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Production of cysteine for bacterial and plant biotechnology: Application of cysteine feedback-insensitive isoforms of serine acetyltransferase

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Summary. The first step of cysteine biosynthesis in bacteria and plants consists in the formation of O-acetylserine catalyzed by serine acetyltransferase (SAT). SAT is highly sensitive to feedback inhibition by cysteine as part of the regulatory circuit of cysteine biosynthesis und thus hampers over-expression and fermentation of cysteine in biotechnological production processes. Since plants contain multiple SAT isoforms with different cysteine feedback sensitivity, this resource was exploited to demonstrate the suitability of plant SATs for the production of cysteine in both bacteria and plants. Three new cDNAs encoding SATs were isolated from *Nicotiana tabacum*. The catalytic activity of SAT4 was insensitive up to 0.6 mM cysteine. Expression of SAT4 in a newly constructed *Escherichia coli* host strain without endogenous SAT activity yielded a significant accumulation of cysteine in the culture medium compared to expression of cysteine sensitive SATs in the same strain. The application of a similarly insensitive SAT isoform from *A. thaliana* demonstrated the suitability of this approach to increase cysteine levels in transgenic tobacco plants.

Keywords: Plant biotechnology – Bacterial fermentation – Sulfur amino acids – Gene cloning

Abbreviations: OAS, O-acetylserine; OAS-TL, O-acetylserine (thiol) lyase; SAT, serine acetyltransferase

Introduction

A two-step process accomplishes cysteine biosynthesis in bacteria and plants. Serine-acetyltransferase (SAT; EC 2.3.1.30) forms the activated thioester O-acetylserine (OAS) from serine and acetyl-coenzyme A. Free sulfide is inserted into OAS to yield cysteine and acetate by catalysis of O-acetylserine (thiol) lyase (OAS-TL; EC 4.2.99.8). SAT represents the rate-limiting component and its activity is exclusively found in association with OAS-TL in the cysteine synthase complex. OAS-TL is present in large excess due to the activity of SAT-free homodimers (Kredich et al., 1969; Droux et al., 1998). Cysteine constitutes the almost exclusive metabolic entrance for reduced sulfur into cell metabolism where it is required for biosynthesis of essential compounds including methionine, several vitamins and Fe/S clusters (Saito, 1999). The production of cysteine is therefore of biotechnological interest for pharmacological processes and as a nutritional supplement for food and feed. It can be produced in large quantities by chemical synthesis, extraction from animal sources like keratin and, more recently, by bacterial fermentation. Production of cysteine in plants is aimed on one hand on agronomical traits such as nutritional quality, on the other hand on production of pharmaceuticals and bioactive substances for plant defence (Hoefgen et al., 2001).

Large-scale production of cysteine in micro-organisms is mainly hampered by intrinsic regulatory mechanisms of the cys-regulon (Kredich, 1996) as well as cysteine toxicity at elevated concentrations (Soerensen and Pedersen, 1991). Several approaches have been undertaken to circumvent these limitations. High yield of cysteine in the growth medium may be achieved by over-expression of an export pump for different metabolites of the cysteine pathway as demonstrated for the *ydeD* gene product from *E. coli* (Daßler et al., 2000). Inhibition of constitutively expressed *E. coli* SAT (CYS E protein encoded by the *cysE* gene) by cysteine is essential for the function of the cys-regulon. It is characterized by a $K_i^{cysteine}$ of about 10^{-6} M in *Salmonella typhimurium* and *E. coli* (Kredich et al., 1969; Kredich, 1996) and thus effec-

tively controls the flux rate of reduced sulfur in a feedback loop. It has been attempted to overcome this mechanism by searching for cysteine insensitive SATs from *E. coli* by random mutagenesis (Denk and Böck, 1987; Takagi et al., 1999b). Alternatively, site-directed mutagenesis was applied to modify amino acid positions near those obtained from random screenings (Nakamori et al., 1998). It was possible to identify up to 15-fold less sensitive feedback mutants as defined by half-maximal inhibition (I_{50}) kinetics and to achieve considerable concentrations of cysteine and cystine in the growth medium. Further and more focused improvement seems difficult, due to the lack of a crystal structure of SAT and insufficient sequence information of cysteine-insensitive SAT forms from other organisms.

An alternative approach makes use of the natural resources of SAT proteins provided by higher plants. They are structurally closely related to the bacterial enzyme and are represented by nuclear encoded isoforms that are targeted to plastids, cytosol and mitochondria. These plant SATs can differ considerably in their feedback-sensitivity to cysteine between compartment-specific isoforms (Noji et al., 1998) as well as between plant species (Urano et al., 2000). They are usually determined via heterologous expression in *E. coli* (Noji et al., 1998; Inoue et al., 1999; Saito, 1999), where they form an active cysteine synthase complex with bacterial OAS-TL (Droux et al., 1998). Domains responsible for cysteine inhibition of plant SATs have been mapped in analogy to *E. coli* SAT (1998; Inoue et al., 1999). A first report shows that application of cDNAs from *Arabidopsis thaliana* encoding organelle-localized SATs results in efficient accumulation of cysteine in the medium of *E. coli* (Takagi et al., 1999a).

