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Subcellular Localization of Spinach Cysteine Synthase Isoforms and Regulation of Their Gene Expression by Nitrogen and Sulfur[1]

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Subcellular localization and regulation of the spinach (*Spinacia oleracea*) cysteine synthase (*O*-acetyl-L-serine[thiol]-lyase, EC 4.2.99.8) isoforms (*CysA*, *CysB*, and *CysC*) were determined in transgenic tobacco (*Nicotiana tabacum*) and in spinach cell cultures. The 5′ regions of *CysB* and *CysC* encoding the chloroplastic (*CysB-TP*) and the putative mitochondrial (*CysC-TP*) transit peptide (TP) sequences were fused to a bacterial β-glucuronidase gene (*gus*) and expressed in tobacco under the control of the cauliflower mosaic virus 35S promoter. Subcellular fractionation of transgenic tobacco showed transportation of β-glucuronidase proteins to chloroplasts by CysB-TP and to mitochondria by CysC-TP, respectively, indicating that both presequences were sufficient to act specifically as chloroplastic and mitochondrial TPs in vivo. The mRNA expression patterns of *CysA* (cytoplasmic form), *CysB*, and *CysC* genes under nitrogen- and sulfur-starved conditions were characterized in spinach cell cultures. In sulfur-starved cells, only slight differences (approximately 1.2- to 1.5-fold) in the mRNA levels of *CysA* and *CysB* were observed during the short-term (0–24 h) cultivation periods compared with cells grown in Murashige-Skoog medium. However, under nitrogen and nitrogen/sulfur double-deficient stress conditions, mRNA levels of *CysC* increased up to 500% of the original level within 72 h.

Higher plants synthesize sulfur-containing amino acids, sulfolipids, and coenzymes using inorganic sulfate in the soil as a major sulfur source. CS (EC 4.2.99.8), which catalyzes the biosynthesis of the first organic sulfur compound, Cys, from *O*-acetyl-L-Ser and free or carrier-bound sulfide, is responsible for the terminal reaction in the sulfur assimilation pathway in higher plants and microorganisms (Anderson, 1980). Since animals do not have this pathway and require organic sulfur compounds as a dietary source, CS has been referred to as the indispensable key enzyme in the sulfur cycle in nature (Giovanelli et al., 1980).

Several CS isoenzymes are known in various plant species, and their subcellular localization is well studied biochemically (Schmidt, 1986; Nakamura and Tamura, 1989; Lunn et al., 1990; Rolland et al., 1992). However, the physiologic roles of each isoform are still unknown. In spinach (*Spinacia oleracea*) leaves, two major activities were found in cytoplasm and chloroplasts, and a third, minor, activity was found in mitochondria (Lunn et al., 1990). Thus, CS has been postulated to exist in all subcellular compartments of plants where protein synthesis takes place. To obtain a comprehensive explanation for the different functional roles of CS isoforms, molecular biologic studies have been initiated. Recently, cDNA clones for the nuclear encoded cytoplasmic, chloroplastic/plastidic, and putative mitochondrial isoforms have been isolated from various plant species (Römer et al., 1992; Saito et al., 1992, 1993, 1994; Hell et al., 1993, 1994; Rolland et al., 1993; Youssefian et al., 1993; Noji et al., 1994). We have designated the three genes encoding CS isoforms in spinach as *CysA* (cytoplasmic) (Saito et al., 1992), *CysB* (chloroplastic/plastidic) (Saito et al., 1993), and *CysC* (putative mitochondrial) (Saito et al., 1994).

Regarding the N-terminal *TP* sequences, deduced amino acid sequences of *CysB* and *CysC* of spinach exhibit several common characteristics for chloroplastic (Keegstra et al., 1989) and mitochondrial (Hartl et al., 1989) targeting TPs: (a) rich in positively charged amino acids (Arg, Lys); (b) rich in hydroxylated amino acids (Ser, Thr); and (c) lacking acidic amino acids. Computer analysis of the putative CysC-TP predicted that the N-terminal 33 amino acids formed the amphiphilic α-helical structure consisting of positively charged amino acids clustered on one face of the helix and hydrophobic amino acids on the other (Saito et al., 1994). This is the unique feature of the secondary structure of mitochondrial TPs (von Heijne et al., 1989). The present study was intended to acquire the direct and conclusive evidence for the subcellular localization of the chloroplastic and mitochondrial isoforms using transgenic plants.

