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RESEARCH PAPER

Improving the nutritive value of rice seeds: elevation of cysteine and methionine contents in rice plants by ectopic expression of a bacterial serine acetyltransferase

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Abstract

With the aim of increasing the cysteine level in rice (*Oryza sativa* L.) and thus improving its nutritional quality, transgenic rice plants were generated expressing an *Escherichia coli* serine acetyltransferase isoform (*EcSAT*), the enzyme synthesizing *O*-acetylserine, the precursor of cysteine. The gene was fused to the transit peptide of the *Arabidopsis* Rubisco and driven by a ubiquitin promoter to target the enzyme to plastids. Twenty-two transgenic plants were examined for transgene protein expression, and five lines with a high expression level and enzymatic activity, respectively, were selected for further analysis. In these lines, the contents of cysteine and glutathione increased 2.4-fold and 2-fold, respectively. More important is the increase in free methionine and methionine incorporated into the water-soluble protein fraction in seeds. Free methionine increased in leaves up to 2.7-fold, in seeds up to 1.4-fold, and bound to seed proteins up to 4.8-fold, respectively, while the bound methionine level remained constant or even decreased in leaves. Notably, the transgenic lines exhibited higher isoleucine, leucine, and valine contents (each up to 2-fold depending on tissue, free, or bound), indicating a potential conversion of methionine via methionine γ-lyase to isoleucine. As the transgenic rice plants overexpressing *EcSAT* had significantly higher levels of both soluble and protein-bound methionine, isoleucine, cysteine, and glutathione in rice they may represent a model and target system for improving the nutritional quality of cereal crops.

Key words: Cysteine and methionine enhancement, nutritional improvement, rice, serine acetyltransferase.

Introduction

Rice is the most important food crop in the world. Almost half of the world's population depends on rice as their staple food (Bajaj and Mohanty, 2005), especially in east and south-east Asia. Rice provides a significant source of grains, and is amongst the most important nutritional sources of protein for mankind world-wide. However, one of the biggest issues is the increase in the world population in developing countries and consequently higher demands from consumers (Bajaj and Mohanty, 2005). Beside challenges such as abiotic or biotic stress tolerance, yield and high nutritional quality in terms of essential amino acids is one of the desired traits (Tyagi and Mohanty, 2000; Bajaj and Mohanty, 2005). However, similar to seed proteins of other cereals, seed proteins of rice are deficient in some essential amino acids such as lysine, tryptophan, and especially methionine (Lee *et al*., 2001), thus limiting the nutritive value of seed protein (Tabe and Higgins, 1998). This limitation reduces, for example, wool growth in sheep, milk production by dairy animals, and meat quality (Lewis *et al*., 1982; Pickering and Reis, 1993; Tabe *et al*., 1995; Prakash, 1996; Xu *et al*., 1998). For the latter, animals are able to convert methionine to cysteine, but not conversely, hence defining methionine as the essential amino acid that can supply the complete

requirement for suphur-containing amino acids (Tabe and Higgins, 1998). To meet the requirements of monogastric animal diets, sulphur-containing amino acids must be added either in a synthetic form or as forage plants containing increased levels of cysteine and methionine. For ruminant animals, however, methionine must be supplied in the form of proteins that are resistant to rumen proteolysis (Galili *et al*., 2002; Hesse and Hoefgen, 2003; Bagga *et al*., 2004; Hesse *et al*., 2004*b*). Increasing the cysteine and methionine contents of seeds and other edible plant parts has been a goal for breeding and agricultural biotechnology. Additional agricultural interest in altering cysteine metabolism comes from its importance as a precursor for glutathione synthesis, being involved in redox regulation of cell metabolism and stress tolerance. Serine acetyltransferase (SAT) and its product *O*-acetylserine (OAS) have been shown to control the enzymes responsible for sulphate reduction and cysteine biosynthesis in plants (Blaszczyk *et al*., 1999; Harms *et al*., 2000; Hesse *et al*., 2001, 2004*a*; Wirtz and Hell, 2003; Hopkins *et al*., 2005; Krueger *et al*., 2009). The production of OAS and cysteine also limits the overall rate of glutathione biosynthesis and the maintenance of an elevated glutathione pool (Barroso *et al*., 1995; Meyer and Fricker, 2002). Thiol levels such as those of cysteine and glutathione consistently increased up to 6-fold independently of the subcellular localization, feedback sensitivity, or activity state of the overexpressed SAT isoform (Blaszczyk *et al*., 1999; Harms *et al*., 2000; Wirtz and Hell, 2003, 2007; Sirko *et al*., 2004). Efforts have been made to improve the sulphur-containing amino acid content of vegetative tissues, for example either by expression of sulphur-rich seed storage proteins (Tabe and Higgins, 1998; Amir and Galili, 2003; Bagga *et al*., 2004) or by expression or reduction of gene expression of key enzymes of cysteine and methionine synthesis, respectively (Harms *et al*., 2000; Zeh *et al*., 2003; Avraham *et al*., 2005). However, the expression of sulphur-rich proteins was not successful either, being unstable in vegetative tissues (Wandelt *et al*., 1992; Ealing *et al*., 1994) or only stable when directed into the endoplasmic reticulum (Wandelt *et al*., 1992; Habben and Larkins, 1995; Khan *et al*., 1996; Christiansen *et al*., 2000; Tabe and Droux, 2001; Bagga *et al*., 2004). Furthermore, recent results obtained for plants overexpressing a sulphur-rich 2S albumin in rice seeds revealed that reduced sulphur is limiting the protein synthesis and thus limiting the approach to increase the level of bound methionine (Tabe and Droux, 2002). Moreover, a major issue with the expression of foreign proteins is their potential allergenicity as was shown for the 2S albumins from seeds of Brazil nut or sunflower (Nordlee *et al*., 1996). To overcome these issues, direct approaches to manipulate the biosynthetic pathway of cysteine or methionine would allow the improvement of the endogenous content of sulphur-containing amino acids in rice (Hesse *et al*., 2001, 2004*b*; Hesse and Hoefgen, 2003).

