF	TGGCATCTCTCAGCACATTCC	55	For qRT-PCR
	ACTINR	TGCACAATGGATGGGTCAGA		

After extraction from T_0 transgenic plants (Murray and Thompson 1980), genomic DNA (4 µg) was digested with *Hin*dIII and transferred to a Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK) for Southern blot analysis. Hybridizations were performed using ^{32}P-labeled partial cDNA fragments of each *AAT* gene and the *β*-glucuronidase (*GUS*) gene as probes and standard procedures (Sambrook and Russell 2001).

qRT-PCR analysis

The expression pattern of the *AAT* gene family was analyzed by qRT-PCR. Twelve tissues were harvested from wild-type (WT) Zhonghua 11 plants (Fig. 2). Leaves from transgenic plants at the three-leaf stage were also harvested for further analysis. RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD, USA). The RNA samples were treated with RNase-free DNase I (Invitrogen) to remove any residual genomic DNA in the preparation. Total RNA (3 µg) was used for first-strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) and oligo dT (Promega, Madison, WI, USA), and the product was diluted to a final volume of 80 µl. Primers for qRT-PCR were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) as shown in Table 1. A total volume of 25 µl solution containing 1 µl of the synthesized cDNA, 1 µl of 10 µM of each primer, and 12.5 µl of the SYBR Green PCR master mix (Applied Biosystems) was used for each real-time PCR reaction. For HotStarTaq DNA polymerase activation, the Applied Biosystems 7500 Real-Time PCR System was programmed as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 30 s; 60°C for 30 s; and 72°C for 1 min. The data collection was carried out during the extension step (72°C for 1 min). As an internal control for calibration of relative gene expression level, expression of the rice gene *Actin1* (accession number X16280) was monitored using the primers ACTINF and ACTINR (Table 1). The relative quantities of the transcripts were calculated using the standard curve method.

Metabolite analysis

For soluble protein analysis, flag leaves (5 g) were homogenized by grinding fresh leaves on ice with 5 ml extraction buffer [5 mM sodium glutamate, 10 mM Trizma (pH 7.5), 10 mM $MgSO_4$, 1 mM dithiothreitol, 10% (v/v) glycerol, and 0.05% (v/v) Triton X-100] followed by centrifugation at 12,000*g* for 20 min at 4°C (Melo et al. 2003). The soluble protein concentration of the 100-fold diluted supernatant was measured by the Bradford (1976) protein assay with the use of Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as the protein standard.

For assessment of total AAT enzyme activities, a colorimetric assay was employed based on the ability of AAT to convert D,L-aspartic acid to α-ketoglutaric acid (Wu et al.

1998). Fresh leaves (200 mg FW ml^{-1} buffer; FW: fresh weight) were ground with an extraction buffer consisting of 20 mM K^+-phosphate buffer (pH 7.4), 5 mM DTT, 10 mM antipain, and 1 mM PMSF. All extraction and purification steps were performed at 4°C. The homogenate was centrifuged at 12,000*g* for 20 min at 4°C and 20 µl of the supernatant was removed and mixed with 100 µl of GOT solution (Wu et al. 1998). The samples were incubated at 37°C for 1 h with occasional mixing and 100 µl of 2,4-dinitrophenylhydrazine solution (0.2 mg l^{-1} with HCl added to help dissolve the 2,4-dinitrophenylhydrazine) and 20 µl of aniline–citric acid solution [aniline (500 ml l^{-1}) and citric acid (500 g l^{-1})] were added to the samples which placed at 37°C for another 20 min. Finally, 1 ml of 0.4 M NaOH was added to adjust the pH (>7.0) and absorbance was measured at 520 nm using a spectrophotometer.

For free NO_3^- and NH_4^+ analysis, flag leaves were ground with cold extraction buffer [10 mM imidazole, 50 mM Tris–HCl (pH 7.0) and 0.5% (w/v) β-mercaptoethanol] and the homogenates were then centrifuged at 12,000*g* for 20 min at 4°C (Oliveira et al. 2002). Free NO_3^- in the supernatant was determined by the Griess method (Walther et al. 1999); the absorbance at 540 nm was determined and NO_3^- contents were calculated from a standard curve of KNO_3. Free NH_4^+ in the supernatant was determined by the Berthelot color reaction method (Gordon et al. 1978); the absorbance at 480 nm was determined using a spectrophotometer and NH_4^+ contents were calculated from a standard curve of NH_4NO_3.

For analysis of total amino acids, about 100 mg samples of ground seed powder were dissolved in 10 ml 4 M HCl at 115°C for 12 h resolution. The samples were then filtered to remove insoluble material and water was added to a final volume of 25 ml per sample and mixed well. Next, aliquots (1 ml) of each sample were evaporated and the dried materials were re-dissolved in 1 ml of 0.02 M HCl. Aliquots (20 µl) were injected into an automatic amino acid analyzer and final readings were collected from the analyzer for further analysis using a software L-8800 ASM (L-8800, Hitachi Instruments Engineering, Tokyo, Japan). All steps were performed according to the manufacturer's instructions (Hitachi Instruments Engineering). Glutamine (Gln) and asparagine (Asn) were hydrolyzed to glutamate (Glu) and aspartate (Asp) under acidic conditions so the final content of glutamate was exactly the sum of the glutamine and glutamate contents and the final content of aspartate was exactly the sum of the aspartate and asparagine contents. Tryptophan could not be measured by this method, so only 17 amino acids contents were shown in the results.