As mentioned above, we have isolated cDNAs encoding three distinct isoforms of different subcellular localization from spinach. Our next interest was how the expression of these *Cys* genes is regulated in the cells under various growing conditions. Although there have been studies of the expression of the cytosolic and the chloroplastic/plastidic isoforms (Hell et al., 1994; Saito et al., 1994), the

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Abbreviations: CS, Cys synthase (*O*-acetyl-L-serine[thiol]-lyase); SOD, superoxide dismutase; TP, transit peptide.

overall regulatory mechanism and responses of the three distinct *Cys* genes to various physiologic stress conditions remain to be clarified. Since CS is found at the junction of nitrogen and sulfur metabolic pathways, one can postulate that its expression would be regulated by both nitrogen and sulfur nutritional conditions in the specific subcellular compartments in different ways. To obtain more insight into the physiologic roles of the three *Cys* isoforms in Cys biosynthesis, we examined their gene expression under sulfur and nitrogen starvation stress in spinach cell cultures.

MATERIALS AND METHODS

Construction of TP-GUS Expression Cassettes

Expression cassettes were constructed in intermediate expression vector pHTT202 (Teeri et al., 1989), which contains the cauliflower mosaic virus 35S promoter, the terminator sequence of the T-DNA-encoded gene 7, and the neomycin phosphotransferase II gene (*nptII*) under control of the nopaline synthase (*nos*) promoter for kanamycin selection (Fig. 1).

For constructions of pCSTP14 and pCSTP15, 5′ regions of *CysB* and *CysC* were amplified by PCR as 226- and 156-bp *Bam*HI-*Hin*dIII-ended fragments from the isolated cDNA clone pCSB11 (Saito et al., 1993) and pCSC19 (Saito et al., 1994), respectively. These fragments were fused to a 1.8-kb PCR-amplified *Hin*dIII-*Bam*HI-ended *gus*-coding sequence and were inserted into the dephosphorylated *Bam*HI site of pHTT202, which generated transcriptional fusion of *TP-gus* cassettes to the 35S promoters. PCR amplifications were performed by *Pfu* DNA polymerase (Stratagene). *Bam*HI and *Hin*dIII sites were created by synthetic oligo-DNA primers: CSP-3, 5′-GCCAAGCTTATGTTACGTCCTGTAGAAACC-3′, and CSP-6, 5′-CTTGGATCCGATTCATTGTTTGCCTCCCTG-3′, for *Hin*dIII-*Bam*HI-ended *gus*; CSP-4, 5′-AATGGATCCGAGAGGAGAGAGAAAGTTGGC-3′, and CSP-5, 5′-GCCAAGCTTGTTAAGGCCTTCAATGGTGGA-3′, for *Bam*HI-*Hin*dIII-ended *CysB-TP*; MTP-1, 5′-AATGGATCCGAAATCATGGCAACT-3′, and MTP-2, 5′-GCCAAGCTTATTAGTCCCAGTAAA-3′, for *Bam*HI-*Hin*dIII-ended *CysC-TP*.

Plant Transformation

Intermediate plasmid vectors pCSTP14 and pCSTP15 were mobilized to *Agrobacterium tumefaciens* C58 C1 harboring the disarmed tumor-inducing plasmid pGV2260 (Deblaere et al., 1985) by electrotransformation. Cointegration of the intermediate vectors with pGV2260 was confirmed by PCR analysis of total DNA extracted from *Agrobacterium*. *Nicotiana tabacum* cv Petit Havana SR1 was transformed with *Agrobacterium* harboring cointegrated tumor-inducing plasmids by the leaf disc method (De Block et al., 1987). The primary transformants of the transgenic tobaccos were selected by kanamycin and were regenerated using methods reported elsewhere (Saito et al., 1991). Regenerated primary transformant plants were clonally propagated by shoot culture in A_1 agar medium (0.5× Murashige-Skoog salts [Murashige and Skoog, 1962], 1% Suc, and 0.8% agar, pH 5.7) under 16-h/8-h light/dark cycles at 25°C. These clonally propagated plants, clones 14–14 and 15–19 obtained by transformation with pCSTP14 and pCSTP15, respectively, were used for all further experiments.