Independent from the beneficial effect of glutathione against oxidative stress, it was thought that increasing cysteine levels by expression of a deregulated SAT could elevate the total levels of free and bound sulphur-containing amino acids in rice, the most important crop world-wide. This consideration makes it a suitable candidate to serve both as a model and as a target plant for testing the potential of improving the nutritional quality of crops.

Materials and methods

Plant materials and growth conditions

The plant material used was *Oryza sativa* L. cv. Taipei 309 (IRGC accession 42576) obtained from the International Rice Research Institute (IRRI, Manila, Philippines). Plants were grown in the greenhouse according to Degenkolbe *et al*. (2009). Seeds were pre-germinated in tap water at 28 °C for 10 d. Plantlets were transferred to a climate chamber with 12 h daylength at a photon flux density of 600 µE m^{-2} s^{-1} (lamps: Iwasaki Eye MT 400 DL/BH E40, DHL Licht, Wülfrath, Germany); temperature was 26 °C in the light and 22 °C at night, with a relative humidity of 75% in the light and 70% at night.

Plasmid construction and plant transformation

The SAT overexpression construct was created with the forward primer 5'-GAC GCT ACT CAA GCA CGA AA-3' and the reverse primer 5'-CCC ATC CCC ATA CTC AAA TG-3', fused to a transit peptide according to Harms *et al*. (2000) and subsequently cloned into the vector pCAMBIA1391Z/Ubiquitin. The pCAMBIA vector has been shown to be ideal for rice transformation (IIag *et al*., 2000). The *Escherichia coli* gene was driven by a ubiquitin promoter in the construct, allowing constitutive expression in plants. The SAT construct was electroporated into *Agrobacterium tumefaciens* LBA4404.

Rice embryonic calli were induced from mature embryos (seeds) and transfected with *A. tumefaciens* LBA4404 containing the construct. The strain has been approved for transformation into Japonica rice varieties (Hiei *et al*., 1997; Malabika *et al*., 2000; Cheng *et al*., 2004). SAT transgenic plants were screened in half-strength Murashige and Skoog (MS) medium containing 75 mg l^{-1} hygromycin (Sigma) and 250 mg l^{-1} cefotaxim. Transgenic plants of the T_0 generation from calli with hygromycin-resistant plants were transplanted into soil and grown in a greenhouse, and screened by real-time-PCR (RT-PCR; not shown).

Extraction and metabolite analysis

Individual soluble thiols were determined as the sum of their reduced and oxidized forms. A 50 mg aliquot of frozen ground leave tissue was added to 25 mg of polyvinylpolypyrrolidone (PVPP) (previously washed with 0.1 M HCl) and 500 µl of 0.1 M HCl. The samples were shaken for 60 min at room temperature. After centrifugation (15 min at 15.777 *g*, 4 °C), the supernatants were frozen at –20.0 °C until reduction/derivatization. The levels of glutathione and cysteine were determined by a high-performance liquid chromatography (HPLC)-based method after reduction and derivatization with monobromobimane, as described by Kreft *et al*. (2003).

Amino acids were determined as described by Kreft *et al*. (2003). A 50 mg aliquot of freshly ground frozen plant tissue was extracted for 20 min at 4 °C sequentially with 400 µl of 80% (v/v), 400 µl of 50% (v/v), and 200 µl of 80% (v/v) aqueous ethanol (buffered with 2.5 mM HEPES-KOH, pH 6.2). Ethanol/water extracts were subjected to HPLC analysis using a Hyperclone C_{18} BDS column (Phenomenex, Aschaffenburg, Germany) connected to an HPLC system (Dionex, Idstein, Germany). OAS was measured by pre-column online derivatization with orthophthaldehyde in combination with fluorescence detection (Lindroth and Mopper, 1979; Kim *et al*., 1997). OAS was eluted similarly to amino acids (Kreft *et al*., 2003), but at pH 6.2 and with 11% (v/v) tetrahydrofuran in 8.5 mM sodium phosphate buffer. OAS stability was tested by determining the recovery rate. More than 90% of OAS was recovered after 10 h at pH 6.2 and 3 °C. Absence of co-eluting compounds was tested with samples incubated with borate buffer at pH 10.7, at which OAS completely converts to *N*-acetylserine which is not accessible for derivatization.

Protein-bound amino acid analyses from leaves and seeds

For the microwave-assisted hydrolysis studies, extracted proteins from 50–100 µg of plant material powder were hydrolysed by microwave irradiation in the presence of 6 M HCl for 40 min at 150 °C with 600 W

using the MARS™ 5 System (CEM GmbH, Germany). Protein standards were also hydrolysed under the same conditions to determine the recovery rate. After hydrolysis, the samples were dried to remove any liquid in the vials by a rotary evaporation for 30 min without heating and dissolved in 200 µl of 0.1 M HCl. The hydrolysates were then analysed using OPA for pre-column modification and separation on the HPLC system as described above. The determination of tryptophan and cysteine is not possible under the chosen conditions due to technical reasons, whereas asparagine and glutamine are completely converted to aspartate and glutamate (Anders, 2002).

Protein-bound methionine, as a percentage of the total protein in rice seeds, was calculated using the following equation: methionine (g $100\,g^{-1}$ protein)=100×methionine (g)/CP (g), where methionine is the protein-bound methionine level (mol)×methionine molecular weight and CP is the total protein weight (relative to tissue dry weight), estimated as 0.008 g (8% protein and 10% dry weight). This estimation is according to NRC tables (cited by Galili *et al*., 2000).