For seed protein content analysis, about 100 mg of ground seed powder and 100 mg catalyst (K_2SO_4:$CuSO_4$:Se in a ratio of 100:10:1) were homogenized in 1 ml H_2O and 2 ml 98% H_2SO_4 at room temperature for 15 h then incubated at 380°C for 5 h. The solution was diluted to 2,500 ml and 5 ml was loaded into a Segmented Flow Analyzer (Futura, Alliance Instruments, Frépillon, France) and NH_4^+ content was determined by the Berthelot color reaction method (Gordon et al. 1978). The protein content in the samples was calculated according to the NH_4^+ content (Sotelo et al. 1990). Each line was measured with three replicates.

Results

Generation and identification of *AAT* transgenic rice

The four constructs, containing *OsAAT1*, *OsAAT2*, *OsAAT3*, or *EcAAT* driven by the *CaMV* 35S promoter, were introduced into Zhonghua 11 by *Agrobacterium*-mediated transformation (Lin and Zhang 2005). Over 600 transgenic plants were generated (>100 plants per construct). T_0 plants were examined by PCR with primers located in the *GUS* gene. PCR results suggested that about 90% of the plants had integrated the construct.

Southern blot hybridization was performed using a *GUS*-specific fragment as a probe to examine the copy number of the transgene in the transformants (PCR primers, GUSF, and GUSR are listed in Table 1). The results showed that ~30% of the transformants examined had a single copy of the transgene which was similar to the ratios in previous reports (Garg et al. 2002; Wu et al. 2003; Xiao et al. 2007). The hybridization pattern of each transgenic plant was unique, suggesting that these plants were derived from independent transformation events.

For each construct, 30 positive transformants were analyzed by RNA gel blot analysis in order to check the transgene expression level. Gene-specific probes for each *AAT* gene were used (Fig. 1). The number transformants overexpressing *OsAAT1*, *OsAAT2*, *OsAAT3*, and *EcAAT* were 7, 8, 25, and 6, respectively.

Expression pattern of *AAT* genes

The expression pattern of *OsAAT1*, *OsAAT2*, and *OsAAT3* genes were analyzed by RT-PCR analysis in various WT rice tissues at different developmental stages. The results showed that all three genes were expressed in all tissues at the different developmental stages examined. The expression of *OsAAT1* and *OsAAT2* was higher than that of *OsAAT3* in all tissues examined (Fig. 2).

qRT-PCR was also performed to check whether the enhanced expression of exogenous *EcAAT* had any effect on expression of the three endogenous *OsAATs*. The results showed that the expression levels of *OsAAT1* and *OsAAT2* were increased in *EcAAT*-OX (transgenic rice

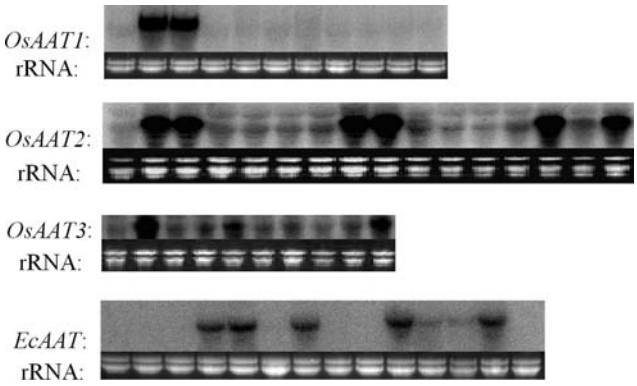

Fig. 1 Northern blot analysis of *OsAAT1*, *OsAAT2*, *OsAAT3* and *EcAAT* mRNA transcriptional levels in different independent primary transgenic plants and wild-type plants (WT). The first *lane* in each gel is WT

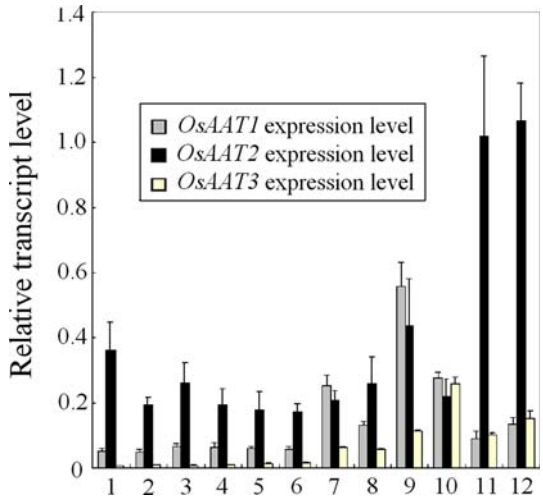

Fig. 2 qRT-PCR analysis of the three *OsAAT* genes in 12 tissues. Tissues: 1, callus at 14 days after subculture; 2–6, spike at 0.5, 1, 2, 3 and 5 cm; 7, sheath when young panicle was at secondary branch primordial differentiation stage; 8, stem at 5 days before heading; 9, leaves at two-tiller stage; 10, leaves at 4–5-cm panicle stage; 11, roots at seedling stage with two tillers; 12, roots at 4–5-cm young panicle stage

over-expressing *EcAAT*), but the expression level of *OsAAT3* was decreased in *EcAAT*-OX (Fig. 3). We also checked whether over-expression of any of the *OsAAT* genes affected expression of the endogenous *OsAAT* genes. qRT-PCR results showed that, except for the increased expression level of the endogenous *OsAAT1* in *OsAAT2*-OX transformants, over-expression of the *OsAAT* genes did not have any effect on expression of the endogenous *OsAAT* genes (Fig. 3).

Accumulation of amino acids and protein in seeds of *AAT* transgenic rice

The total amino acid content was examined in the T_1 families over-expressing the different *AAT* genes. In the seeds of the *OsAAT1*-OX, *OsAAT2*-OX, and *EcAAT*-OX T_1 families, the total amino acid content was 166.80, 153.80, and 171.56 mg g^{-1}, respectively. These values are significantly higher ($P < 0.01$) than that in WT plants (124.48 mg g^{-1}) (Fig. 4a). However, no significant difference in total amino acid content between *OsAAT3*-OX (129.36 mg g^{-1}) and WT plants was found.