Subcellular Fractionation

All procedures were carried out at 4°C. Plant materials were cut into small particles and ground gently in the grinding buffer (0.5 M D-sorbitol, 1 mM EDTA·2Na, 0.1% BSA, 2 mM sodium isoascorbate, and 50 mM Hepes-KOH, pH 7.2) with a pestle in a mortar. Crude extracts were obtained by passing homogenized cells through nylon cloths. Filtrates were centrifuged at 2,000*g* for 2 min to yield crude plastidic pellets. Supernatants were centrifuged again at 8,000*g* for 5 min to give crude mitochondrial pellets. Crude organelle pellets were suspended in the grinding buffer and layered on silicasol continuous gradients (Percoll, Pharmacia) formed by ultracentrifugation at 50,000*g* for 40 min. Gradients were kept in osmotic conditions with the grinding buffer. The initial density of the solution (Percoll) was adjusted to 1.065 g mL^{-1}. Subcellular particles in the crude organelle fraction were separated by centrifugation of the gradients at 8,000*g* for 20 min, and equivalent volumes were collected from the bottom. All subcellular fractions were assayed for GUS enzyme ac-

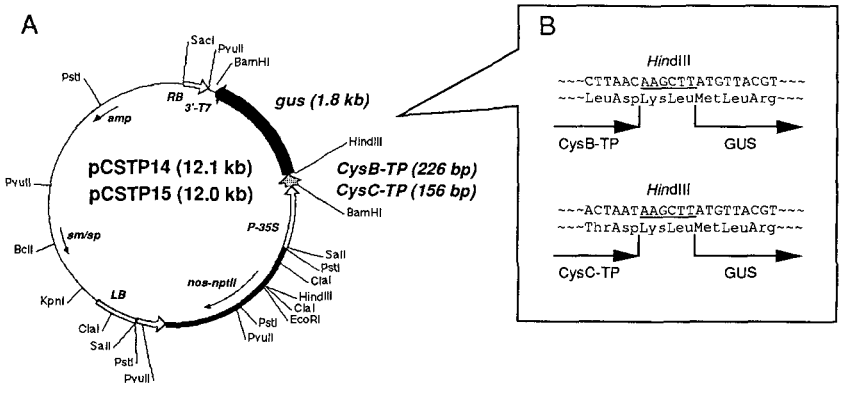

Figure 1. Construction of expression vectors harboring *TP-gus* genes. A, Expression vectors pCSTP14 and pCSTP15 derived from the intermediate vector pHTT202. B, Partial nucleotide and amino acid sequences of translationally fused junctions at the *Hin*dIII sites. *amp*, ampicillin-resistant gene; *CysB-TP*, 226-bp 5′ region of *CysB*; *CysC-TP*, 156-bp 5′ region of *CysC*; *gus*, coding region of GUS; *LB*, left border; *nos-nptII*, neomycin phosphotransferase II gene flanked with the nopaline synthase promoter and terminator; *P-35S*, 35S promoter sequence of cauliflower mosaic virus; *RB*, right border; *sm/sp*, streptomycin/spectinomycin-resistant gene; *3′-T7*, 3′ end of T-DNA-encoded gene 7.

tivity by the methods of Jefferson et al. (1987) with some modifications.

For large-scale preparation of chloroplasts and mitochondria for protease protection assay and western blot analysis, step gradient methods were performed as described previously (Jackson et al., 1979; Mills and Joy, 1980; Baumgartner et al., 1989) with minor modifications.

Protease and Detergent Protection Assay

Chloroplasts isolated from the expanding green leaves of 14–14 tobacco and mitochondria from the calli of 15–19 tobacco were used. Protein concentrations of organelle fractions were adjusted to 1.0 mg mL^{-1}. Incubation was carried out at 25°C for 30 min in the presence and absence of 0.2% Triton X-100 (Sigma) and 1.0 mg mL^{-1} proteinase K (Boehringer Mannheim). Reaction mixtures were kept in osmotic conditions with the grinding buffer. Proteolysis was terminated by the addition of leupeptin (0.2 mM). The remaining organelles were collected after the treatment by centrifugation at 1000*g* for 1 min for chloroplasts and 8000*g* for 3 min for mitochondria. Resultant pellets and supernatants were assayed for GUS activity.