In this study we present the successful screening for new cDNAs encoding cysteine-insensitive SAT isoforms from *Nicotiana tabacum* and the suitability of this approach to improve cysteine production in bacteria and plants. Isoform SAT4 from tobacco exceeded the existing SATs with respect to feedback-insensitivity by far and caused a strong accumulation of cysteine in the growth medium *of E. coli* as a host. Over-expression of the insensitive SAT isoform A (Bogdanova et al., 1995; Noji et al., 1998) from *A. thaliana* in tobacco plants created a significant increase of cysteine levels in leaves.

Material and methods

DNA cloning, analysis and plant transformation

General cloning and PCR procedures were carried out according to Sambrook et al. (1989). DNA sequences were obtained with a 373A sequencer (Perkin-Elmer). The *N. tabacum* cv. Samsun cDNA library was generated from source leaves in λZAPII and generously provided by U. Sonnewald, IPK Gatersleben. *In vivo* excision followed the protocol of the supplier (Stratagene). Medium expression levels of SAT A from *A. thaliana* (EMBL accession no. X80936) were achieved with the pBluescript (Stratagene) based plasmid pBS-ΔSAT1-6 (Bogdanova et al., 1995). Low expression levels of SAT A were mediated by plasmid pACYC-ΔSAT1-6 that was constructed by ligation of the 1.3 kb *Eco*RV fragment from pBS-ΔSAT1-6 into the *Eco*RV site of pACYC. Strong expression of *A. thaliana* OAS-TL A was provided by plasmid pEXP-OAS A (Hell et al., 1994).

Southern analysis of *E. coli* genomic DNA was carried out as described (Hell et al., 1994) with the 2.2 kb *cysE* fragment (see below) as a probe. DNA sequences were analyzed and compiled with the MacVector/AssemblyLign software (IBI Kodak) and DNAStar (Lasergene). The evolutionary tree was constructed from amino acid sequences of mature proteins without signal peptides after translation from the nucleotide sequence using CLUSTALX v. 1.64b and DNAStar.

For plant transformation SAT A cDNA without mitochondrial transit peptide was cloned as described above behind the cauliflower mosaic virus promoter into the *Bam*HI/*Sal*I restriction sites of pBIN-AR (Höfgen and Willmitzer, 1992) and its derivative pBINAR-TkTp equipped with the transit peptide of plastid transketolase from tobacco (R. Badur, PhD Thesis, University of Göttingen, 1998), both generously provided by U. Sonnewald, IPK Gatersleben. Transformation of *Agrobacterium tumefaciens* C58 with binary vectors and subsequent transformation and selection of tobacco (*Nicotiana tabacum* cv. SNN) were carried out as described by Henkes et al. (2001).

Insertional mutagenesis and complementation of E. coli

Insertional inactivation of the wild-type *cysE* gene from *E. coli* C600 (*thr leu thi lac* (λ)-P1+F′; Clontech) was carried out according to Hamilton et al. (1989). The *cysE* gene including its flanking regions was amplified by PCR with genomic DNA from strain C600 and primers ECS155 (5′-CGTGGATCCTTAGGCGATCAAATTCC-3′) and ECS156 (5′-GGGGAGTCGACGGCGCTGTATGTACT CCCT-3′). The resulting 2.2 kb DNA fragment was ligated into *Sal*I and *Bam*HI sites of pUC18. In this plasmid the *cysE* gene was restricted by *Cla*I at position 522 relative to the open reading frame and inactivated by insertion of a 2.2 kb *Cla*I/*Cla*I fragment from pACYC184-Gm (generously provided by W. Klipp, Microbiology Dept., Ruhr-Universität Bochum, Germany) that carried a gentamycin resistance gene, yielding plasmid pUC18*cysE*-Gm. Thus, at most 174 amino acids of the *E. coli* SAT can be translated. The *cysE*-Gm cassette was excised with *Sal*I and *Bam*HI as a 4.4 kb fragment and ligated into the same sites of pMAK705 that carries a kanamycin resistance gene and a temperature-sensitive origin of replication (pHO1; Hamilton et al., 1989; generously provided by R. Eichenlaub, Dept. Microbiol., Universität Bielefeld, Germany). The resulting plasmid (pMW1) was used for gene replacement via homologous recombination. Strain C600 was transformed with pMW1 to select for Cm resistant colonies at non-permissive temperature (44°C). Cointegrates were cleaned from plasmid first by growth under permissive conditions (30°C) followed by non-permissive conditions and the resulting cysteine auxotrophic strain was termed MW1 (*thr leu thi lac* (λ)-P1+F′ *cysE* Gm^R).