Assay for SAT enzyme activity

Soluble protein extracts were prepared using 150 mg of frozen *Arabidopsis* leaf or root material or fractions of lyophilized powder from non-aqueous gradients and 600 µl of extraction buffer [50 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride (PMSF)]. After centrifugation, the supernatant was desalted and total protein quantified according to Bradford (1976). SAT activity was assayed according to Toda *et al*. (1998) by measuring either the disappearance of the 232 nm absorbance peak of acetyl-CoA or the appearance of the 412 nm absorbance peak of thionitrobenzoic acid according to Kredich and Tomkins (1966). A 20 µg aliquot of protein extract was incubated in a 90 µl reaction mixture containing 50 mM TRIS-HCl pH 7.5, 0.1 mM acetyl-CoA, and 1.25 mM EDTA, or additionally in the presence of 1 mM DTNB [5,5'-dithio-bis-(2-nitro-benzoic acid)]. The reaction was started by adding 10 mM L-serine and measured every 20 s at a wavelength of 412 nm. CoA solutions were used as standards

Statistical analysis

Heatmap presentation was performed on the data sets obtained from metabolite profiling with the software package TMEV (Saeed *et al*., 2003). The data were $\log_2$ transformed before analysis. The term 'significant' is used in the text only when the change in question has been confirmed to be significant ($P < 0.05$, at least). The *t*-tests were performed using the algorithm embedded in Microsoft Excel.

Results

Generation and selection of transgenic rice plants expressing EcSAT

To investigate the capability of EcSAT for the synthesis of cysteine and glutathione in rice plants, the full-length bacterial *EcSAT* gene was fused to an *rbcS* signal sequence according to Harms *et al*. (2000) to target the protein to the chloroplasts. Expression of the fused gene was under the control of a ubiquitin promoter which was successfully used in rice (Wang and Oard, 2003). The clone was inserted into the binary vector pCAMBIA, which was used to transform the rice cultivar Taipei 309. The transformation by using mature seeds was adapted from Hiei *et al*. (1994). Twenty-two rice plants expressing *EcSAT* were identified upon hygromycin selection. No morphological differences were noticed between the control, non-transformed plants and transgenic plants expressing *EcSAT* (data not shown). The expression level of *EcSAT* in the transgenic plants was determined by quantitiative RT-PCR (data not shown) and the SAT activity was determined from five selected lines. Total activity increased in transgenic plants up to 12-fold in line 51 (Fig. 1A). Coincidently the steady-state OAS content increased up to 10-fold as shown for example for line 39 [5.89±0.98 pmol mg^{-1} fresh weight (FW)], line 47 (6.43±0.13 pmol mg^{-1} FW), and line 51 (5.62±0.96 pmol mg^{-1} FW) in Fig. 1B in comparison with the OAS level in wild-type plants (0.63±0.11 pmol mg^{-1} FW). Consequently, the contents of cysteine and glutathione in transgenic lines (line 39, cysteine 1.23±0.14 nmol mg^{-1} FW, glutathione 11.1±0.88 nmol mg^{-1} FW; line 40, cysteine 1.08±0.06 nmol mg^{-1} FW, glutathione 10.22±0.356 nmol mg^{-1} FW; line 47, cysteine 1.05±0.17 nmol mg^{-1} FW, glutathione 9.01±1 nmol mg^{-1} FW; line 48, cysteine 1.33±0.11 nmol mg^{-1} FW, glutathione 9.8±0.17 nmol mg^{-1} FW; line 50, cysteine 0.87±0.08 nmol mg^{-1} FW, glutathione 8.7±0.66 nmol mg^{-1} FW; line 51, cysteine 0.99±0.07 nmol mg^{-1} FW, glutathione 9.13±0.52 nmol mg^{-1} FW) increased too, but levelled out on up to 2.5-fold higher than the respective levels of control plants (cysteine 0.51±0.22 nmol mg^{-1} FW, glutathione 4.91±2.22 nmol mg^{-1} FW) (Fig. 1C, 1D).

Transgenic rice plants accumulated soluble methionine and isoleucine in their leaves

In order to investigate whether the expression of the *EcSAT* gene in transgenic rice plants influences the endogenous levels of other amino acids, their content were determined in non-transformed and transformed plants. Young, green, and fully expanded leaves of 8-week-old plants were harvested and the amino acid content was determined via HPLC. Data of measured contents were converted into heatmap presentations (Fig. 2; Supplementary Table S1 available at JXB online). The most important result is the increase in methionine content in all investigated lines. Transgenic lines significantly accumulated comparable levels of free methionine, exhibiting an up to a 2.7-fold higher level compared with wild-type plants. While wild-type plants have a methionine content of ~1.48 nmol g^{-1} FW, for all transgenic lines the methionine level ranges from 3.36 nmol mg^{-1} FW to 4.03 nmol g^{-1} FW (Supplementary Table S1). Methionine accumulation in leaves was accompanied by a significant elevation in isoleucine (2-fold), but leucine and valine also slightly increased in their respective contents (up to 1.5-fold), thus forming a cluster in the heatmap presentation (Fig. 2). In contrast, the asparagine content dropped in nearly all transgenic plants. Notably, aromatic amino acids such as trypthophan, tyrosine, and phenylalanine increased slightly in their respective contents. Especially in line 39, tryptophan accumulated 2-fold (Fig. 2; Supplementary Table S1). Amino acids such as glycine, serine, alanine, glutamate/glutamine, arginine, histidine, and also amino acids of the aspartate family, aspartate, threonine, and lysine, did not respond to the altered internal cysteine levels.