To confirm this result for the T_1 generation, T_2 lines with (positive) and without (negative) the constructs were identified by PCR using the GUSF/GUSR primers and were measured for individual amino acid content. In the positive T_2 seeds of *OsAAT1*-OX and *OsAAT2*-OX, the content of each of the 17 amino acids, except for Glu in *OsAAT1*-OX and Cys in *OsAAT2*-OX, was significantly increased (ranging from 10.3 to 39.1%) compared with negative plants. The total amino acid content in T_2 seeds positive for *OsAAT1*-OX, *OsAAT2*-OX, and *EcAAT*-OX was 119.36, 115.36, and 113.72 mg g^{-1}, respectively. These values are significantly higher than that in the corresponding negative transgenic T_2 seeds (102.84, 103.00, and 107.92 mg g^{-1}, respectively). The total amino acid content increased 16.1% in *OsAAT1*-OX ($P < 0.01$), 12.0% in *OsAAT2*-OX ($P < 0.01$), and 5.4% in *EcAAT*-OX ($P < 0.05$) (Table 2; Fig. 4b). No significant difference was found for amino acid content between *OsAAT3*-OX and the corresponding negative plants.

The seed protein contents in T_2 seeds positive for *OsAAT1*-OX, *OsAAT2*-OX, and *EcAAT*-OX were 71.06, 74.56, and 71.00 mg g^{-1}, respectively. These values are significantly higher than that of the corresponding transgenic negative T_2 seeds (58.18, 61.57, and 63.88 mg g^{-1}, respectively). The seed protein content increased 22.2% in *OsAAT1*-OX ($P < 0.01$), 21.1% in *OsAAT2*-OX ($P < 0.01$), and 11.1% in *EcAAT*-OX ($P < 0.05$) (Table 2). No significant difference was found in seed protein content between *OsAAT3*-OX positive and negative plants.

Increase of AAT activities in leaves of *AAT* transgenic rice

T_1 families of the four *AAT* over-expressing plants and WT plants were harvested 6 weeks after germination for measurement of total AAT activities in leaves. The enzymatic activities in *OsAAT1*-OX (26.6 A min^{-1} mg^{-1} FW; A: units of activity, defined as increase of absorbency per min per mg; FW: fresh weight), *OsAAT2*-OX (23.6 A min^{-1} mg^{-1} FW) and *EcAAT*-OX (19.6 A min^{-1} mg^{-1} FW) transgenic homozygotes were significantly higher ($P < 0.05$) than that in WT (17.7 A min^{-1} mg^{-1} FW). In keeping with the results of the amino acid content analysis, the total leaf AAT activity (19.1 A min^{-1} mg^{-1} FW) for *OsAAT3*-OX showed no significant difference compared with WT (Fig. 5). Both the biomass and the seed production of AAT over-expressors and WT were not significantly different (data not shown).

