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Transgenic tobacco plants overexpressing the *Met25* gene of *Saccharomyces cerevisiae* exhibit enhanced levels of cysteine and glutathione and increased tolerance to oxidative stress

I. Matityahu[1], **L. Kachan**[1], **I. Bar Ilan**[1], and **R. Amir**[1,2]

[1] Plant Science Laboratory, Migal – Galilee Technology Center, Kiryat Shmona, Israel
[2] Tel-Hai Academic College, Upper Galilee, Israel

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Summary. The cysteine biosynthesis pathway differs between plants and the yeast *Saccharomyces cerevisiae*. The yeast *MET25* gene encoded to *O*-acetylhomoserine sulfhydrylase (AHS) catalyzed the reaction that form homocysteine, which later can be converted into cystiene. *In vitro* studies show that this enzyme possesses also the activity of *O*-acetyl(thiol)lyase (OASTL) that catalyzes synthesis of cysteine in plants. In this study, we generated transgenic tobacco plants expressing the yeast *MET25* gene under the control of a constitutive promoter and targeted the yeast protein to the cytosol or to the chloroplasts. Both sets of transgenic plants were taller and greener than wild-type plants. Addition of SO_2, the substrate of the yeast enzyme caused a significant elevation of the glutathione content in representative plants from each of the two sets of transgenic plants expressing the yeast gene. Determination of non-protein thiol content indicated up to four-folds higher cysteine and 2.5-fold glutathione levels in these plants. In addition, the leaf discs of the transgenic plants were more tolerant to toxic levels of sulphite, and to paraquat, an herbicide generating active oxygen species.

Keywords: Cysteine biosynthesis – Glutathione – *O*-Acetylhomoserine sulfhydrylase – *O*-Acetyl(thiol)lyase – Oxidative stress – Transgenic tobacco plants

Introduction

Plants and bacteria incorporate inorganic sulfate from the soil and derivatives of sulphite/sulphide from the atmosphere as a source of sulphur (Leustek and Saito, 1999; Saito, 2000). Cysteine, the first organic sulphur-containing metabolite in plants, is generated through this assimilation pathway and then serves as a precursor for the synthesis of various sulphur-containing metabolites, including methionine and glutathione. Glutathione represents the major storage and transport form of reduced sulphur and has a critical role in protection against abiotic/biotic stresses, acting as a scavenger of reactive oxygen species (Leustek and Saito, 1999). The two enzymes that catalyze the final step in cysteine biosynthesis, serine acetyltransferase (SAT; EC 2.3.1.30) and *O*-acetylserine(thiol)lyase (OASTL; EC 4.2.99.8) (Fig. 1), play an important role in the regulation of cysteine synthesis (Droux et al., 1998; Hell et al., 2002). SAT catalyzes the formation of *O*-acetylserine (OAS) from serine and acetyl-coenzyme A, while OASTL, inserts the reduced sulphide into OAS, a three-carbon chain, to yield cysteine. Both SAT and OASTL are located in all three protein producing compartments of the plant cell: the cytosol, chloroplasts, and mitochondria (Hell et al., 2002; Saito, 2000). These two enzymes form a complex, known as cysteine synthase, that regulates the enzymatic activities of its constituent enzymes (for a review see Hell et al., 2002). OASTL is active only in a free form, being allosterically inactivated in the complex, while SAT activity relies on the association with OASTL, since free SAT loses its catalytic activity when the SAT/OASTL complex is disrupted (Droux et al., 1998; Hell et al., 2002). The stability of the complex is affected by its substrates: OAS disrupts the complex while sulphide stabilizes it. It has been suggested that OASTL functions as a regulatory subunit that regulates SAT in response to sulphide and OAS. The ratio between OASTL and SAT is different in the three compartments, ranging from 3:1 to 300:1, indicating that only a small fraction of the total OASTL is associated with the complex (Droux et al., 1998; Hell et al., 2002). The excess of OASTL over SAT suggests that SAT is a suitable target for a manipulation aiming to increase cysteine

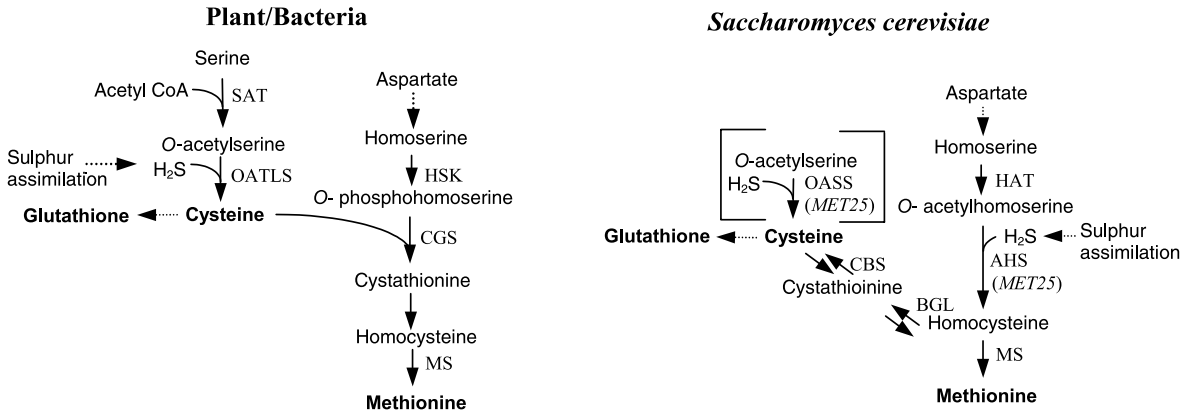

Fig. 1. The cysteine and methionine biosynthesis pathways in bacteria and plants (left panel) and in the yeast *Saccharomyces cerevisiae* (right panel). Dashes arrows represent more than one biochemical step; the brackets in the yeast pathway show additional activity of the enzyme encoded by the *MET25* gene that does not occur *in vivo*. *SAT*, serine acetyltransferase; *OASTL*, *O*-acetyl(thio)lyase; *CGS*, cystathionine γ-synthase; *MS*, methionine synthase; *OASS*, *O*-acetylserine sulfhydrylase; *HSK*, homoserine kinase; *HAT*, homoserine *O*-acetyltransferase; *AHS*, *O*-acetylhomoserine sulfhydrylase; *CBS*, cystathionine β-synthase; *CGL*, cystathionine γ-lyase