Expression of EcSAT *modifies the composition of protein-bound amino acids in leaves*

In order to test whether the enhanced production of soluble methionine in leaf tissues was associated with increased incorporation into leaf proteins, proteins were hydrolysed and quantified

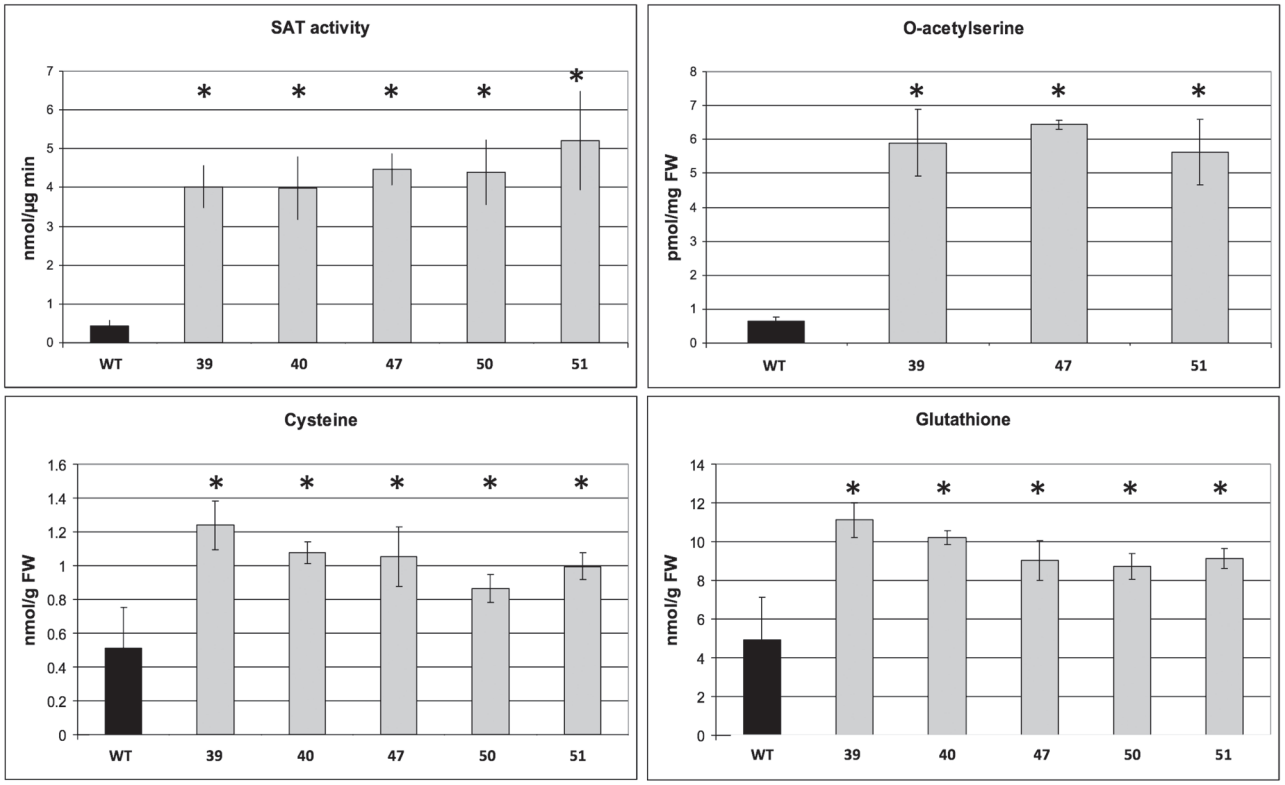

Fig. 1. Analysis of transgenic rice plants expressing *EcSAT* in plastids. Serine acetyltransferase (SAT) activity in transgenic plants was analysed in leaves of 8-week-old plants from crude extracts. From the same plant material, metabolic analyses of transgenic and wild-type plants were performed. The obtained data represent mean values and the SD for *O*-acetylserine (OAS), cysteine, and glutathione concentrations in leaves of 3–4 biological replicates for each transgenic plant and the wild type. Asterisks mark significant differences between the wild type and transgenic plant lines (*$P < 0.05$). FW, fresh weight.

by HPLC. The amino acid contents obtained were converted into a heatmap (Fig. 3; Supplementary Table S2 at JXB online). The heatmap was generated by using $\log_2$-transformed fold changes, being normalized to the respective amino acid content in wild-type plants. First, no major differences in the protein amino acid content were detected despite slight alterations in comparison with wild-type levels. Secondly, methionine in particular did not accumulate in leaf protein despite the increased availability of free methionine, as shown in Fig. 3 (Supplementary Table S2). For several lines, even a decrease in methionine content down to 0.7-fold of the wild-type level could be observed (Fig. 3; Supplementary Table S2). A decrease could also be observed for the aromatic amino acids tyrosine and phenylalanine (both down to 0.6-fold of the wild-type level). In contrast, levels of arginine and isoleucine increased in most plants (both up to 1.3-fold of the wild-type level). The other amino acids stayed constant in content, although a few exceptions could be observed for threonine (decrease in lines 39 and 40) and aspartate (increase in line 50), and in line 40 (decrease in histidine and lysine).

Does the expression of EcSAT *influence thiol and free amino acid levels in seeds?*

In plants, glutathione is one of the major transport forms of reduced sulphate. Since *EcSAT* was expressed in leaves, transport of cysteine and glutathione to rice seeds was investigated. As seen in leaves, the levels of both the metabolites cysteine and glutathione increased significantly in the five transgenic plants tested (Fig. 4). Cysteine increased up to 1.7-fold (line 51) and glutathione increased up to 2-fold (line 40), indicating that part of the excess thiols was delivered to the sink organ.