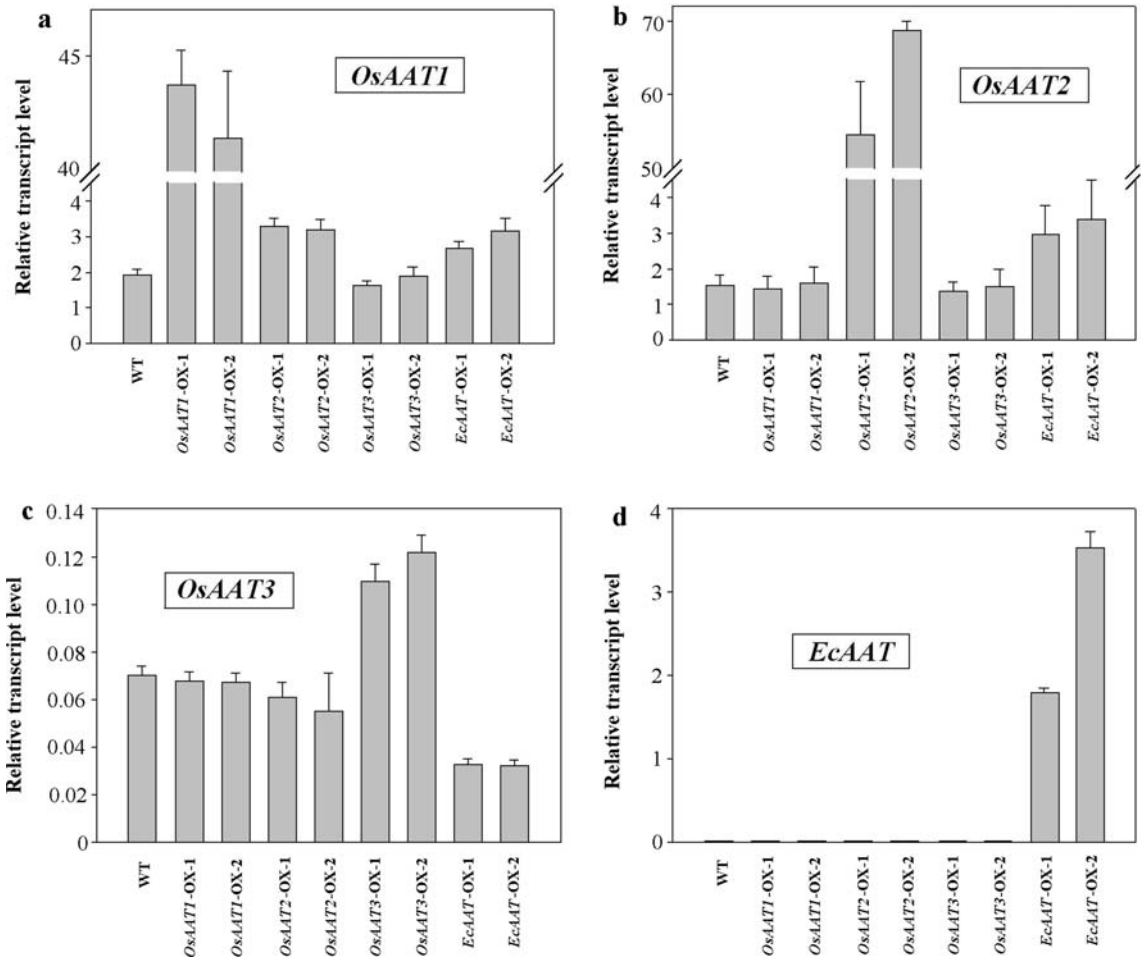

Fig. 3 qRT-PCR analysis of *AAT* expression levels in pairs of independent transformants **a** *OsAAT1*, **b** *OsAAT2*, **c** *OsAAT3*, and **d** *EcAAT*

To evaluate the effects of over-expression of *AAT* genes on nitrogen assimilation, we also analyzed the concentration of soluble proteins, free NO_3^-, and free NH_4^+ in the leaves of *AAT* over-expressing T_2 plants grown in the field. Leaf NO_3^- concentration in *OsAAT1*-OX, *OsAAT2*-OX, and *EcAAT*-OX plants showed an increase of 35.2, 31.3, and 11.0%, respectively, compared with WT (Fig. 6a). However, no change was found for the leaf NO_3^- concentration in the *OsAAT3*-OX plants. In addition, no significant difference was found for free NH_4^+ concentrations (Fig. 6b) and soluble protein (Fig. 6c) in leaves between any of the four transgenic plants and WT.

Discussion

Aspartate aminotransferase, a key enzyme involved in nitrogen and carbon metabolism, catalyzes the reversible transamination reaction between aspartate and α-ketoglutarate to form oxaloacetate and glutamate. In plants, AAT usually exists as multiple isoenzymes and plays an important role in numerous metabolic processes. Three isoenzymes of AAT in rice are localized in the chloroplast (OsAAT1), cytosol (OsAAT2) and mitochondrion (OsAAT3), respectively. Whether these different isoenzymes serve different metabolic roles in plant growth or have functional redundancy is unknown. One approach to examining the role of these isozymes is the creation and evaluation of transgenic lines in which each individual gene is over-expressed or knocked out.

In mammals, it has been suggested that transcription of the cytosolic and mitochondrial isozymes of AAT is coordinately regulated (Setoyama et al. 1990). In transformed tobacco plants, over-expression of *cAspAT* (located in cytosol) may induce transcription of endogenous *mAspAT* (located in mitochondrion) (Sentoku et al. 2000). At the same time, some reports have shown that the antisense expression of AAT-P2 (plastidial *AspAT* of *Lotus corniculatus*) in transgenic *L. corniculatus* did not change the expression of another *AspAT* (Mett et al. 1996). In this study, we found enhanced expression of the bacterial *EcAAT* gene can induce expression of endogenous *OsAAT1*

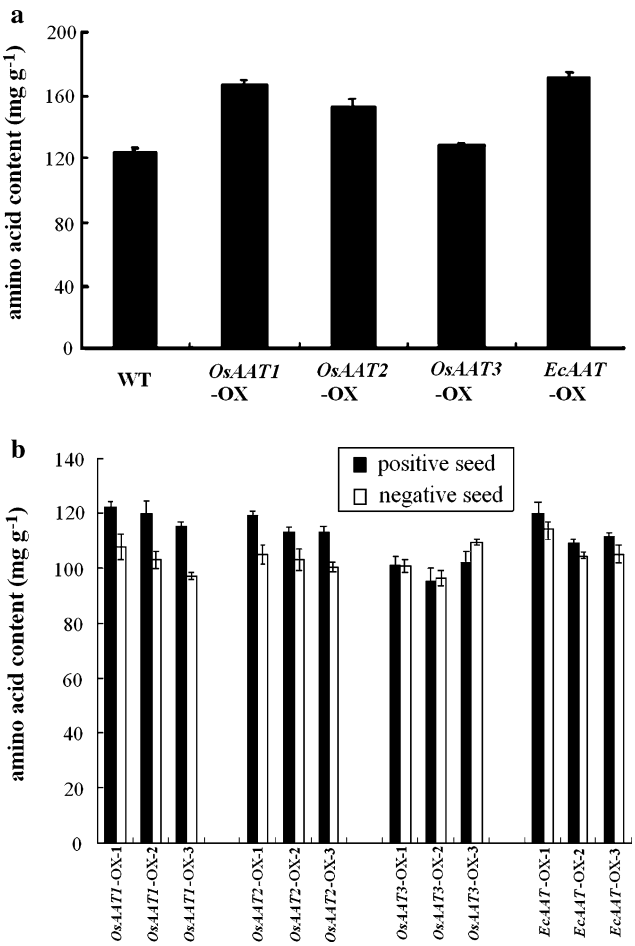

Fig. 4 Increased amino acid content of seeds in *OsAAT1*, *OsAAT2*, and *EcAAT* over-expressed plants. **a** Seed amino acid content in T_1 family members *OsAAT1*-OX, *OsAAT2*-OX, *OsAAT3*-OX, and *EcAAT*-OX plants and wild-type plants. **b** Seed amino acid content in positive and negative T_2 family members *OsAAT1*-OX, *OsAAT2*-OX, *OsAAT3*-OX, and *EcAAT*-OX plants. Values are mean ± SD from three independent transgenic lines

and *OsAAT2*, but suppresses expression of *OsAAT3*, whose protein is localized to the mitochondria. Interestingly, over-expression of any one of the three *OsAAT* genes had no effect on expression of the endogenous copies of the other two *OsAAT* genes except for the expression of *OsAAT1*, which was up-regulated in *OsAAT2*-OX. Differences in expression patterns of the *OsAAT* genes and in the subcellular localization of the AAT isoenzymes suggest distinct functional roles in plant metabolism.