Strain *E. coli* NK3 represents a cysteine auxotrophic double mutant (Δ*trpE5 leu6 thi hsdR hsdM cysK cysM*) generously provided by N. Kredich, Duke University, USA). The hitherto unknown molecular nature of the mutations was elucidated by PCR cloning of the *cysK* and *cysM* genes of NK3 using gene-specific primers. Both structural genes were fragmented by numerous random DNA deletions that completely and stably destruct any open reading frame of these genes (data not shown). NK3 is thus devoid of OAS-TL activity and was transduced with MW1 to integrate the inactivated *cysE* gene. MW1 cells were lysed by phage P1kc and the phage lysate was employed to transduce NK3. Colonies with the inactivated *cysE* gene were selected on gentamycin containing medium and yielded the cysteine auxotrophic triple mutant strain MW2 (Δ*trpE5 leu6 thi hsdR hsdM cysK cysM cysE* Gm^R).

Complementation and auxotrophy assays were carried out on solid M9 minimal medium supplemented with IPTG (1 mM), ampicillin (100 μg/ml), gentamycine (50 μgm/l), thiamine (0.1 mM) and 1 mM of each proteinogenic amino acid except methionine and cysteine. Single plasmids or the tobacco cDNA library were electroporated (BioRad) into MW1 or MW2 and colonies selected by incubation at 37°C for up to 48 hours.

Protein expression analysis and enzyme assays

Expression of SATs in all constructs was induced with isopropylthiogalactoside in full (LB) or minimal medium (M9) supplemented with ampicillin or gentamycin or both as described (Bogdanova et al., 1995; Wirtz et al., 2000). After collection of cells and lysis by french press the soluble supernatant (10 min at 30.000 × g) was desalted by gel filtration on a PD 10 column (Amersham) and kept at −80°C until further use. SAT A from *A. thaliana* was essentially obtained according to Droux et al. (1998) after S-tag affinity purification. SAT activity from purified or crude fractions was assayed in 250 μl containing 50 mM Tris-HCl pH 7.5, 0.2 mM acetyl-Coenzyme A, 2 mM dithiothreitol and 5 mM serine in the presence or absence of varying cysteine concentrations as indicated. Absorbance at 232 nm was recorded for several minutes according to Kredich and Becker (1971). SAT and OAS-TL acitivities from crude plant and bacterial protein extracts were determined according to Nakamura et al. (1987). Kinetic analysis was performed using Sigma Plot software, allowing hyperbolic fits that were based on the Michaelis-Menten equation ($v = (K_M + [S])/v_{max} \times [S]$). Determination of protein concentrations, SDS-PAGE separation, immunoblotting followed standard protocols (Sambrook et al., 1989). Polyclonal antibodies against SAT A from *A. thaliana* were raised in rabbits by established procedures.

Quantification of thiols

Cysteine and glutathione were determined from bacterial culture media or plant tissue after extraction with 0.1 N HCl in a 1 : 5 ratio. After reduction with dithiothreitol the sulfhydryl groups were derivatized with monobromobimane (Calbiochem). Separation, detection and quantification of fluorescent adducts was achieved by a reversed-phase column (Waters Nova-Pak C18, 4.6 × 250 mm) and a Waters HPLC system as described (Hell and Bergmann, 1990). Tobacco plants were grown in soil under standard greenhouse conditions and leaves of six-week-old plants were harvested for analysis.

Results

Construction and verification of cysteine auxotrophic E. coli *mutant strains*

Screening for genes in functional complementation assays largely relies on the stability of the mutation used for selection in the host organism. This is particularly the case for heterologous complementation between distant taxa and the requirement of large numbers of transformants when the donor organism is of high genomic complexity. In order to efficiently screen for plant cDNAs involved in cysteine biosynthesis, two new mutant strains of *E. coli* were created that showed virtually no reversion of phenotype. First, the *cysE* gene of *E. coli* strain C600 was inactivated by insertional mutagenesis using a gentamycin resistance cassette, yielding *E. coli* MW1. Second, the existing *E. coli* strain NK3 that is mutated in the OAS-TL A and B genes *cysK* and *cysM* was transduced with MW1, resulting in the cysteine auxotroph and triple mutant strain MW2. No revertants of both strains were observed in any experiment, in contrast to experimental experience with existing mutant *cysE* strains such as JM15, JM39 and EC1801 (*E. coli* Stock Center, Yale University, USA). Consequently, the screening for components of the cysteine synthesis pathway from heterologous sources could be carried out under any condition without interference of unwanted genetic events. The functional identity of these genotypes was verified 1) biochemically by enzyme assays, 2) genetically by DNA-DNA hybridization and 3) physiologically by complementation with known plant cDNAs.