It was further tested whether the observed increase in methionine is also reflected in the pool size of free methionine in seeds on the assumption that the excess methionine is not incorporated into proteins in leaves but is delivered from source to sink tissues. The pool sizes of free amino acids were evaluated (Fig. 5; Supplementary Table S3 at JXB online). In the heatmap, amino acids are clustered into two major groups, separating amino acids such as glutamine, alanine, valine, phenylalanine, isoleucine, and leucine, which showed a general elevated level from >1.3-fold up to 2.7-fold in the investigated transgenic lines, from those which remained nearly constant or decreased in content such as glutamate and asparagine. The methionine levels for transgenic lines increased 1.36-fold and 1.44-fold in line 40 and line 51, respectively. Lysine, threonine, and aspartate group together with methionine (Fig. 5). Intriguingly, again the amino acids valine, isoleucine, and leucine group together, as observed in leaves. Tryptophan increased exclusively 2-fold in line 39.

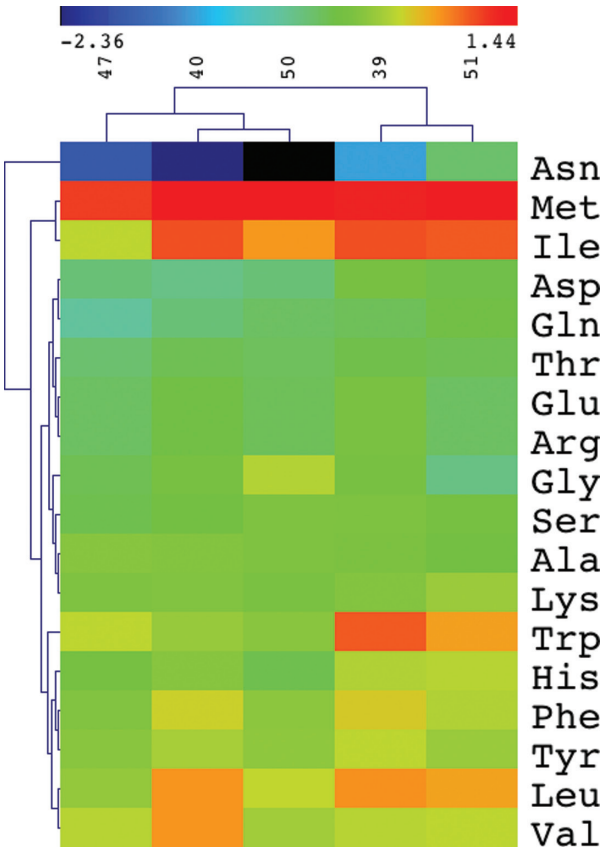

Fig. 2. Heatmap visualization and cluster tree representations of free amino acid contents in leaves and genotypes (lines 47, 40, 50, 39, and 51). Amino acid data were obtained from 8-week-old plants. The heatmap was generated by using $\log_2$-transformed fold changes and normalized to the respective amino acid content in wild-type plants. Each amino acid is represented by a single row and each genotype by a single column. Red indicates increased relative metabolite content, whereas blue indicates decreased relative contents of amino acids compared with the wild type. See Supplementary Table S1 at *JXB* online.

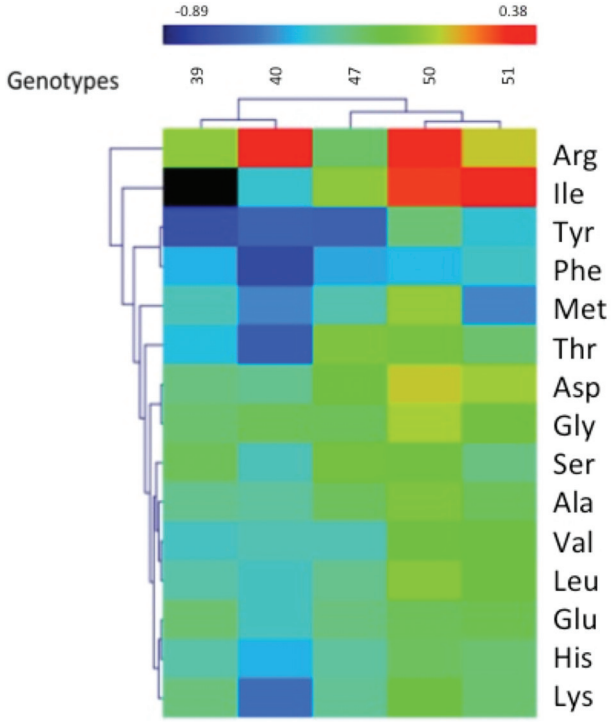

Fig. 3. Heatmap visualization and cluster tree representations of amino acid levels of 8-week-old leaves of wild-type and transgenic rice plants expressing *E. coli* serine acetyltransferase (genotypes: lines 39, 40, 47, 50, and 51). Proteins were hydrolysed and contents were calculated from the amino acids as detected by high-performance liquid chromatography (HPLC). The heatmap was generated by using $\log_2$-transformed fold changes and normalized to the respective amino acid content in wild-type plants. Each amino acid is represented by a single row and each genotype by a single column. The data are presented as the means obtained from four independent measurements. See Supplementary Table S2 at *JXB* online.

Does the increase of free methionine content change the protein-bound amino acid composition in seeds of transgenic SAT lines?

The analysis of the content of free methionine in seeds of transgenic SAT lines revealed an increase in its level. To provide further proof that the genetic manipulation leads to elevated methionine-bound levels, seed protein was extracted and analysed via HPLC analysis. Figure 6 illustrates the content of detectable protein-bound amino acids (data available in Supplementary Table S4 at JXB online). Generally the amino acids cluster in two groups. The first cluster includes tyrosine and methionine, both elevated in their respective level. Methionine levels in particular increased up to 4.7-fold in line 47, 2.7-fold in line 50, and 1.4-fold in line 51, indicating that the excess methionine is indeed incorporated into proteins. Tyrosine increased in line 47 up to 5.3-fold, and 2.1-fold in line 50. The second cluster comprises all other measurable amino acids, revealing a decrease in their respective content. However, in line 47, arginine and threonine tend to increase slightly. Most other amino acids stayed constant or decreased even down to 50% of the respective wild-type level. The data on protein-bound amino acid contents in seeds show that expression of *EcSAT* in rice significantly influenced the amino acid composition in seeds of these transgenic plants.