Murooka et al. (2002) reported that the contents of several free amino acids in seeds of *Arabidopsis* were increased by the introduction of the *AAT5* gene from soybean. Miesak and Coruzzi (2002) observed reduced root length and dramatic decreases in aspartate and asparagine content in leaves of an *Arabidopsis AAT* mutant. In another case, a significant increase in the accumulation of some amino acids was observed in ferredoxin-dependent glutamate synthase (Fd-GOGAT, a key enzyme in nitrogen metabolism) antisense plants (Ferrario et al. 2000). These results suggest that altering the expression of genes encoding enzymes involved in nitrogen metabolism will result in modification of the nitrogen metabolism cycle which can alter the accumulation of amino acids.

Rice is one of the most important crops in the world. It provides a staple food for more than half of the world population. Thus, improving the nutritional quality of the rice grain should be a useful strategy for improving nutritional status, especially for the poor. In this study, the total amino acid content of the rice grain was increased by 16.1% in *OsAAT1*-OX ($P < 0.01$) and by 12.0% in *OsAAT2*-OX ($P < 0.01$) transgenics. The contents of all 17 amino acids, except for Glu in *OsAAT1*-OX and Cys in *OsAAT2*-OX, were significantly increased (from 10.3 to 39.1%) compared with that of negative controls, indicating that over-expression of these genes could provide an effective means for increasing the nutritional quality of the rice grain. However, our results differ from those observed in *Arabidopsis* where transgenics exhibited 10–50-fold increases in free asparagine and glutamine and 2–4-fold increases in free alanine and glycine while free aspartate, glutamate, tyrosine, valine, isoleucine and phenylalanine levels were reduced from 20 to 90% compared to control plants (Murooka et al. 2002). In addition, Murooka et al. (2002) did not report any changes in the total amino acid content of transgenic seeds.

Analysis of free NO_3^- and NH_4^+ concentrations in *AAT* over-expressed plant leaves showed an increase of free NO_3^- level but not NH_4^+. An increased level of leaf free NO_3^- was also observed in the transgenic rice over-expressing a glutamine synthetase (GS) gene encoding a key enzyme in nitrogen metabolism (Cai et al. 2008). The increased AAT or GS activity may have a feedback effect on accelerating the NO_3^- absorption in plants and bears further investigation.

Our results show that over-expressing *OsAAT1* (encoding chloroplastic AAT) and *OsAAT2* (encoding cytoplasmic AAT) increases AAT activity in leaves and amino acid content in seeds, but no significant changes were found when *OsAAT3* (encoding mitochondrial AAT) was over-expressed. Our failure to see these effects in *OsAAT3* over-expressors is unlikely to be due to positional effects of the transgene because we see the same failure in seven other independent transformants with elevated levels of *OsAAT3* transcripts (data not shown). The much higher expression level of the endogenous *OsAAT1* and *OsAAT2* compared with *OsAAT3* in WT plants may suggest the important function of these two members in transfer of the amino group in rice plant (Fig. 3). As reported in wheat, changes in chloroplast and cytosol AAT enzymatic activity have a significant impact on AAT activity, whereas changes in

Table 2 Amino acid and protein contents in seeds of positive and negative T_2 transgenic plants