synthesis (Hofgen et al., 2001). Indeed, overexpression of the *E. coli* SAT gene or the *Arbidopsis* feedback-insensitive SAT in transgenic plants resulted in significantly increased levels of cysteine and glutathione (Blaszczyk et al., 1999; Harms, et al., 2000; Wirtz and Hell, 2003). Overexpression of OASTL, however, caused only minor changes in the concentration of thiols (Liszewska et al., 2001; Noji et al., 2001; Saito et al., 1994; Youssefian et al., 2001), although these transgenic plants were more tolerant to abiotic stress and to toxic levels of sulphide (Liszewska and Sirko, 2003; Noji et al., 2001; Saito et al., 1994; Youssefian et al., 2001, 1993).

The biosynthesis pathway of cysteine in the yeast *Saccharomyces cerevisiae* is different from that found in bacteria and plants (Hacham et al., 2003) (Fig. 1). In yeast, sulphide is incorporated into the four-carbon chain of *O*-acetylhomoserine (OAH), which subsequently yields homocysteine. This reaction is catalyzed by OAH sulfhydrylase (AHS), the product of the *MET25* gene. Homocysteine then serves as a substrate for cysteine and methionine biosynthesis (Cherest et al., 1993) (Fig. 1). Two enzymes, cystathionine β-synthase and cystathionine γ-lyase, encoded by the *STR4* and *STR1* genes, respectively, catalyze the formation of cysteine from homocysteine. The biosynthesis pathway of cysteine in *S. cerevisiae* was unraveled by genetic analysis of two cysteine auxotrophs (*str1* and *str4*) that failed to grow on methionine or homocysteine, demonstrating that in yeast the conversion of homocysteine into cysteine is the only means of *de novo* cysteine synthesis (Antoniewski et al., 1973; D'Andrea et al., 1987; Thomas and Surdin-Kerjan, 1997). However, the enzyme that catalyzed the homocysine synthesis, AHS, was found in *in vitro* studies to be bifunctional: it sulfhydrylated both OAH and OAS using sulphide to yield homocysteine and cysteine, respectively (Yamagata et al., 1975, 1994). Therefore, this enzyme has also been called OAH-OAS sulphydrylase (Yamagata et al., 1994).

In the present study we describe the data obtained from transgenic tobacco plants overexpressing the yeast *MET25* gene encoding OAH-OAS sulphydrylase. In its activity as OAS- sulphydrylase, the yeast enzyme is expected to act like the OASTL enzyme in bacteria and plants, yielding higher levels of cysteine and glutathione. The yeast enzyme was targeted to the cytosol or to the chloroplasts, two compartments that naturally synthesize cysteine and glutathione. We found that the transgenic plants indeed had enhanced levels of cysteine and glutathione, indicating that the yeast enzyme was active in the cysteine biosynthesis pathway. In addition, these plants were more tolerant to oxidative stress and to high levels of sulphite, a common environmental pollutant.

Materials and methods

Engineering the binary vector

The coding DNA sequence of the *MET25* gene on plasmids pEMBLYr25 was kindly provided by Prof. Yolande Surdin-Kerjan. *MET25* was amplified by PCR using primer 1: 5′-GGCATGCCATCTC ATTTCGATACTG-3′ (which includes an *Sph*I restriction site containing the ATG translation-initiation codon) and primer 2: 5′-TCATGGTTTTTGG CCAGCGAAAACAG-3′. The amplified fragment was ligated to the PCR-vector pGEMT (Promega). After digestion with *Sph*I and *Pst*I, the PCR fragment was subcloned into pCE and pCD vectors (Shaul and Galili, 1992) that were digested by the same enzymes. These vectors contained the 35S promoter of the cauliflower mosaic virus and an Ω DNA sequence

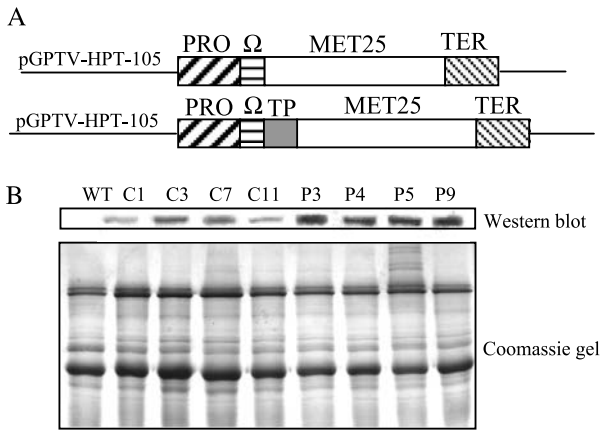

Fig. 2. Expression of the *MET25* gene of *Saccharomyces cerevisiae* in plants. **A** A schematic diagram of the chimeric *MET25* gene utilized in the present study. PRO, the 35S promoter; Ω, the DNA encoding the Ω mRNA leader sequence; TP, the DNA encoding the pea rbcS-3A chloroplast transit peptide targeting the protein to the chloroplasts; MET25, the DNA sequence of the yeast *MET25* gene; TER, the DNA sequence of the octapine synthase 3′ terminator. **B** Expression of the yeast protein in various homozygous tobacco transgenic plants in which the yeast protein is localized to the cytosol (C1–C7) or in the plastids (P3–P9). Wild type plants (*WT*) are shown as a negative control. Western blot analysis (upper panel): Total soluble protein (40 μg) was separated by SDS-PAGE and subjected to immunoblot analysis using antiserum against the yeast protein. The lower panel shows coomassie blue staining of gels loaded with 15 μg of total soluble protein

from the coat protein gene of the tobacco mosaic virus for translation enhancement. pCE also had the transit peptide of pea rbcS-3A chloroplast (Shaul and Galili, 1992) (Fig. 2A). These vectors were then cut with *Sma*I and *Sac*I and the fragment was subcloned into the binary Ti plasmid pGPTV-HPT-105 carrying the gene for hygromycin resistance (Becker et al., 1992).