Discussion

The present results provide evidence that with elevation of the cysteine level, the methionine level also increases. Furthermore, the level of methionine appears to be co-ordinately regulated. Most probably the excess methionine is converted to isoleucine which increases in content in the transgenic plants. Rice plants accumulating excess free methionine due to expression of *EcSAT* show further increased incorporation of carbon from methionine into isoleucine, valine, and leucine. The results presented here support those of previous studies showing that SAT in plants is a rate-limiting factor for cysteine biosynthesis. Expression of SAT can contribute to increased cysteine and glutathione accumulation (Saito *et al*., 1994; Blaszczyk *et al*., 1999; Harms *et al*.,

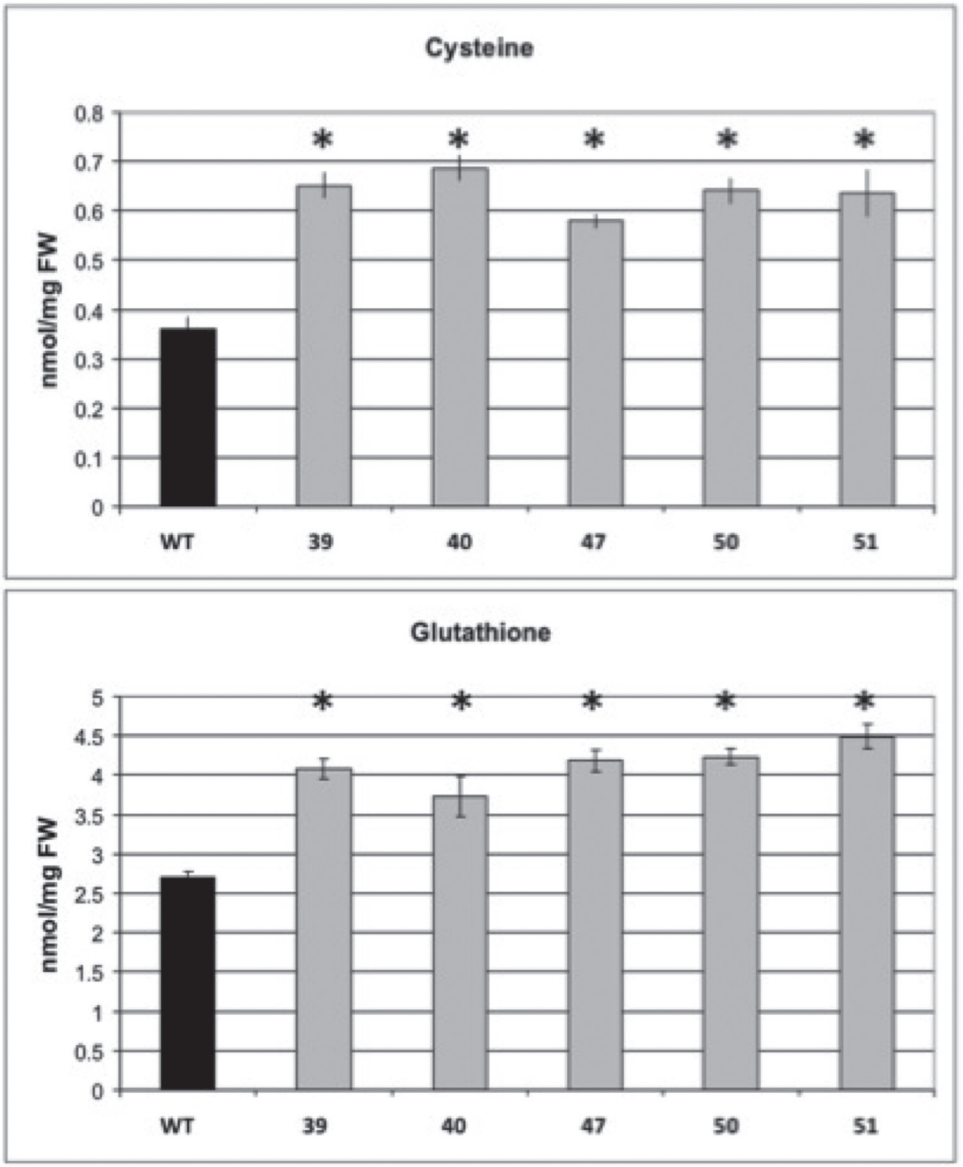

Fig. 4. Contents of cysteine (upper panel) and glutathione (lower panel) in seeds of wild-type and transgenic rice plants expressing *E. coli* serine acetyltransferase. Cysteine and glutathione levels were measured by high-performance liquid chromatography (HPLC). The data are presented as the means ±SE obtained from four independent measurements. Asterisks mark significant differences between wild type and transgenic plant lines (*$P < 0.05$). FW, fresh weight.