Amino acid	*OsAAT1*-OX			*OsAAT2*-OX			*OsAAT3*-OX			*EcAAT*-OX		
	Positive (mg g^{-1})	Negative (mg g^{-1})	%	Positive (mg g^{-1})	Negative (mg g^{-1})	%	Positive (mg g^{-1})	Negative (mg g^{-1})	%	Positive (mg g^{-1})	Negative (mg g^{-1})	%
Asp	11.32 ± 0.32**	10.00 ± 0.16	13.2	10.96 ± 0.32**	9.88 ± 0.20	10.9	8.96 ± 0.32	9.60 ± 0.68	−6.7	10.52 ± 0.32*	10.28 ± 0.36	2.3
Thr	4.88 ± 0.16**	4.40 ± 0.08	10.9	4.76 ± 0.08**	4.24 ± 0.02	12.3	3.96 ± 0.12	4.04 ± 0.28	−2.0	4.52 ± 0.12	4.52 ± 0.08	0.0
Ser	7.80 ± 0.20**	6.84 ± 0.12	14.0	7.12 ± 0.16**	6.28 ± 0.12	13.4	6.16 ± 0.16	6.44 ± 0.40	−4.3	7.16 ± 0.28	7.00 ± 0.16	2.3
Glu	19.68 ± 0.52	19.36 ± 0.44	1.7	19.88 ± 3.96**	17.64 ± 1.60	12.7	21.4 ± 0.56	19.84 ± 1.24	7.9	21.52 ± 0.96*	20.68 ± 1.24	4.1
Gly	10.12 ± 0.36**	8.68 ± 0.28	16.6	9.68 ± 0.32**	8.60 ± 0.16	12.6	8.04 ± 0.24	8.08 ± 0.56	−0.5	9.20 ± 0.32	9.08 ± 0.28	1.3
Ala	10.60 ± 0.36**	8.84 ± 1.60	19.9	9.80 ± 0.48**	8.80 ± 0.20	11.4	8.08 ± 0.24	8.40 ± 0.40	−3.8	9.72 ± 0.84**	8.48 ± 1.28	14.6
Cys	1.24 ± 0.08**	1.04 ± 0.08	19.2	1.16 ± 0.28*	1.08 ± 0.24	7.4	0.76 ± 0.04*	0.72 ± 0.04	5.6	1.04 ± 0.08**	0.96 ± 0.04	8.3
Val	7.72 ± 0.24**	6.40 ± 0.20	20.6	7.56 ± 0.04**	6.84 ± 0.04	10.5	6.16 ± 0.16	6.64 ± 0.40	−7.2	7.12 ± 0.40*	6.84 ± 0.52	4.1
Met	2.24 ± 0.12**	1.80 ± 0.04	24.4	2.00 ± 0.20**	1.80 ± 0.12	11.1	1.88 ± 0.04	1.80 ± 0.12	4.4	1.96 ± 0.04*	1.76 ± 0.16	11.4
Ile	4.56 ± 0.12**	3.84 ± 0.16	18.8	4.56 ± 0.04**	4.08 ± 0.04	11.8	3.64 ± 0.08	4.08 ± 0.24	−10.8	4.32 ± 0.20*	4.04 ± 0.28	6.9
Leu	9.96 ± 0.28**	7.96 ± 0.36	25.1	9.64 ± 0.16**	8.64 ± 0.16	11.6	7.60 ± 0.20	8.64 ± 0.42	−12.0	9.04 ± 0.48*	8.44 ± 0.64	7.1
Tyr	3.12 ± 0.16**	2.40 ± 0.16	30.0	3.32 ± 0.24**	2.88 ± 0.32	15.3	2.40 ± 0.08	2.64 ± 0.12	−9.1	2.84 ± 0.16**	2.40 ± 0.28	18.3
Phe	5.16 ± 0.16**	4.20 ± 0.12	22.9	5.00 ± 0.16**	4.48 ± 0.16	11.6	3.96 ± 0.12	4.40 ± 0.24	−10.0	4.68 ± 0.24*	4.44 ± 0.24	5.4
Lys	4.20 ± 0.16**	3.72 ± 0.12	12.9	3.96 ± 0.16**	3.52 ± 0.20	12.5	3.52 ± 0.16	3.48 ± 0.28	1.1	3.88 ± 0.04	3.88 ± 0.08	0.0
His	2.72 ± 0.08**	2.28 ± 0.08	15.2	2.56 ± 0.08**	2.32 ± 0.16	10.3	2.00 ± 0.08	2.16 ± 0.20	−7.4	2.60 ± 0.08*	2.48 ± 0.01	4.8
Arg	7.68 ± 0.28**	5.52 ± 0.48	19.3	7.48 ± 0.08**	6.56 ± 0.08	14.0	5.88 ± 0.20	6.48 ± 0.48	−9.3	7.12 ± 0.28*	6.84 ± 0.24	4.1
Pro	6.36 ± 0.36**	5.52 ± 0.20	39.1	5.92 ± 0.32**	5.36 ± 0.28	10.4	5.16 ± 0.20	4.96 ± 0.28	4.0	6.48 ± 0.20**	5.80 ± 0.84	11.7
Total	119.36 ± 3.48**	102.84 ± 5.32	16.1	115.36 ± 3.48**	103.00 ± 2.28	12.0	99.56 ± 3.68	102.40 ± 6.56	−2.8	113.72 ± 5.56*	107.92 ± 5.24	5.4
Protein	71.06 ± 1.81**	58.18 ± 2.38	22.2	74.56 ± 2.05**	61.57 ± 1.63	21.1	63.76 ± 2.51	62.39 ± 3.13	2.2	71.00 ± 3.20*	63.88 ± 2.01	11.1

Values are mean ± SE ($n = 3$; three independent lines per T_2 family). Data on the difference between each positive and negative plant of over-expressing *OsAAT1*, *OsAAT2*, *OsAAT3*, and *EcAAT* were examined by the *t* test

Significant differences at the levels of *$P = 0.05$ and **$P = 0.01$, respectively

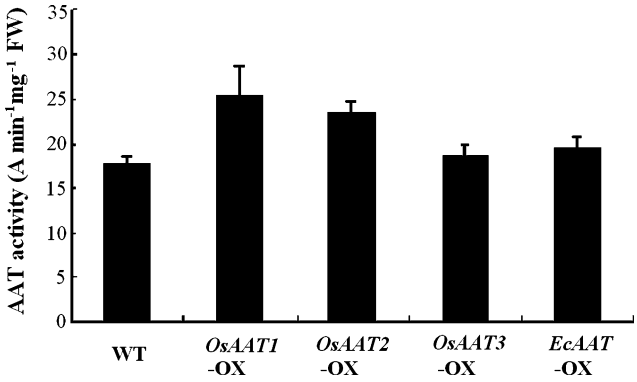

Fig. 5 Increased total leaf AAT activities in *OsAAT1*-OX, *OsAAT2*-OX, and *EcAAT*-OX plants. Values are mean ± SD from three independent experiments

mitochondrial AAT activity do not have any dramatic impact (Marcin and Andrzej 2004). This difference could partially be explained by differences in the structure and activity of these isoenzymes (Karcicio and Izbirak 2003). The expression level of *OsAAT3* in OsAAT3-OX was much lower than that of *OsAAT1* and *OsAAT2* in *OsAAT1*-OX and *OsAAT2*-OX, respectively. This might be due to difference in the cloning of the three genes. The *OsAAT3* used for transformation was derived from cDNA sequence in which all introns were removed while both *OsAAT1* and *OsAAT2* used for transformation were derived from genomic sequences. The absence of introns might have some negative effect on the transcription of the *OsAAT3* transgene.