Strain MW1 showed no detectable SAT activity but retained 47% of the wild-type OAS-TL activity (Table 1). This reduction appeared to be a consequence of the lack of induction of *cysK/cysM* induction by OAS in the cys-regulon. The identity of MW1 and MW2 strains was demonstrated by complementation with *A. thaliana* cDNAs encoding SAT and OAS-TL, respectively, on minimal medium without cysteine (Fig. 1A). SAT deficiency in MW1 was functionally complemented by low and intermediate expression levels of SAT A using vectors with different copy numbers. Lack of SAT and OAS-TL activity could only be complemented by co-expression of SAT and OAS-TL using vectors with different origins of replication but not by SAT or OAS-TL alone. No false positive colonies were observed in several repetitions of the experiment. Finally, hybridization of genomic DNA from wild-type strain C600, MW1 and MW2 with the *cysE*

Table 1. SAT and OAS-TL activities of *E. coli* strains used in this study. Compared were wild type, auxotrophic cysteine synthesis mutant strains and mutant strains complemented with plasmid-encoded plant cDNAs. Mean values ± standard errors of 3 independent preparations of desalted protein extracts with 4 repetitions each are given

E. coli strain (genotype)	SAT [nmol/min mg protein]	OAS-TL [nmol/min mg protein]
C600 (*cysE*$^+$/*cysK*$^+$/*cysM*$^+$)	2.50 ± 0.41	1,100 ± 400
MW1 (*cysE*$^-$/*cysK*$^+$/*cysM*$^+$)	0	520 ± 70
MW2 (*cysE*$^-$/*cysK*$^-$/*cysM*$^-$)	0	0
MW1 (pBS-SAT A)	3,760 ± 810	–
MW2 (pACYC-SAT A + pEXP-OAS A)	46.0 ± 9.0	46,300 ± 7,030

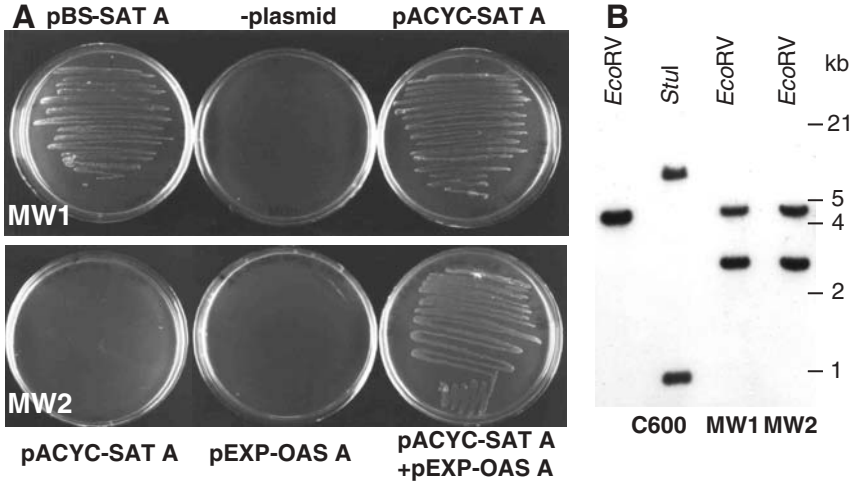

Fig. 1. Functional and genomic verification of cysteine synthesis deficient *E. coli* strains. **A** MW1 (*cysE*$^-$) and MW2 (*cysE*$^-$/*cysK*$^-$/*cysM*$^-$) were transformed with the plasmids indicated and cultured on plates containing minimal medium without reduced sulfur source. Cysteine auxotrophy was complemented by high and low copy number plasmids pBS and pACYC. **B** Southern blot of genomic DNA from wild-type (C600), MW1 and MW2 strains hybridized with a full-length *cysE* probe

gene as a probe confirmed the positional basis of the homologous recombination (Fig. 1B). Restriction with *Eco*RV that cuts outside *cysE* and with *Stu*I that cuts within the *cysE* gene yielded a 4.6 kb and 7.4/1.0 kb signals, respectively, as predicted from the *E. coli* genome map around 81.44 min. Insertion of the 2.2 kb gentamycin-cassette would increase the *Eco*RV genomic fragment to 6.8 kb. But an internal *Eco*RV site in the cassette gives rise to signals of 4.7 kb and 2.1 kb in DNA-DNA hybridization, confirming the location, orientation and identity of the insertion. The data provide proof-of-function for the implementation of the two-step process of plant cysteine synthesis into *E. coli* without any endogenous background. MW1 consequently will allow screening for large numbers of SAT encoding cDNAs by complementation with expression libraries from various sources (see next section). Furthermore, the triple-mutant MW2 provides the possibility to optimize cysteine production using combinations of SATs and OAS-TLs from different origins by simultaneous complementation using plasmids with compatible origins of replication.