2000; Hesse *et al*., 2004*a*; Hopkins *et al*., 2005; Tabe *et al*., 2010). However, the regulatory role of SAT in cysteine biosynthesis probably differs in different plant species. For example, potato plants expressing the same construct showed a high transgene RNA level and up to an 80-fold increase in SAT activity, but the cysteine and glutathione levels differ in content between leaves and tubers and between two potato varieties Désirée and White Lady, respectively (Harms *et al*., 2000; Stiller *et al*., 2007). When EcSAT was expressed in potato tubers exclusively, the level of cysteine and glutathione in tubers increased 3-fold (Stiller *et al*., 2007). In rice even a moderate increase in SAT activity of up to 13-fold caused an elevated OAS steady-state level (up to 13-fold). Consequently, as described before, cysteine and glutathione levels increased up to 2.4-fold probably approaching an internal upper threshold. Intriguingly, OAS accumulated to a high level without conversion to cysteine. This result might indicate that despite elevated OAS levels the sulphur assimilation rate is not influenced and reduced sulphur is not delivered in appropriate amounts. On the other hand, the elevated OAS level might reflect sulphur depletion symptoms due to accelerated flux towards cysteine and methionine synthesis, as observed in seeds of transgenic chickpea plants ectopically expressing a methionine-rich 2S albumin (Chiaiese *et al*., 2004). Here an elevated OAS level was also observed. However, phenotypically no morphological changes or sulphur depletion symptoms were observed for the investigated rice plants (data not shown).

Tabe *et al*. (2010) expressed SAT ectopically in seeds of lupine. They observed up to 5-fold higher concentrations of

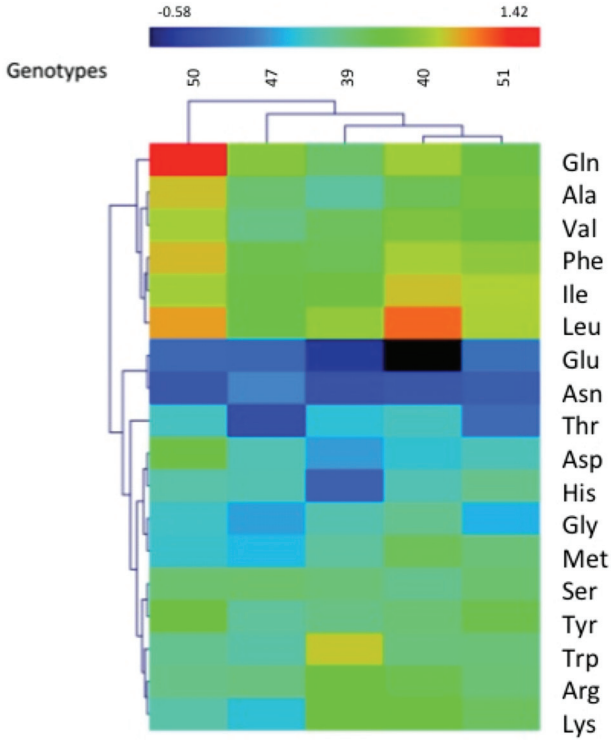

Fig. 5. Free amino acid content of seeds from wild-type and transgenic rice plants. The free amino acid content of seeds is visualized as a heatmap according to the legend of Fig. 2 (genotypes: lines 39, 40, 47, 50, and 51). Each amino acid is represented by a single row and each genotype by a single column. See Supplementary Table S3 at *JXB* online.

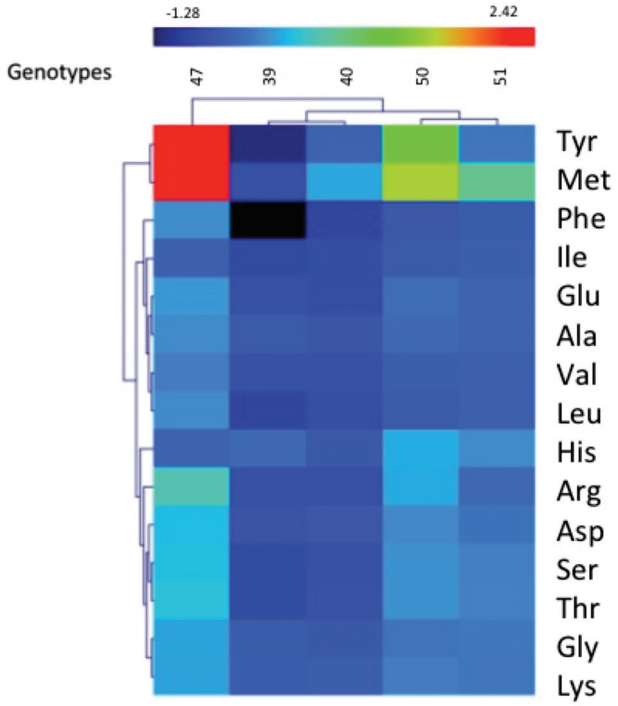

Fig. 6. Content of amino acids incorporated into the soluble protein fraction of seed proteins. The respective amino acid content is visualized as a heatmap according to the legend of Fig. 2 (genotypes: lines 39, 40, 47, 50, and 51). Each amino acid is represented by a single row and each genotype by a single column. See Supplementary Table S4 at *JXB* online.

OAS and up to 26-fold higher cysteine levels in developing embryos. However, an increase in methionine content was not observed. From their data they concluded that cysteine supply is not limiting for methionine biosynthesis in maturing lupine embryos in conditions of adequate sulphur nutrition. In contrast, in the present study, the expression of SAT resulted in up to 2.7-fold higher methionine levels in leaves in comparison with control plants. One reason might be the choice of the plant material. Lupine belongs to the group of legumes which are relatively rich in levels of sulphur-containing compounds in contrast to cereals such as rice (Hesse *et al*., 2004*b*, and references therein). Thus, it might be possible that in transgenic rice plants the availability of cysteine limits the synthesis of methionine. Furthermore, the ectopic expression of SAT might be responsible for the different levels of methionine. While SAT was ectopically expressed in lupine seeds, in rice SAT was constitutively expressed. The highest levels were obtained in rice leaves, while in seeds the methionine level was not changed. However, in seeds, the level of protein-bound methionine was high. This might be caused by the biosynthetic capacity of leaves to synthesize methionine from cysteine in leaves, which is then delivered to seeds and finally incorporated into protein. This finding supports results by Locke *et al*. (1997) who by expressing cystathionine γ-synthase in maize produced up to a 5-fold higher methionine content in seeds, indicating that in cereals SAT activity might limit cysteine biosynthesis and thus methionine in conditions of adequate sulphur nutrition (Amir, 2008).