To determine the individual AAT isozyme functions in vivo, we also constructed three vectors to inhibit expression of *OsAAT* genes one at a time. Unfortunately, after construction of the vectors, the transformed plants were not available because nearly all callus cells had died at the differentiation stage. At the same time, we searched the rice mutant database but found no mutant lines for these three genes. The mutant lines are probably unavailable because AAT plays a very important role in plant growth and development. If each isoenzyme has a specific metabolic role, absence of any one of the three might be lethal for rice.

The metabolic engineering method, which involves modification of key enzymes involved in metabolism, provides a strategy for improving amino acid production in plants. Better results may be obtained with cooperative regulation of some key enzymes in the nitrogen metabolism pathway within the same transgenic plant. We are currently crossing these individual *OsAAT* over-expressors to develop transgenic plants that over-express all three genes with a view to looking for additive or synergistic effects on seed amino acid content and nitrogen metabolism. We are also crossing recently generated transgenic plants that over-express glutamine synthetase, glutamate synthase, or

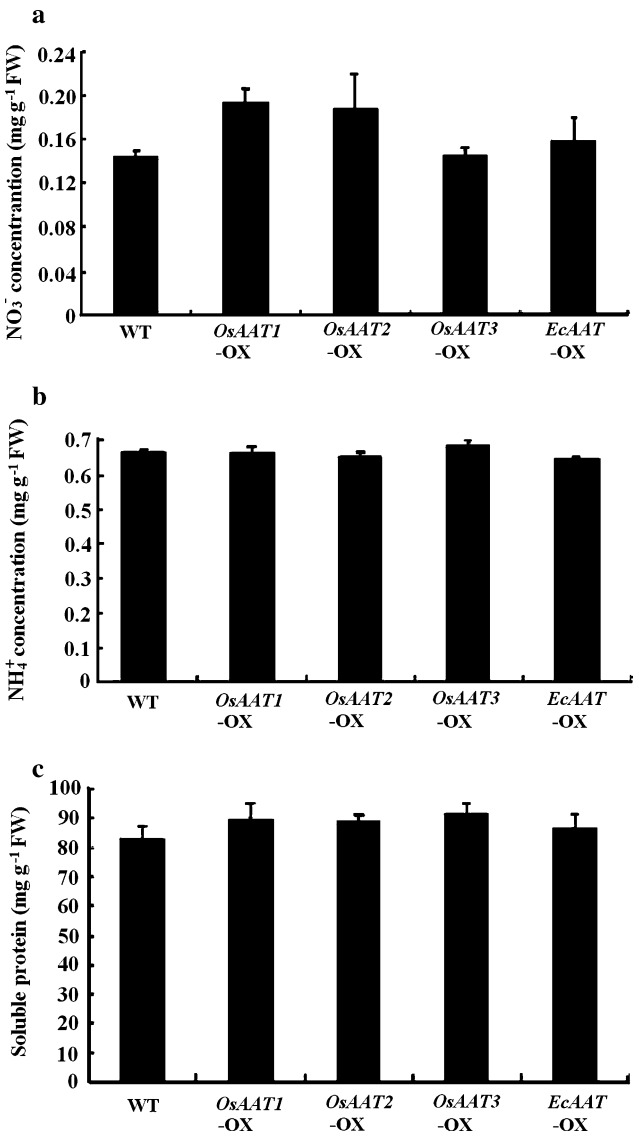

Fig. 6 Metabolite contents in leaves of the *AAT* over-expressed transgenic and wild-type plants. **a** Free NO_3^- concentration. **b** Free NH_4^+ concentration. **c** Soluble protein content. Values are mean ± SD from three independent experiments

glutamate dehydrogenase to assess the impact of these transformants on nitrogen use efficiency.

Acknowledgments This research was supported in part by grants from the National Basic Research Program of China (2005CB120905), the National Special Key Project of China on Functional Genomics of Major Plants and Animals, the National Natural Science Foundation of China and the Cultivation Fund of the Key Scientific and Technical Innovation Project, Ministry of Education of China (No. 707045). We thank Lizhong Xiong and John Bennett for helpful comments on the manuscript.

References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. Anal Biochem 72:248–254

Bryan JK (1980) Synthesis of the aspartate family and branched-chain amino acids. In: Stumpf PK, Conn EE (eds) The biochemistry of plants: a comprehensive treatise. Academic Press, New York, pp 403–452

Cai HM, Zhou Y, Xiao JH, Li XH, Zhang QF, Lian XM (2008) Over-expressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. Plant Cell Rep (in press)

Chavan JK, Duggal SK (1978) Studies on the essential amino acid composition, protein fractions and biological value (BV) of some varieties of rice. J Sci Food Agric 29:225–229

Chu ZH, Peng KM, Zhang LD, Zhou B, Wei J, Wang SP (2003) Construction and characterization of a normalized whole-life-cycle cDNA library of rice. Chin Sci Bull 48:229–235

Coruzzi GM (2003) Primary N-assimilation into amino acids in Arabidopsis. In: Somerville CR, Meyerowitz EM (eds) The *Arabidopsis* book. American Society of Plant Biologists, Rockville, pp 1–17

Coruzzi GM, Last R (2000) Amino acids. In: Buchanan BB, Gruissem W, Jones RL (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, pp 358–410

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Webber P (1995) Transgenic canola and soybean seeds with increased lysine. Bio/Technology 13:577–582

Ferrario MS, Suzuki A, Kunz C, Valadier MH, Roux Y, Hirel B, Foyer CH (2000) Modulation of amino acid metabolism in transformed tobacco plants deficient in Fd-GOGAT. Plant Soil 221:67–79

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99:15898–15903

Gelfand DH, Rudo N (1977) Mapping of the aspartate and aromatic amino acid aminotransferase genes *tyrB* and *aspC*. J Bacteriol 133:441–444