Construction of the yeast expression vector containing the MET25 yeast gene and yeast complementation

The pGMT plasmid containing the *MET25* gene was digested with *Nco*I and *Sal*I and the sites were blunted by the Klenow enzyme (Bohringer). The fragment was subcloned into the expression yeast vector, pFL61, which was digested with *Not*I and blunted by Klenow. The yeast gene, in the sense and antisense orientation, was transformed into CC365-1A (MATa, *his3*, *ura3*, *met25*), and the transformants were grown with shaking at 30°C in 0.67% Yeast Nitrogen Base (YNB, Difco) supplemented with 2% glucose and the appropriate amino acids, without methionine and cysteine. Yeast transformation was performed as described in the standard protocol (Vanoni et al., 1983).

Plant transformation and growth

The chimeric gene was used for transformation into *Nicotiana tabcum* (cv Samsun, NN) using the leaf-disc protocol (Horsch et al., 1985). Transgenic plants were selected on the basis of their ability to root and regenerate on media containing 15 μg ml^{-1} hygromycin. Four-week old plants were transferred to medium containing 30% tuff, 30% vermiculite and 40% peat. The plants were fertilized every 10 days with 20-20-20 fertilizer (0.1 g per pot).

Seedling growth in B5 medium

Twenty-day-old seedlings of wild type and transgenic plants were transferred to B5 medium (DuShefa). The seedlings were grown in light for ten days, at 25°C and with continuous shaking. Na_2SO_3 was then added to half of the cultures, to a final concentration of 10 mM, for five hours, after which the cultures were harvested.

Antibody preparation against the yeast protein

The yeast gene was amplified using primers 1 and 2 (as described above), where the *BamHI* site in the forward primer replaced the *SphI* site and the reverse primer contained a *Not*I site. The PCR fragments were digested by these enzymes and cloned into the pGEX-5x-1 vector (Pharmacia, Biotech). The plasmid was used to transform the *E. coli* BL21 strain, and these bacteria were grown at 25°C to reduce the formation of inclusion bodies and aggregates. The yeast protein, after being purified according to the manufacturer's instructions, was used for rabbit immunization with three injections of 200 μg each every three weeks.

Western blot analysis

Proteins were extracted from 100 mg of tobacco leaf samples with 0.5 ml phosphate buffer saline (PBS) supplemented with a protease inhibitor cocktail (Boehringer). Extracts were centrifuged at 15,000 g for 30 min at 4°C. Protein concentrations were measured using the Bradford reagent (Bio Rad). Equal amounts (40 μg) of proteins were fractionated on 10% SDS-PAGE (Laemmli, 1970). Fractionated proteins were transferred onto nitrocellulose membranes, stained with Ponceau-S, and reacted with the serum raised against the yeast protein using an ECL kit (Amersham), as recommended by the manufacture.

Determination of cysteine and glutathione content

Thiols were prepared as described by Harms et al. (2000). Separation and quantification were performed by reverse phase HPLC after derivatization with monobromobimane. The reduction of disufides was carried out with bis-2-mercaptoethylsulfone, and the labeling reaction with monobromobimane was terminated with 15% HCl.

Frozen leaves (200 mg) were ground in a mortar and pestle and then extracted for 20 min in 2 ml 0.1 N HCl at 4°C. After centrifugation of the mixture at 4°C (20 min, 11,000 g), 120 μl of the supernatant was added to 200 μl of 0.2 M 2-(cyclohexylamino) ethanesulfonic acid (pH 9.3). Reduction of total disulfides was performed by adding 10 μl of bis-2-mercaptoethylsulfone in 9 mM Tris-HCl, and 5 mM EDTA (pH 8.0). After 40 min at room temperature, free thiols were labeled with monobromobimane by adding 20 μl of 15 mM monobromobimane in acetonitirile to the mixture and placing it for 15 min at room temperature in the dark. The reaction was terminated by adding 250 μl of 15% HCl and the mixture was kept on ice for at least 2 h in the dark. The reaction mixture was re-centrifuged at 4°C (20 min, 11,000 g). The resulting supernatant was filtered through a 0.2 μm nylon filter and separation of thiols was carried out on a RP18 column (MERC) using HPLC (Hp model 1050) system. The elution protocol (RT, 1.2 ml min^{-1}) employed a linear gradient of solvents A (0.25% aqueous acetic acid + 10% methanol pH 3.9) and B (methanol) as follows: 0 to 8% B for 10 min, 8 to 14% B for 5 min, 14 to 100% for 2.5 min, 100% B for 11 min, 100% to 0% B for 1 min, and 0% B for 1.5 min. The bromobimane thiol derivatives were detected fluorometrically (excitation 380 nm; emission 480 nm). Using as reference mixed standards treated like the sample supernatants, peaks at approximately 7 min and 11 min were identified as cysteine and glutathione, respectively.