Functional screening for cysteine insensitive SATs from tobacco

A cDNA expression library prepared from *Nicotiana tabacum* leaf mRNA was transformed into MW1 cells. Prototrophic colonies were selected on minimal medium without organic sulfur source. Positive selection of the plasmids contained was verified by retransformation into MW1 and plating on minimal medium (data not shown). Six strains prototrophic for cysteine remained and were tested positive for SAT enzymatic activity. DNA sequencing revealed three independent cDNAs (Fig. 2A). Since the presence of 10 μM cysteine is sufficient to completely inhibit bacterial SAT activity, protein extracts of the three strains were assayed again to distinguish between feedback sensitive and insensitive SAT forms. Inhibition be-

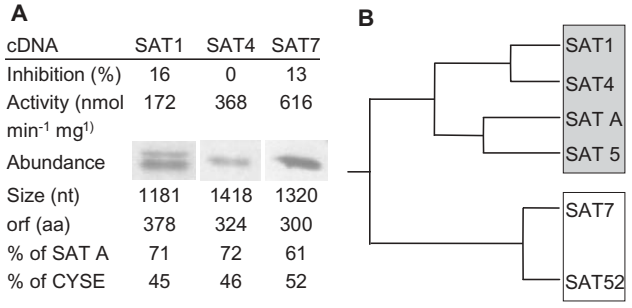

Fig. 2. Properties of SAT cDNAs and their encoded proteins from tobacco. **A** Characteristics include inhibition of activity (%) by 10 μM cysteine assayed from crude desalted protein extracts, protein levels in *E. coli* shown by immuno-blotting (see Fig. 5) and amino acid identity to *A. thaliana* SAT A and *E. coli* CYS E. **B** Evolutionary tree of *N. tabacum* SAT1 (EMBL accession AJ414051), SAT4(AJ414052) and SAT7 (AJ414053) together with presumably cytosolic SAT52 (U30298) and organelle-localized SAT5 (Z34888) as well as SAT A (X82888) from *A. thaliana*

tween 0 and 18% was observed, indicating that all encoded proteins belong to the insensitive group of SATs.

The cDNA inserts of SAT1, 4 and 7 varied between 1,182 and 1,418 bp (Fig. 2A), wherein SAT4 and SAT7 displayed unusually long 3′ untranslated regions of 454 and 438 bp, respectively. DNA sequences were deposited in the EMBL database as accessions AJ414051 (SAT1), AJ414052 (SAT4) and AJ414053 (SAT7). Start codons were present near the 5′-ends but since no stop codons were found in the sequences upstream it cannot be excluded that some of the clones were not full-length. Alignment of the derived amino acid sequences with SAT1 (Roberts and Wray, 1996, U22964; equivalent to SAT A, X80938) representing the longest known open reading frame of a SAT, and *E. coli* SAT (CYS E; Denk and Böck, 1987) revealed significant homology. In particular, the C-terminus was highly conserved that contains the domains for transferase activity, SAT/OAS-TL protein-protein interaction and essential residues for feedback inhibition. The putative acetyl-CoA binding motif DRH (Wirtz et al., 2001) at position 309 and the methionine residue involved in feedback-sensitivity at position 377 relative to SAT A (X80938) were conserved in all three tobacco SATs (not shown). Interestingly, the cysteine-insensitive SAT4 differed from all known SATs at two positions: 1, an insertion of two cysteine and serine residues at position 190 of the variable region and 2, a three amino acid deletion at position 380 close to the otherwise very conserved C-terminal end (relative to SAT A; data not shown). SAT 7 comprised only the minimal size required for SAT activity as defined by the bacterial sequence (Bogdanova and Hell, 1997), while SAT1 and 4 carried N-terminal extensions that may function as organelle targeting peptides.

A phylogenetic tree of the three tobacco proteins and three *A. thaliana* SATs was based on the core size of the proteins to eliminate the influence of sequence length and putative transit peptides (Fig. 2B). Indeed, tobacco SAT1 and SAT4 were closest related to the putative mitochondrial and plastid localized *A. thaliana* isoforms SAT A and SAT3 (Noji et al., 1998), while tobacco SAT7 was more similar to cytosolic SAT52 (Howarth et al., 1997) from *A. thaliana*. Northern analysis showed that all three SATs were expressed at similar levels in leaves of tobacco. Estimated mRNA sizes were 1,300 bp (SAT1), 1,500 bp (SAT4) and 1,400 bp (SAT7), indicating that the isolated cDNAs were close to full-length size.