Givan CV (1980) Aminotransferases in higher plants. In: Stumpf PK, Conn EE (eds) The biochemistry of plants. Academic Press, New York, pp 329–357

Gordon SA, Fleck A, Bell J (1978) Optimal conditions for the estimation of ammonium by the Berthelot reaction. Ann Clin Biochem 15:270–275

Hatch MD, Osmond CB (1973) Activity, location, and role of aspartate aminotransferase isoenzymes in leaves with C4 pathway photosynthesis. Arch Biochem Biophys 156:195–206

Heber U, Heldt HW (1981) The chloroplast envelope: structure, function and role in leaf metabolism. Annu Rev Plant Physiol 32:139–168

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Joachim J, Richard AP, Ursula S, Johan NJ (1994) Three-dimensional structure of a mutant *E. coli* aspartate aminotransferase with increased enzymic activity. Protein Eng 7:605–612

Karcicio M, Izbirak A (2003) Isozyme variations in some *Aegilops* L. and *Triticum* L. species collected from Central Anatolia. Turk J Bot 27:433–440

Lin YJ, Zhang QF (2005) Optimising the tissue culture conditions for high efficiency transformation of *indica* rice. Plant Cell Rep 23:540–547

Marcin M, Andrzej P (2004) Genetic control of aspartate aminotransferase isoenzymes in *Aegilops* and *Triticum* species. J Appl Genet 45:411–417

Melo PM, Lima LM, Santos IM, Carvalho HG, Cullimore JV (2003) Expression of the plastid-located glutamine synthetase of Medicago truncatula. Accumulation of the precursor in root nodules reveals an in vivo control at the level of protein import into plastids. Plant Physiol 132:390–399

Mett VL, Podivinsky E, Tennant AM, Lochhead LP, Jones WT, Reynolds PHS (1996) A system for tissue-specific copper controllable gene expression in transgenic plants: nodule-specific antisense of aspartate aminotransferase-P2. Transgenic Res 5:105–113

Miesak BH, Coruzzi GM (2002) Molecular and physiological analysis of *Arabidopsis* mutants defective in cytosolic or chloroplastic aspartate aminotransferase. Plant Physiol 129:650–660

Murooka Y, Mori Y, Hayashi M (2002) Variation of the amino acid content of Arabidopsis seeds by expressing soybean aspartate aminotransferase gene. J Biosci Bioeng 94:225–230

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plan DNA. Nucleic Acids Res 8:4321–4325

Oliveira IC, Brears T, Knight TJ, Clark A, Coruzzi GM (2002) Overexpression of cytosolic glutamine synthetase: relation to nitrogen, light, and photorespiration. Plant Physiol 129:1170–1180

Rawstone S, Michin FR, Summerfield RJ, Cookson C, Coombs J (1980) Carbon and nitrogen metabolism in legume root nodules. Phytochemistry 19:341–355

Rudman D, Meister A (1953) Transamination in *Escherichia coli*. J Biol 200:591–604

Ryan E, Fottrell PF (1974) Subcellular localization of enzymes involved in the assimilation of ammonia by soybean root nodules. Plant Mol Biol 19:2647–2652

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 1.31–1.119

Sentoku N, Taniguchi M, Sugiyama T, Ishimaru K, Ohsugi R, Takaiwa F, Toki S (2000) Analysis of the transgenic tobacco plants expressing *Panicum miliaceum* aspartate aminotransferase genes. Plant Cell Rep 19:598–603

Setoyama C, Ding S, Choudhury BK, Joh T, Takeshima H, Suzuki T, Shimada K (1990) Regulatory regions of the mitochondrial and cytosolic isoenzymes genes participating in the malate–aspartate shuttle. J Biol Chem 265:1293–1299

Shaul O, Galili G (1992) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia coli*. Plant Physiol 100:1157–1163

Song J, Yamamoto K, Shomura A, Yano M, Minobe Y, Sasaki T (1996) Characterization and mapping of cDNA encoding aspartate aminotransferase in rice, *Oryza sativa* L. DNA Res 3:303–310

Sotelo A, Sousa V, Montalvo I, Hernandez M, Hernandez AL (1990) Chemical composition of different fractions of 12 Mexican varieties of rice obtained during milling. Cereal Chem 67(2):209–212

Walther E, Kerstin B, Hubert K (1999) Regulation of inducible nitric oxide synthase expression in β cells by environmental factors: heavy metals. Biochem J 338:695–700

Wang WM, Zhao Q, Yu JJ, Zhu DY, Ao GM (2005) Transfer of high lysine gene sb401 into rice and analysis for protein and amino acid content in transgenic rice seeds. Acta Agronom Sinica 31:603–607

Wilkie SE, Roper JM, Smith AG, Warren MJ (1995) Isolation, characterisation and expression of a cDNA clone encoding plastid aspartate aminotransferase from *Arabidopsis thaliana*. Plant Mol Biol 27:1227–1233

Wu LH, Jiang SH, Tao QN (1998) The method of mensurate enzyme activity of plant GOT and GPT. Chin J Soil Sci 29(2):136–138

Wu CY, Li XJ, Yuan WY, Chen GX, Kilian A, Li J, Xu CG, Zhou DX, Wang SP, Zhang QF (2003) Development of enhancer trap lines for functional analysis of the rice genome. Plant J 35:418–427

Xiao BZ, Huang YM, Tang N, Xiong LZ (2007) Over-expression of a *LEA* gene in rice improves drought resistance under the field conditions. Theor Appl Genet 115:35–46

Yoshida S, Forno D, Cock J, Gomez K (1976) Laboratory manual for physiological studies of rice. International Rice Research Institute, Manila, pp 61–65