GC-MS analysis of soluble amino acid content

For analysis of soluble amino acid content, replicate samples of 100 mg were ground to powder (kept frozen with liquid nitrogen),

and extracted in 1400 μl methanol. 4.6 μl of 2 mg/ml norleucine was added as an internal standard. After vortex, the mixture was extracted for 15 minutes at 70°C, and mixed with 750 μl water. To separate the polar phase, 375μl chloroform was added, the mixture was vortexed briefly and separated by 15 minutes centrifugation at 2200 × g. 150 μl of the upper polar phase of each sample were reduced to dryness under vacuum, and then dissolved and treated for 2 hours with 40 μl 20 mg/ml methoxyamine hydrochloride in pyridine, followed by derivatization for 30 minutes in N-methyl-N(trimethylsilyl)-trifluoro-acetamide, at 37°C, with vigorous shaking. Sample volumes of 1μl were injected to a GC-MS system, with a split ratio of 1:1. Along with the samples, amino acid standards of 200, 100, 50, 25 μM were injected to establish quantification curves. The GC-MS system included an HP6890 autosampler injector, HP5890 series II plus gas chromatograph, and an HP5972 series selective detector mass spectrometer (Hewlett Packard, USA). GC was performed on a 30 m Rtx-5SIL MS column with 0.25 mm inner diameter and 0.25μm film thickness (Restek, USA, Cat # 12723-124). Injection temperature was 230°C, the interface set to 250°C. Helium carrier gas flow was 1 ml/min, and the temperature was set to 5 min heating at 70°C, followed by a 5°C/min increase in temperature to 310°C, with an additional 1 min heating at 310°C. Prior to injection of the next sample the system was equilibrated for 6 min at 70°C. Mass spectra was recorded at two scans per second, with a m/z 50–600 scanning range. The mean quantity of each amino acid was calculated from calibration curves of standards.

Determination of the sulfate content

One hundred mg of fully expanded leaves of 8-week old control and transgenic plants expressing the yeast protein were homogenized with 1 ml of deionized water, and the mixture was incubated at 80°C. The mixture was mixed by vortex every 20 min, and after four hours the extract was centrifuged for 5 min at 12,000 g and then filtered using a 20 μm syringe filter. Twenty microliters were injected for ion-chromatography (Dionex, ED50 Electrochemical detector with ASRS-ultra II-4 mm suppressor). Results were calculated to mg sulfate per Kg fresh weight of the leaves (given as ppm).

Exposure of leaf discs to SO_3^{2-} and to paraquat

Leaf discs (7 mm in diameter) cut from fully expanded leaves of 12-week old plants were cultured in 0.5 MS medium (Murashige and Scoog medium, DuShefa) with or without 10 mM sodium sulphite for 20 h under constant illumination (25 $\mu E\,m^{-2}\,S^{-1}$). The chlorophyll content of the leaf discs was then measured. For paraquat test, the discs were immersed in a solution containing 10^{-5} μM paraquat (methyl viologen, 1,1-dimethyl-4, 4-bipyridinium dichloride; Sigma, St. Louis) as described in (Noji et al., 2001). Discs were then placed under sunlight (1400 $\mu E\,m^{-2}\,S^{-1}$) at 25°C for 3 h and then examined visually for damage. The content of chlorophyll in leaf discs was determined as described previously (Noji et al., 2001). The values of remaining chlorophyll content after the stress treatment were calculated as follows: the amount of chlorophyll per leaf disc treated with SO_3^{2-} or paraquat was divided by the amount of chlorophyll per leaf disc treated without sulphite or paraquat and expressed as a percentage.

Results

Complementation test

The yeast *MET25* gene was PCR amplified from the plasmid pEMBLYr25 and its ability to form an active enzyme was examined by a complementation test using the *met25* mutant. Sense and antisense forms of the PCR fragment were cloned into the yeast expression vector, pFL61. Colonies developed on minimal medium only when their harbored construct was in the sense orientation, demonstrating that the yeast enzyme did not lose its activity following PCR amplification.

Engineering plants expressing the MET25 yeast gene

To study whether the *MET25* gene of *S. cerevisiae* can enhance the cysteine and glutathione levels in the transgenic plants, two chimeric constructs were prepared (Fig. 2A). In the first, the yeast gene was targeted into the cytosol, while in the second it was fused in frame to DNA encoding the plastid transit peptide of pea rbs-3A, in order to target the mature protein into the chloroplasts. The 35S promoter of the cauliflower mosaic virus was used to obtain a constitutive expression of the yeast gene. About 20 independent hygromycin-resistant transformants from each group were generated and selected based on the steady-state protein level as determined by western blot analysis (data not shown). Four lines from each of the plastidic (P) or cytosolic (C) series with the highest expression level of the yeast protein were selected for detailed analysis (Fig. 2B). Homozygous lines were obtained by self-pollination.

Results obtained from western blot analysis of the eight selected lines showed that the foreign protein co-migrated in both series of transgenic plants; evidently, in the chloroplastic-type, the transit peptide had been removed when the enzyme was translocated into the chloroplasts. Generally, plants in which the yeast enzyme was transported into the plastids showed higher steady state levels than plants expressing this enzyme in the cytosol (Fig. 2B). The transcript levels in the two series of plants were similar (data not shown), suggesting that the yeast protein is less stable in the cytosol. From these two series of transgenic plants, we selected two representatives, designated C7 and P3 as parental lines. Homozygous plants of lines C7 and P3 were crossed to generate the plants of first filial generation, designated X_1.