Strains carrying SAT 1, 4 and 7 from tobacco were further analyzed in detail for the relationship between specific activity and the concentration of cysteine (Fig. 3). Determination of activity in the presence of up to 0.6 mM cysteine revealed graphical functions that could be modelled using the Michaelis-Menten equation for SAT 1 and 7, yielding I_{50} constants in the order of 50 μM cysteine. Both kinetics corresponded well to that of SAT A, the mitochondrial SAT isoform from *A. thaliana* that had previously been defined as

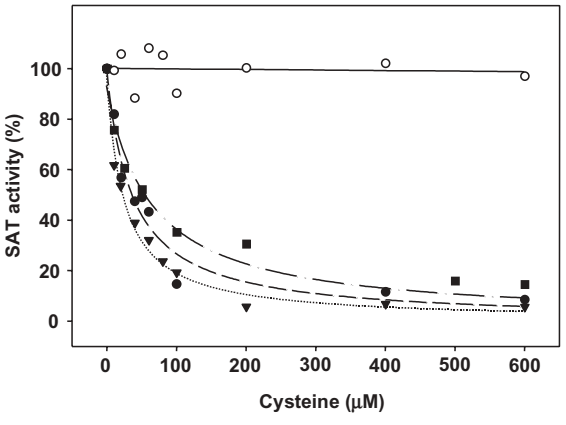

Fig. 3. Inhibition of tobacco SATs by cysteine. cDNAs encoding *N. tabacum* SAT1 (●), 4 (○) and 7 (▼) were expressed in *E. coli* strain MW1 and crude desalted protein extracts were assayed for enzymatic activity in the presence of increasing cysteine concentrations. 100% activity refers to the specific values given in Fig. 2. Inhibition of purified *A. thaliana* a SAT A protein (■) is shown for comparison. Each data point represents 3 repetitions

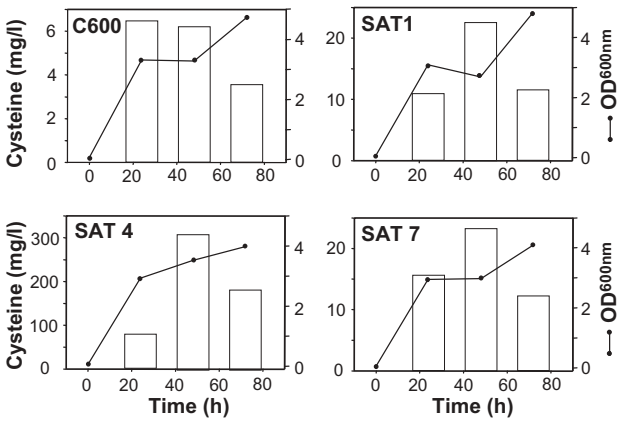

Fig. 4. Accumulation of cysteine in *E. coli* cultures expressing tobacco SATs. *N. tabacum* SAT 1, 4 and 7 were expressed in strain MW1. Cell density (black dots) and cysteine levels in the culture medium were determined during growth as indicated. Each data point given represents the mean value obtained from two independently conducted experiments

cysteine insensitive (Noji et al., 1998). Most importantly, however, SAT4 could thus not be described with kinetic terms, since it was not inhibited by cysteine at all. These analyses were based on comparable specific activities and protein abundance in immunoblots of the tobacco SATs (Fig. 2A). Taken together, the data demonstrate that new improved SAT isoforms with respect to reduced feedback inhibition can be selected from plants.

Production of cysteine in bacteria

The suitability of the isolated tobacco SATs was tested for production of cysteine by *E. coli* fermentation. Since a membrane-bound facilitator protein mediates efficient efflux of cysteine, the release of cysteine into the surrounding growth medium as a result of overproduction by the heterologous expression of SATs was analysed during growth of MW1 in sulfur-rich culture medium (Fig. 4). The growth curves of all three SAT strains were comparable to wild-type *E. coli* C600. SAT 1 and SAT 7 caused only 3–4-fold improvement of cysteine accumulation in the medium. Expression of SAT4, however, resulted in about 50-fold increase compared to wild type and reached 300 mg total cysteine per liter medium. Changes in the accumulation of cysteine during the 80-hour culture period are probably due to still insufficient sulfate supply and the consequent uptake of reduced sulfur compounds to sustain growth during prolonged cultivation. Glutathione was also found in the medium in concentrations similar to cysteine (data not shown). The data therefore demonstrate that the selection of highly active and cysteine-insensitive SATs from plant is a suitable way to improve cysteine fermentation.