Western Blot Analysis

Subcellular fractions of 14–14 plants containing 5 μg of protein and those of 15–19 plants containing 30 μg of protein were separated by SDS-PAGE. Western blotting and immunostaining were carried out on PVDF membranes (Immobilon P, Millipore) with rabbit anti-GUS serum (Clontech, Palo Alto, CA), phosphatase-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine, and nitroblue tetrazolium chloride (GIBCO-BRL). Rabbit primary antibody was used at 1:400 and 1:100 dilutions for 14–14 plants and 15–19 plants, respectively.

Northern Blot Analysis

Total RNA was extracted in the buffer (200 mM Tris-HCl, pH 9.0, 100 mM NaCl, 10 mM EDTA·2Na, 1.0% SDS, and 14 mM β-mercaptoethanol) by the phenol/SDS method and precipitated by LiCl as described elsewhere (Sambrook et al., 1989). Total RNA (20 μg) was separated under denaturing conditions by formaldehyde-agarose gel electrophoresis and transferred onto nylon membranes (Hybond-N+, Amersham). RNA blots were probed with ^{32}P-labeled DNA probes synthesized by random oligonucleotide priming of cDNA insert fragments of pCS4 (*CysA*) (Saito et al., 1992), pCSB11 (*CysB*) (Saito et al., 1993), and pCSC19 (*CysC*) (Saito et al., 1994). To verify the equivalent amounts of RNA blots, membranes were probed with ^{32}P-labeled rice rDNA (pRR217) (Takaiwa et al., 1985). Hybridization was carried out at 65°C in 5× SSPE (0.9 M NaCl, 0.05 M sodium phosphate, and 5 mM EDTA), 0.5% SDS, 5× Denhardt's solution, and 25 mg mL^{-1} salmon sperm DNA. Washing was conducted at 65°C for 15 min in 0.1× SSPE and 0.1% SDS (Sambrook et al., 1989). Hybridization signals were detected and quantified by a BAS-2000 image analyzer (Fuji Film, Tokyo, Japan).

Spinach Cell Cultures

Spinach cell cultures were grown under continuous light at 25°C in Murashige-Skoog medium (Murashige and Skoog, 1962) containing 0.5 mg L^{-1} naphthalene acetic acid, 1.0 mg L^{-1} 6-BA, and 2% Suc as described by Nakagawa et al. (1985). Nutritional starvation was forced by reducing all sulfate, nitrate, and ammonia elements in the medium to 1% of the Murashige-Skoog salts. The concentrations of metal ion components were maintained by replacement with the chloride compounds.

Miscellaneous Techniques

GUS enzyme activities were determined fluorometrically using 4-methylumbelliferyl β-glucuronide as a substrate by the method of Jefferson et al. (1987) with some modifications. Protein concentration was determined with a kit (Bio-Rad, Tokyo, Japan) using the methods of Bradford (1976).

RESULTS

Expression of TP-GUS Fusion Protein in Tobacco Cells

The 226-bp 5′ region of *CysB* encoding 66 N-terminal amino acids (CysB-TP) and the 156-bp 5′ region of *CysC* encoding 48 N-terminal amino acids (CysC-TP) were fused to the entire coding sequence of a bacterial GUS as a reporter gene (Fig. 1). The N-terminal sequence of CysB-TP used here contained the TP portion of 52 amino acids cleaved out in the mature CysB protein (Saito et al., 1993) and an additional 14 amino acids. For CysC-TP, the corresponding N-terminal part (48 amino acids) deduced from the alignment of *CysA*, *CysB*, and *CysC* (Saito et al., 1994) was used for TP. *TP-gus* fusion genes were expressed in tobacco under the control of the cauliflower mosaic virus 35S promoter as described in "Materials and Methods." Among several kanamycin-resistant and GUS-positive transgenic tobacco regenerants, numbers 14–14 and 15–19, representing CysB-TP-GUS plants and CysC-TP-GUS plants, respectively, were arbitrarily chosen for further analysis. These transformants apparently exhibited no phenotypic changes compared with a wild-type plant.