Plants expressing the yeast enzyme were taller and greener

Eight-week old transgenic plants expressing the cytosolic or chloroplatic forms of the yeast gene were 1.4- to 2.2-fold taller than wild type plants or tobacco plants

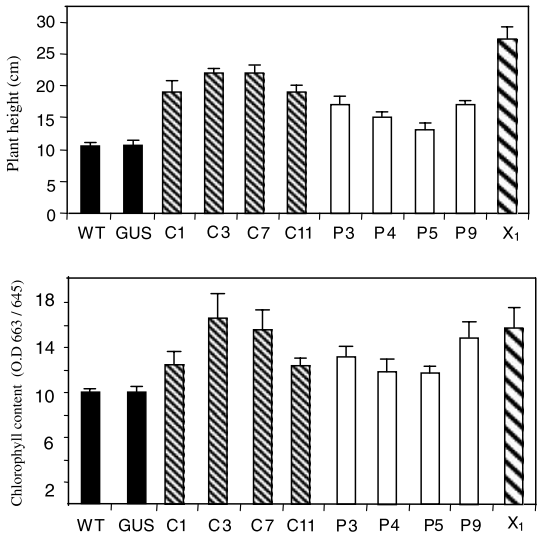

Fig. 3. Height (cm) (upper panel) and chlorophyll content (lower panel) in 8-week-old control wild type (*WT*) and tobacco plants expressing the 35S-β-glucuronidase (*GUS*), and in transgenic homozygous plants expressing the *MET25* yeast gene whose product was targeted to the cytosol (C-plants) or to the chloroplasts (P-plants). X_1, is the heterozygous progeny of C-7 and P-3. Values represent mean $\pm$ SD of eight plants from each transgenic line

expressing the 35S-β-glucuronidase (GUS) (Fig. 3, upper panel). Notably, the X_1 plants were significantly taller than their parents and 2.5-fold taller than control plants (Fig. 3). In addition, all transgenic plants had a higher number of nodes, compared to control plants, and their leaf size was significantly larger (data not shown). The transgenic plants were also greener than the control plants, as evident by the chlorophyll content. The chlorophyll content of C3 and P9, was $\sim$1.7 and $\sim$1.5 fold higher, respectively, than control plants (Fig. 3, lower panel). However, this phenotype was lost as plants became older (18–20 weeks old), and only slight differences were detected when the plants flowered.

Plants expressing the yeast enzyme can use SO_2 as a substrate

As SO_2 is a substrate of OAH-OAS sulphydrylase, we examined whether adding this substrate enables the transgenic plants to produce higher levels of cysteine and glutathionine. When SO_2 is taken up by plants, it is converted rapidly into SO_3^{2-}, therefore Na_2SO_3 can serve as a source for SO_2 (Noji et al., 2001). Twenty two day old seedlings of homozygous P3, C7 and wild type plants were grown in B5 liquid culture for 8 days and Na_2SO_3 was than added for 5 hr before harvesting. Levels of cystine and glutathione in these plants were measured by

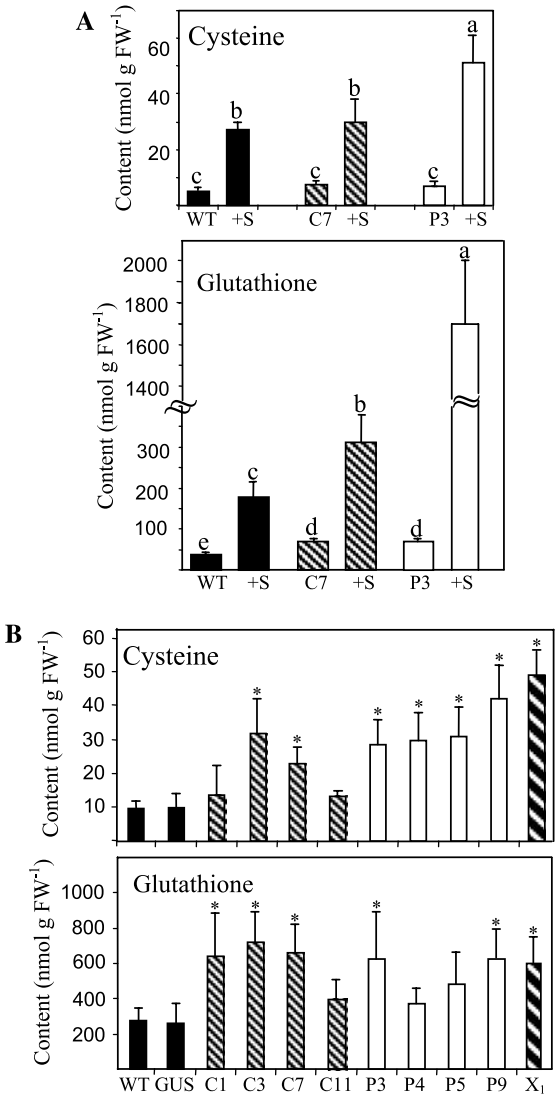

Fig. 4. Cysteine (upper panel) and glutathione (lower panel) content in wild type (WT) and homozygous transgenic plants expressing the yeast *MET25* gene. **A** Cysteine and glutathione content in twenty-day-old representative homozygous seedlings expressing the yeast protein in cytosol (*C7*) or in the chloroplasts (*P3*). The seedlings were grown for 8 days in B5 medium, supplemented with 10 mM Na_2SO_3 five hours before harvesting. **B** Cysteine and glutathione content in fully expanded leaves of wild type and 35S-β-glucuronidase (*GUS*) and in homozygous transgenic plants expressing the yeast *MET25* gene whose product was targeted to the cytosol (C-plants) or to the chloroplasts (P-plants). X_1, is the heterozygous progeny of C7 and P3 plants. The level of the thiol compounds was measured using HPLC. Values are mean $\pm$ SD of five replicates. Statistically significant changes ($p < 0.05$, using Student's test) are identified by an asterisk or letters

HPLC. As shown in Fig. 4A, addition of 10 mM Na_2SO_3 to the seedlings significantly increased the cysteine and glutathionine content in all tested plants. No significant differences in the content of these two sulphur metabolites were found between the wild type C7 plants after the

addition of Na_2SO_3. However, in P3 seedlings, levels of cysteine and glutathione were increased to a much greater degree (8.5 fold and 28-fold, respectively) by addition of Na_2SO_3 to the growth medium, compared with the increase observed in wild type or C7 seedlings (Fig. 4A). This corresponds with the high expression level of the yeast gene in P3 plants (Fig. 2B). Taken together, these results imply that the yeast enzyme is active, at least in P3 plants.