Expression of feedback-insensitive SAT in tobacco

A complementation to food and feed additives is the direct augmentation of amino acids in plants. The strategy of over-expression of a feedback-insensitive SAT was therefore applied to tobacco as a model for transgenic plants. The cDNA encoding SAT A from *A. thaliana* is cysteine insensitive and was selected because its enzymology and subcellular localization is well characterized (Noji et al., 1998; Wirtz et al., 2001) and because of the low risk of co-suppression effects in tobacco as compared to the homologous over-expression of tobacco SAT4. SAT A was constitutively expressed under the control of the cauliflower mosaic virus 35S promoter using two constructs: cytosolic over-expression without fusion peptide and plastid targeting by fusion with a transketolase transit peptide. Young leaves of two independent transgenic tobacco lines of the second generation after transformation were analysed for SAT and OAS-TL total activities and compared to wild-type grown under the same conditions (Fig. 5A). SAT activities were increased between 10- and 40-fold in cytosolic and plastid over-expression lines, indicating successful expression of both transgenes. Enzymatic activities correlated with transgenic SAT A abundance as shown by immuno-blotting (Fig. 5B). The presence of two protein bands in plastid constructs probably resulted from incomplete processing of SAT A during chloroplast import due to overload of the import system. At the same time this finding supports the correct targeting of the SAT A fusion protein. As expected, the activity of OAS-TL was much higher than that of SAT and was not significantly affected in any transgenic line as compared to wild-type plants.

Accumulation of cysteine in transgenic tobacco plants

The accumulation of cysteine as a consequence of the strongly enhanced SAT activities in cytosol and plastids in leaves of transgenic tobacco plants was analysed (Fig. 6). The transgenic plants showed a wild-type phenotype (not shown). The variation of cysteine accumulation between the three tested individuals

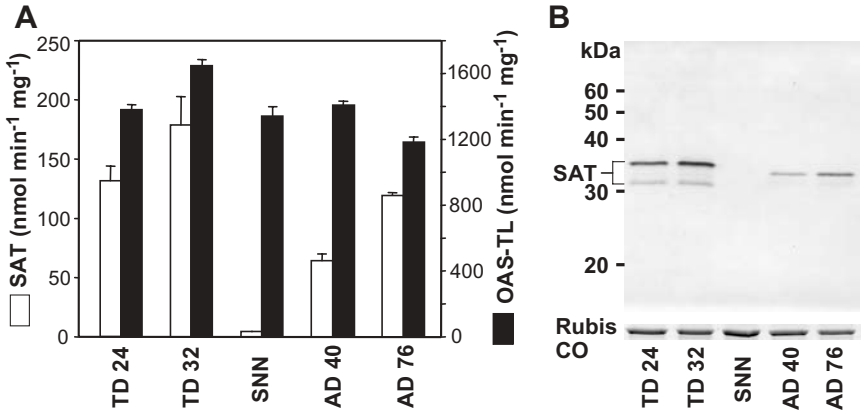

Fig. 5. **A** Enzymatic activity of SAT and OAS-TL in leaves of transgenic tobacco plants. Specific activities of two independent T2 generation lines transformed with *A. thaliana* SAT-A either targeted to the plastid (*TD*) or to the cytosol (*AD*) compared to non-transformed controls (*SNN*). Open bars: SAT; black bars: OAS-TL. Shown are mean values from 3 determinations. **B** Immuno-blot of tobacco leaf extracts decorated with anti-SAT A polyclonal antibody. The same blot was decorated with an antibody against ribulose-1,5-bisphosphate carboxylase/oxygenase (*RubisCO*) as a loading control. In all cases crude protein extracts were assayed after desalting

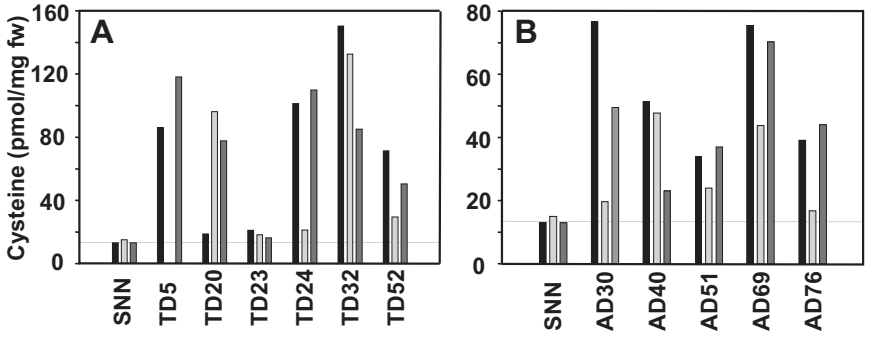

Fig. 6. Cysteine accumulation in leaves of transgenic tobacco plants. Binary constructs of *A. thaliana* SAT A cDNA targeted the enzyme (**A**) to the plastid or (**B**) to the cytosol. Bars indicate individual T2 generation plants from each line. Non-transformed controls (SNN) are shown for comparison

from each line suggested that at least lines TD20, TD24, AD30 and AD76 were not homozygous for the transgene in the T2 generation. This observation is corroborated by the segregation patterns of these lines in germination experiments in the presence of kanamycin (not shown). On average, cysteine contents were elevated by 3-fold cytosolic and 6-fold by plastid targeting of SAT A. Strongest accumulation was reached by plastid constructs with 10-fold cysteine levels compared to wild-type grown under the same conditions. Glutathione contents were also increased in all transgenic lines. Wild-type tobacco leaves contained 423 $\pm$ 48 nmol glutathione per g freshweight. The relative increases varied considerably between the transgenic lines but were 3-fold on average for both targeted compartments (data not shown). The enzymatic activity of transgenic SAT exceeded the increase of cysteine contents in all cases, indicating that factors other than OAS-TL might have limited further cysteine formation under these conditions. It is concluded that expression of plant SAT proteins with native reduced sensitivity to cysteine is a useful strategy to improve cysteine formation in bacterial cultures as well as in plants.