Subcellular Localization of GUS in Transgenic Tobaccos

For precise analyses of subcellular localization of the reporter gene products, silicasol continuous gradients (Percoll, Pharmacia) formed by ultracentrifugation were used to separate chloroplasts, mitochondria, and other particles having intermediate densities between these two organelles (Figs. 2 and 3). To determine the location of chloroplasts and mitochondria in the gradients, transgenic tobacco plants expressing *TP-gus* fusion genes containing the TP sequences of chloroplastic stroma localizing the Rubisco small subunit from *Pisum sativum* (*rbcSS-TP*) (Van den Broeck et al., 1985) and of the mitochondrial matrix localizing MnSOD of *Nicotiana plumbaginifolia* (*MnSOD-TP*) (Bowler et al., 1989) were analyzed as positive indicators. SOD-1 and SOD-2 in Figure 3 represent the two constructs

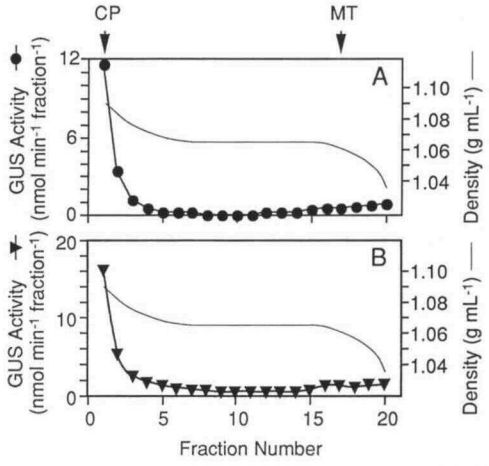

Figure 2. Subcellular distribution of GUS activities in CysB-TP-GUS (A) and rbcSS-TP-GUS (B) transgenic tobaccos. Fraction numbers indicate subcellular fractions collected from the bottom of the gradients. CP, Chloroplasts; MT, mitochondria. Total GUS activities applied onto the gradients: A, 23.0 nmol min^{-1}; B, 40.0 nmol min^{-1}.

with different TPs originating from the two MnSOD isoforms of *N. plumbaginifolia*. Concerning the targeting specificity of the positive indicators, Creissen et al. (1995) recently showed the transport of the foreign protein, glutathione reductase, by these TPs to the appropriate organelles in transgenic tobacco. We have also confirmed that two mitochondrial marker enzymes, fumarase and Cyt *c* oxidase, are localized in the same fraction (number 17) in the gradient.

As shown in Figures 2 and 3, the subcellular distribution pattern of GUS enzyme activities in 14–14 tobacco was the same as that in rbcSS-TP-GUS tobacco and that in 15–19 tobacco was the same as that in MnSOD-TP-GUS tobacco. The absolute coincidence of GUS localization with the positive indicators confirmed correct transportation of TP-GUS fusion proteins to chloroplasts in 14–14 tobacco and to mitochondria in 15–19 tobacco, respectively. In all experiments, 85 to 90% of GUS activities applied on the gradients were recovered after the fractionation, and more than 70% of them were estimated to be localized around the particular fractions (numbers 1 and 17). Transgenic tobacco expressing GUS under the cauliflower mosaic virus 35S promoter without TPs exhibited accumulation of GUS only in the cytosolic fraction as shown by Kavanagh et al. (1988) (data not shown). The results clearly indicated that N-terminal TP sequences of the two CS isoforms used in our experiments were sufficient to transport the passenger protein, GUS, toward specific organelles in vivo.