Expression of the yeast gene led to increased endogenous levels of cysteine and glutathione in leaves

Next, we investigated whether the expression of the *MET25* gene in tobacco plants influenced the endogenous levels of cysteine and glutathione. Fully expanded leaves of eight-week-old transgenic and wild type plants were harvested and their thiol content was determined by HPLC. Some of the transgenic lines exhibited significantly increased levels of cysteine and glutathione (Fig. 4B). For example, the cysteine content of lines C3 and P9 was ∼three fold and ∼four fold higher, respectively, than that of control plants. Glutathione was increased in line C3 ∼two and half-fold and in line P3 ∼two-fold. In the X_1 line, the cysteine and the glutathione levels increased up to five-fold and three-fold, respectively, relative to control plants. These enhanced levels of thiol-compounds suggest that the yeast enzyme was active in the cysteine biosynthesis pathway in both sets of transgenic plants.

Levels of soluble methionine in the transgenic plants did not alter

A higher level of cysteine causes an increase in the content of the cysteine metabolite, glutathione, but may also affect the level of methionine, to which cysteine contributes its sulfur group. Therefore, the level of methionine, homocysteine, OAS and other amino acids of the asparatate family, were determined in leaves of transgenic and wild type plants using GCMS. The results (Table 1) demonstrated that the levels of these amino acids did not significantly differ between the wild type and transgenic plants. The level of OAS was below the detection level (below 10 μmol/mg fresh weight) in all tested plants.

Expression of the yeast gene led to increased endogenous levels of sulfate in leaves

Since changes in thiol content may affect the level of sulfate in the transgenic plants the level of sulfate was determined using ion-chromatography. The results (Fig. 5) demonstrated that in general, transgenic plants expressing the yeast gene have higher levels of sulfate then the control plants. Lines C3 and C7, in which the yeast protein is targeted to the cytosol, had the highest level of sulfate (about three-fold higher then the control). Line P9, in which the yeast protein is targeted to the chloroplasts, had a two-fold increase in the sulfate level (Fig. 5).

Tolerance to SO_3^{2-}

SO_2 causes foliar damage, such as chlorosis and necrosis, but it can also serve as a substrate for OAH-OAS sulphydrylase. We therefore expected that the transgenic plants would become more tolerant to toxic levels of this compound. Accordingly, leaf discs from lines P3 and C7 as well as from line X_1 were cultured with or without 10 mM Na_2SO_3 under constant illumination for 20 h, and their level of the remaining chlorophyll was determined. The transgenic leaf discs showed higher tolerance to 10 mM SO_3^{2-} (higher percentage of remaining chlorophyll) than

Table 1. Levels of sulphur and aspartate related amino acids in wild-type and in representative homozygous transgenic plants expressing the yeast protein in cytosol (C7) or in the chloroplasts (P3). Soluble amino acid levels were measured by GCMS analysis of leaf extracts, presented as μmol/g fresh weight. X_1, is the heterozygous progeny of C7 and P3 plants. Five plants of each type were analyzed and the data are presented as the mean ± SE

Amino acid	Wild-type	P3	C7	X_1
Methionine	0.049 ± 0.019	0.039 ± 0.011	0.045 ± 0.009	0.042 ± 0.015
Homocysteine	1.363 ± 0.335	1.333 ± 0.461	1.161 ± 0.265	1.421 ± 0.321
Aspartate	7.162 ± 2.917	6.989 ± 3.738	6.716 ± 1.369	7.778 ± 3.470
Threonine	3.276 ± 1.866	3.154 ± 1.211	3.327 ± 2.312	2.984 ± 1.754
Lysine	0.846 ± 0.314	0.673 ± 0.159	0.833 ± 0.236	0.967 ± 0.212
Isoleucine	0.262 ± 0.081	0.425 ± 0.098	0.367 ± 0.116	0.271 ± 0.043

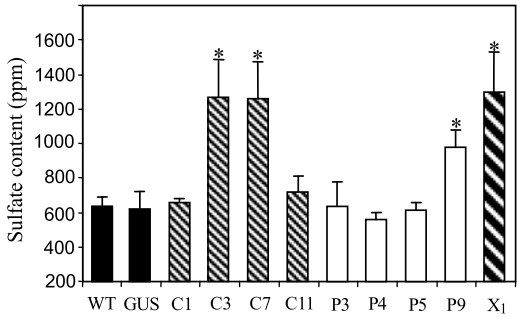

Fig. 5. Content of sulfate in fully expanded leaves of wild type (*WT*) and 35S-β-glucuronidase (*GUS*) and in homozygous transgenic plants expressing the yeast *MET25* gene whose product was targeted to the cytosol (C-plants) or to the chloroplasts (P-plants). Values are mean $\pm$ SD of four replicates of two different experiments. Statistically significant changes ($p < 0.05$, using Student's test) are identified by an asterisk