Discussion

SAT has an essential role in the biosynthesis of cysteine in bacteria in plants. For the production of cysteine either as free amino acid or bound in protein, the intrinsic feedback inhibition of SAT by cysteine has to be circumvented. The improvement of biotechnological methods for cysteine production therefore requires feedback-insensitive SAT proteins with high specific activity. Using tobacco and *A. thaliana* as model systems we show that plants provide a source of SAT proteins with potential for biotechnological use in both bacteria and plants.

The construction of *E. coli* strains either without endogenous SAT or without SAT and OAS-TL activities allows the single or combined selection of plant cDNAs encoding this enzyme free of any background and is essentially applicable to all genetic resources. *E. coli* strain MW2 was developed as a proof-of-concept for metabolic engineering of the two-step pathway of cysteine synthesis in bacteria. It will enable the implementation, screening and optimisation of cysteine synthesis genes from bacterial and plant origin in homologous or heterologous combinations. Strain MW1 provided the tool to isolate three new SAT cDNAs from tobacco, of which SAT4 exhibited complete insensitivity up to 0.6 mM cysteine. This exceeded the properties of all known native SAT isoforms from *A. thaliana* and other plants (Noji et al., 1998; Saito, 1999; Urano et al., 2000) by far as indicated by the direct comparison of homogenously purified mitochondrial SAT A from *A. thaliana* (Wirtz et al., 2001). If functional conservation exists between SAT isoforms from different plant species, this might suggest that tobacco SAT4 is localized in chloroplasts or mitochondria. Its association with organelle-localized SATs in a phylogenetic tree and its large mRNA size support this assumption. Despite high overall amino acid identity, SAT4 differed from all known SATs by two unique amino acid insertions and deletions, respectively. These positions might be important for future analysis to understand the mechanism of cysteine inhibition as described for bacterial and plant SATs (Noji et al., 1998; Takagi et al., 1999b).

Expression of cysteine-insensitive SATs was applied to bacteria and plants. In *E. coli* a facilitator protein mediates the efflux of cysteine thereby protecting the cytoplasm from toxic cysteine concentrations (Daßler et al., 2000). Indeed, the presence of cysteine in culture media correlated with cysteine insensitivity of the SAT protein in the respective *E. coli* strain in the order C600 wild type < MW1/SAT1, MW1/SAT7 < MW1/SAT4. Concentrations of up to 0.3 g/l were achieved in simple shaking cultures without any optimisation of growth conditions. Aeration, increased sulfur sources or other medium components combined with fermentation are likely to improve yield, as indicated by the moderate cell densities reached under the current conditions. The observed cysteine amounts were still low, but already in the range of previous reports using different bacterial and plant SATs (Takagi et al., 1999a and b; Nakamori et al., 1998).

Constitutive over-expression of *E. coli* SAT (CYS E) in tobacco and potato had previously been shown to increase steady-state levels of cysteine and glutathione 2–3-fold (Blaszczyk et al., 1999; Harms et al., 2000). Interestingly, expression of a CYS E mutant protein with reduced feedback-sensitivity to cysteine (Denk and Böck, 1987) did not significantly improve cysteine accumulation neither by cytosolic nor plastid targeting. It was assumed that the mutant protein is catalytically less active that the wild-type CYS E protein (Blaszczyk et al., 1999). Using the insensitive SAT A isoform from *A. thaliana* an average cysteine increase of 3-fold and 6-fold was observed for the cytosol and plastid targeted proteins, confirming the better fit of suitable plant proteins for this task. The results furthermore showed that cysteine could apparently be synthesized independently in both compartments and that at least for tobacco a putative cysteine-insensitivity of endogenous plastid SAT could easily be topped by the heterologous SAT A. Since SAT activities of transgenic tobacco lines were much higher than the concomitant cysteine accumulation it cannot be excluded that other factors such as sulfate supply or the sulfate assimilation pathway limited further elevation of cysteine formation. Alternatively, cysteine turnover could account for this discrepancy, including glutathione formation, protein synthesis or degradation. In future experiments such transgenic approaches shall thus be transferred to crop plants, specified for expression in plant organs of interest and combined with sinks for cysteine, such as sulfur rich proteins to make efficient use of the strongly enhanced cysteine synthesis capacity.

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