The approach presented here not only provides a new tool for studying the regulation of cysteine biosynthesis in plants through the expression of a heterologous *EcSAT*, but may also open up a new avenue for the breeding of crop plants with a more balanced level of this essential amino acid. Transgenic plants produced in this study exhibiting higher cysteine levels had significantly higher levels of both soluble and bound methionine, as well as increased levels of isoleucine, valine, and leucine. Partially, transgenic lines had higher contents of aromatic amino acids. However, transgenic lines differed in their soluble and bound contents of methionine and cysteine, as well as in the proportions between methionine, and protein-bound methionine. This suggests that different plants accumulate sulphur compounds in a slightly different manner, probably reflecting small changes in their response to growth conditions. It is intriguing that levels of other aspartate-derived amino acids (except isoleucine) did not change significantly. In particular, threonine remained relatively constant while the isoleucine level increased significantly, indicating that the conversion pathway from threonine to isoleucine is not used for isoleucine synthesis alone. It is likely that methionine γ-lyase (EC 4.4.1.11), an enzyme that converts methionine to methanethiol and 2-ketobutyrate, which has been studied extensively in microbes and protozoa (Inoue *et al*., 1995; Faleev *et al*., 1996; Hori *et al*., 1996; Dias and Weimer, 1998; McKie *et al*., 1998; Tokoro *et al*., 2003; Manukhov *et al*., 2005),

might be responsible. More recently, methionine γ-lyase activity has also been demonstrated in plants. Nuclear magnetic resonance (NMR) metabolite profiling of *Arabidopsis thaliana* cell suspension cultures labelled with [^{13}C]methionine showed that methionine γ-lyase produces 2-ketobutyrate for isoleucine biosynthesis (Rebeille *et al*., 2006). Details of the contribution of methionine γ-lyase to the degradation of methionine have been published recently (Joshi and Jander, 2009; Jander and Joshi, 2010). The presence and role in regulating the methionine content might explain the up to 2-fold elevated isoleucine levels due to the increased internal methionine content. This assumption is supported by published research providing indirect evidence that there is significant metabolic flux from methionine to isoleucine in plants under certain conditions. Tubers from potato plants expressing antisense threonine synthase accumulated elevated amounts of methionine and isoleucine, without any apparent change in threonine content (Zeh *et al*., 2001), suggesting threonine-independent isoleucine synthesis. Similarly, overexpressing cystathionine γ-synthase, the committing enzyme of methionine biosynthesis, increased both methionine and isoleucine content of potato tubers (Dancs *et al*., 2008). A 7-fold increase in the isoleucine level in transgenic tobacco plants expressing bacterial feedback-insensitive aspartate kinase and methionine-insensitive *A. thaliana* cystathionine γ-synthase genes was suggested to result from methionine degradation (Hacham *et al*., 2008). The increase in isoleucine might also cause the increased levels of valine and leucine. In plants, isoleucine, leucine, and valine share four common enzymes in their biosynthesis pathways and thus are co-ordinately regulated (Joshi *et al*., 2010). The degradation product of methionine, 2-ketobutyrate, can be readily used as a precursor for the synthesis of isoleucine and through the co-ordinated regulation of the branched chain amino acids leucine and valine as well increased in content in transgenic rice plants (Höfgen *et al.,* 1995).

There are potential practical applications to take advantage of the observed co-regulation of methionine and isoleucine levels in crop plants. Since isoleucine abundance in some of the world's major food crops, including maize and rice, is suboptimal for mammalian diets (Lewis *et al*., 1982; Prakash, 1996), increasing the content of essential amino acids in seeds and other edible plant parts has been a goal for breeding and agricultural biotechnology. Thus, expressing *EcSAT* in rice demonstrates that it is possible to increase not only the pool sizes of cysteine and glutathione but also the content of soluble and bound methionine and isoleucine, two important essential amino acids, without this being at the expense of the content of other essential amino acids. According to the World Health Organization (WHO) (cited by Shewry, 2000), the level of these two amino acids should be increased; for example, the methionine content in legumes should be increased from about 2 g 100 g^{-1} protein to 3.5 g 100 g^{-1} protein. Considering that rice seeds contain 8–15% of crude protein compared with other cereals (http://www.fao.org/inpho/content/documents//vlibrary/t0567e/T0567E0g.htm#Protein%20requirements%20of%20preschool%20children%20and%20adults%20on%20rice%20diets; Shewry and Halford, 2002), the percentage methionine increased from 0.6% to ~3.0% in the transgenic line 47 (calculated according to the equation described in the Materials and methods). Thus, one can conclude that the transgenic plants produced in this work contain adequate methionine content to feed animals without requiring any additional synthetic methionine. Thus, expression of *EcSAT* could be an appropriate approach to improve the nutritional quality of rice plants in particular, and of other forage crops in general.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. The level of free amino acids in the leaves of the wild type and rice plants expressing E. coli serine acetyltransferase.

Table S2. The level of bound amino acids in the leaves of the wild type and rice plants expressing E. coli serine acetyltransferase.

Table S3. The level of free amino acids in the seeds of the wild type and rice plants expressing E. coli serine acetyltransferase.

Table S4. The level of bound amino acids in the leaves of the wild type and rice plants expressing E. coli serine acetyltransferase.

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