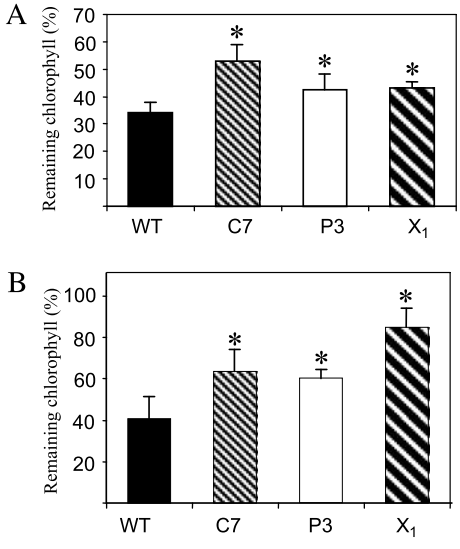

Fig. 6. Tolerance to sodium sulphite toxicity (**A**) and effects of paraquat (**B**) on leaf discs of wild type (*WT*) and representative transgenic plants expressing the yeast protein in the cytosol (*C7*) or in the chloroplasts (*P3*). Tolerance is expressed as the percentage of remaining chlorophyll content in leaf discs after cultivation in the presence of 10 mM Na_2SO_3 (A) or after treatment with 10^{-5} M paraquat (**B**). Means and $\pm$SD were calculated from six independent leaf discs that were taken from each of the five plants. Statistically significant changes ($p < 0.05$, using Student's test) are indicated by an asterisk. The data represent three independent repeats of this experiment

non-transformed wild-type plants (Fig. 6A). Line C7 showed higher tolerance to SO_3^{2-} compared to P3 and wild-type plants. These results suggest that overexpression of the yeast gene in the cytosol contributes to the tolerance towards SO_3^{2-} more than its expression in the chloroplasts. The relatively low tolerance level of line X_1 may be a result of its state of heterozygosity compared to lines C7 and P3 that were homozygous to the yeast gene.

Effect of photooxidative stress on transgenic plants

An elevated glutathione level in plants was reported to correlate well with their increased resistance to aboitic stress, including oxidative stress (Blaszczyk et al., 1999). In addition, the toxicity of SO_2 is thought to result from generation of active oxygen species (Noji et al., 2001; Youssefian et al., 2001). We therefore determined whether the transgenic plants better tolerated the oxidative stress induced by methyl viologen (paraquat), a reagent that generates active oxygen species in chloroplasts under constant illumination (Youssefian et al., 2001). After three-hours of cultivation with or without 10^{-5} mM paraquat in full sunlight (1400 μE m^{-2} S^{-1}) the remaining chlorophyll was determined. The transgenic plants were significantly more resistant to paraquat than wild- type plants (Fig. 6B) while the content of the remaining chlorophyll in X1 plants was doubled relative to wild-type plants.

Discussion

In our long-term goal to increase the level of cysteine and thus of glutathione in transgenic plants, we overexpressed the yeast *MET25* gene of *S. cerevisiae* in transgenic tobacco plants. *In vitro* studies have previously shown that this gene encodes a bifunctional enzyme (OAH-OAS sulphydrylase) that promotes homocysteine and cysteine biosynthesis. However, in yeast, this enzyme functions only in the homocysteine biosynthesis pathway as shown by the auxotrophy feature of *str1* and *str4* mutants, which suffer from lack of cysteine (Fig. 1) (D'Andrea et al., 1987; Thomas and Surdin-Kerjan, 1997). We assumed that this bifunctional enzyme would be active in plants only in the cysteine pathway, since OAH, the substrate for homocysteine synthesis, is not produced in most plant tissues and to date, has only been found in pea pods (Rochat and Boutin, 1991). Two lines of evidence obtained in this study suggest that the yeast enzyme was active in cysteine biosynthesis both in the cytosol and chloroplasts: (i) Elevated levels of cysteine and glutathione were demonstrated in the two sets of the transgenic plants (Fig. 4B); and (ii) the transgenic plants contained significantly higher levels of glutathione when SO_3^{2-}, the substrate of the yeast enzyme, was added (Fig. 4A). The observation that the transgenic plants were more tolerant to toxic levels of SO_3^{2-}, as well as to oxidative stress (Fig. 6) also suggests that they have higher levels of glutathione as a result of the increase in levels of cysteine.

The levels of cysteine and glutathione found in transgenic plants expressing the yeast enzyme were generally

similar to those found in plants overexpressing the SAT enzyme (Harms et al., 2000; Blaszczyk et al., 1999) on the whole, these levels were higher than in plants overexpressing OASTL (Dominguez-Solis et al., 2001; Noji et al., 2001; Saito et al., 1994; Sirko et al., 2004; Youssefian et al., 2001). Recently it was suggested that in addition to its role in cysteine synthesis the plant's OASTL can catalyze a reaction that degrades cysteine to pyruvate, ammonia and H_2S; and that these two activities play a major role in controlling cysteine level in plants (Riemenschneider et al., 2005). The higher level of cysteine and glutathione found in the transgenic plants overexpressing the yeast enzyme compared to those overexpressing the plant's enzyme suggests that the yeast enzyme does not have cysteine-degradation activity. Thus, the results of the present study support the assumption that the expression of heterologous enzymes of the sulfate assimilation pathway from organisms that are evolutionary distant from plants might be a good approach to enhance the levels of thiols in plants (Blaszczyk et al., 1999; Harms et al., 2000; Sirko et al., 2004). Such an approach could overcome the intrinsic regulatory mechanisms that are responsible for the tight control of these metabolic pathways.

Previous data suggests that, in general, high concentration of cysteine and glutathione repress sulphur-assimilation and uptake activities, while sulphur-starvation results in increased activities of key enzymes in the sulphur-transport assimilatory pathway (Buchner et al., 2004; Hesse et al., 2004). For example, expression of sulfate transporters and ATP-sulphurylase is enhanced when levels of cysteine and glutathione are reduced. OAS plays a major role in this regulation, since it causes both an elevation of the transcript level and the activity of sulfate transporters, as well as other enzymes in the sulphur-assimilation pathway, as shown in OAS-feeding experiments (for reviews see Hesse et al., 2004; Saito, 2000). Notably, the transgenic plants C3, C7 and P9 that contain high levels of cysteine and gluathione also show significantly higher sulfate content. This suggests increased activity of sulfate transporters in the leaves of the transgenic plants; however, the sulfate may be accumulating in the vacuole so that its level in the chloroplasts and in the cytosol is still kept low. In any case, the existence of high levels of sulfate in the transgenic plants is not easily explained, and further studies are required to clarify this point.