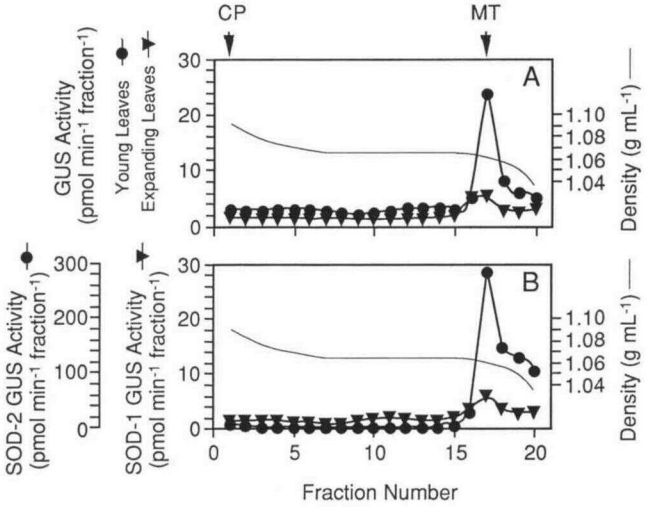

Figure 3. Subcellular distribution of GUS activities in CysC-TP-GUS (A) and MnSOD-TP-GUS (B) transgenic tobaccos. All experimental conditions were the same as reported for Figure 2. Total GUS activities applied onto the gradients: A, 65.0 pmol min^{-1} for young leaves and 28.0 pmol min^{-1} for expanding leaves; B, 25.3 pmol min^{-1} for SOD-1 and 805.0 pmol min^{-1} for SOD-2. SOD-1 and SOD-2 represent different TP structures.

Western blot analyses with rabbit anti-GUS serum exhibited exclusive accumulation of GUS proteins (70 kD) in the chloroplasts of 14–14 tobacco and in the mitochondria of 15–19 tobacco (Fig. 4A). Relative values of immunoreactive signals nearly corresponded to in vitro GUS enzyme activities of each subcellular fraction (Fig. 4B). Expanding green leaves of 14–14 tobacco and calli of 15–19 tobacco were chosen as materials, since the highest activities were found in these tissues. No immunoreactive protein was detected in the mitochondria of 14–14 tobacco or in the plastidic fractions of 15–19 tobacco (including chloroplasts isolated from leaves). The molecular masses of immunoreactive signals were identical with the predicted size (70 kD) of the mature protein, indicating that the correct proteolytic processing of primary TP-GUS translational products occurred in the specific subcellular compartments. The same results were observed in the positive control plants of rbcSS-TP-GUS and MnSOD-TP-GUS constructs (data not shown).

Translocation of GUS in the Interior of Chloroplasts and Mitochondria

To obtain the evidence for the accumulation of mature GUS proteins in the chloroplastic stroma and mitochondrial matrix, protection assays against externally added

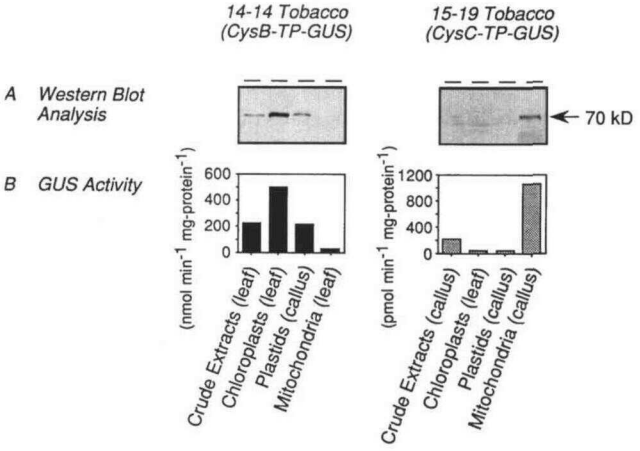

Figure 4. Western blot analysis and GUS enzyme assay of subcellular fractions of transgenic tobacco.

protease and detergents were carried out for the isolated organelles (Fig. 5). Chloroplasts from the expanding green leaves of 14–14 tobacco and mitochondria from the calli of 15–19 tobacco were isolated by step gradients (Percoll, Pharmacia) and were treated with proteinase K with or without Triton X-100 (Sigma).

After the proteinase K treatments, GUS enzyme activities remained in chloroplastic and mitochondrial pellets. The loss of GUS activity in the mitochondrial pellet after the proteinase K treatment was presumably caused by the degradation of BSA, which is necessary to keep the intactness of the isolated mitochondria (Bonner, 1967). When Triton X-100 was added, GUS activities were found in the supernatants; however, they were completely abolished in the presence of proteinase K. These data suggest that enzymatically active GUS accumulates in soluble parts inside the organelles. Therefore, a series of recognition and processing steps required for pre-protein transport to specific subcellular and suborganeller compartments functioned properly for the TP-GUS fusion proteins in transgenic tobacco. We confirmed that CysB-TP and CysC-TP were the specific targeting TPs for chloroplastic stroma and mitochondrial matrix, respectively.