Plants grown with insufficient levels of sulfate exhibited symptoms of sulphur deficiency, including chlorosis of young leaves and growth retardation (Hesse et al., 2004). Thus, it is reasonable to assume that the vigorous growth and greener leaves found at the early developmental stages of plants expressing the yeast *MET25* gene resulted from the higher level of thiols produced in these plants. However, such phenotypic changes have not been reported for other transgenic plants that had higher levels of thiols. Conversely, tobacco plants expressing the bacterial OASTL in chloroplasts showed bleaching of older leaves in young plants, a phenomenon that prompted the authors to suggest that enhanced OASTL activity in the chloroplasts had a harmful effect on chlorophyll content (Sirko et al., 2004). Such differences in the phenotype of transgenic plants expressing the bacterial versus the yeast gene suggests that although both share the same basic activity (function similarly) in cysteine biosynthesis, the two enzymes have different effects in plants.

In general, plants expressing the yeast enzyme in the cytosol were taller and had higher levels of glutathione and sulfate than plants expressing the enzyme in chloroplasts. In addition, these C-plants were more resistant to toxic levels of Na_2SO_3 despite their lower steady state expression level of the yeast enzyme (Fig. 2B), and the lower increase in cysteine and glutathionine levels when Na_2SO_3 was applied (Fig. 4A). This suggests that the activity of the yeast enzyme in the cytosol was more effective in maintaining the resistance to oxidative stress and vigorous growth of these plants. These differences between the P and the C plants are probably due to the different ratios between the activity of SAT and OASTL in these two compartments, being lower in the cytosol (200-fold) than in the chloroplasts (300-fold) (Hell et al., 2002). Thus, elevation in the OASTL activity in the cytosol may contribute to cysteine synthesis more than elevation of this enzyme in the chloroplasts. Similar results were obtained when spinach OASTL was expressed in either of these two compartments of tobacco plants (Noji et al., 2001). In the plants in which the enzyme was targeted to the cytosol, the transgene protein level was lower, compared to the plants in which the enzyme was targeted to the chloroplast, although the glutathione level was higher (Noji et al., 2001). In our study, a positive correlation was generally found between the expression level of the transgenic plants (detected by higher band intensity, Fig. 2) and the thiols content. For example, lines C3, C7, P3 and P9, which showed high expression levels, had a higher level of thiols and were taller and greener than lines expressing the yeast enzyme at lower levels (e.g., lines C1, C11, P4, P5).

Glutathione and cysteine function as important antioxidants in both enzymatic and non-enzymatic reactive oxygen species scavenging reactions (Leustek and Saito,

1999; Youssefian et al., 2001). Indeed, as previously reported, plants that had higher levels of glutathionine were more resistant to abiotic stress, such as oxidative stress (Blaszczyk et al., 1999; Youssefian et al., 2001), toxic levels of hydrogen sulphide gas (Youssefian et al., 1993, 2001), sulphite (Noji et al., 2001), and heavy metals like cadmium (Dominguez-Solis et al., 2001; Kawashima et al., 2004). The transgenic plants described in this study were more resistant to paraquat and to sulphite, two compounds that generate reactive oxygen species in plants. Expression of the yeast enzyme in both compartments (X_1 plants) significantly contributed to the tolerance of these transgenic plants to paraquat compared to their parents (lines C7 and P3). Similarly, Noji et al. (2001) pointed to the importance of simultaneous elevation in thiol content in both compartments for the tolerance of transgenic plants towards oxidative stress. Expression of the yeast enzyme in both compartments also contributed to the vigorous growth of the X_1 plants, which were significantly taller than their parents and 2.5-fold taller than wild-type plants. The enhanced tolerance to stress observed in the transgenic plants may result directly from the function of the yeast protein and the elevation of glutathione levels that it caused. However, one cannot exclude the possibility that their improved stress tolerance relates indirectly to their vigorous growth and their higher chlorophyll content. Thus further studies are required to reveal this point.

Cysteine is a substrate for synthesis of glutathione and for the other sulphur containing amino acid-methionine. Therefore, levels of methionine might increase in transgenic plants oeverexpressing the yeast gene. However, the level of soluble methionine was not significantly different between the transgenic and wild type plants. Levels of homocysteine and other aspartate family amino acids also did not differ. Theoretically synthesis of methionine might be increased if an additional yeast enzyme, homoserine *O*-acetyltransferase, that produces OAH, the main substrate for AHS (Fig. 1), will be co-expressed with AHS. These two enzymes can complete the yeast pathway for methionine synthesis in plant cells (Thomas and Surdin-Kerjan, 1997; Hacham et al., 2003). However, yeast homoserine *O*-acetyltransferase is heat labile and tends to form inactive aggregates, when temperatures increase above 25°C (Gamrasni et al., 2005), and therefore cannot be used to elevate methionine synthesis in plants.

Finally, the transgenic plants described in this work are good candidates for producing plants with high tolerance to a variety of biotic and abiotic stresses. In addition, plants with a high content of thiols could be used to enhance the level of important sulphur-containing metabolites, such as vitamins like biotin, lipoic acid, and thiamine.

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Authors' address: Dr. Rachel Amir, Migal – Galilee Technology Center, P.O. Box 831, Kiryat Shmona, 11016, Israel,
Fax: 972-4-6944980, E-mail: rachel@migal.org.il