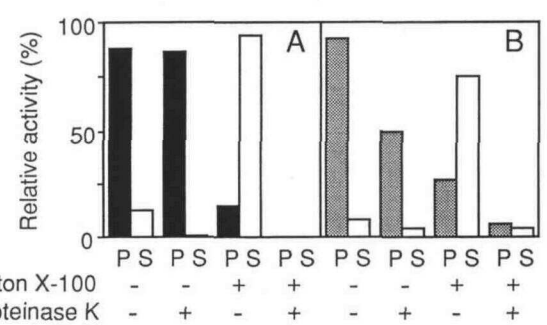

Figure 5. GUS localized in the interior of chloroplasts and mitochondria. Chloroplasts in expanding green leaves of 14–14 tobacco (A) and mitochondria in calli of 15–19 tobacco (B) were isolated and treated with Triton X-100 (0.2%, Sigma) and proteinase K (1.0 mg mL^{-1}) at 25°C for 30 min. GUS activities were assayed for the organelle pellets (P) and the supernatants (S) after the centrifugation of treated chloroplasts at 1000*g* for 1 min and mitochondria at 8000*g* for 3 min. +, Presence of additives; −, absence of additives. A, 543.3 nmol min^{-1} mg^{-1} protein; B, 952.4 pmol min^{-1} mg^{-1} protein.

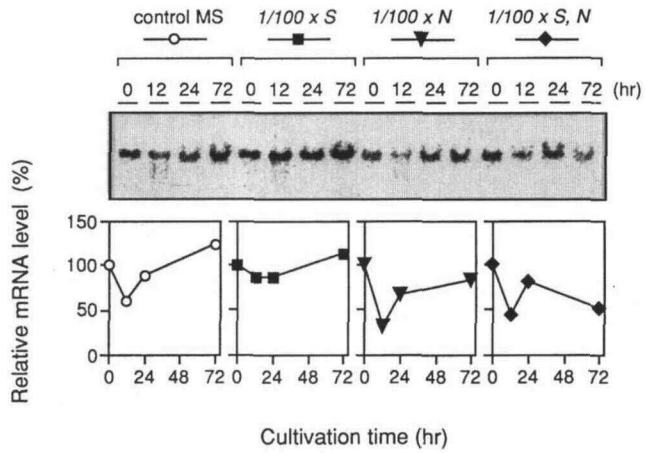

Figure 6. mRNA expression patterns of cytosolic CS (*CysA*) in spinach cell cultures under sulfur and nitrogen starvation. Total RNAs (20 μg) extracted from cell cultures were separated under denaturing conditions, transferred onto the nylon membrane (Hybond-N+, Amersham), and hybridized with the ^{32}P-labeled *CysA* cDNA insert fragment. Hybridization signals were quantified and normalized by the amounts of 25S and 17S rRNAs. MS, Murashige-Skoog medium.

Expression of the Three CS Isoform Genes under Nutritional Stress

As shown above, we determined that *CysB* and *CysC* are chloroplastic and mitochondrial isoforms, respectively, and subsequently confirmed the existence of individual CS enzymes in all subcellular compartments where protein synthesis occurs. Our next interest was in characterizing their functional roles in the specific subcellular compartments.

Northern blot analyses were carried out for *CysA*, *CysB*, and *CysC* genes in spinach cell cultures under various sulfur and nitrogen nutritional conditions. Spinach suspension cells were precultivated for 5 d under the normal condition in Murashige-Skoog medium (Murashige and Skoog, 1962; Nakagawa et al., 1985) and then subjected to nitrogen- and sulfur-deficient stress for 3 d (reducing sulfate and/or nitrate/ammonium levels to 1% of those present in Murashige-Skoog medium). Total RNAs extracted from various cell cultures were probed with cDNAs of *CysA*, *CysB*, and *CysC* (Saito et al., 1992, 1993, 1994). Distinct hybridization signals representing the expression of *Cys* mRNAs were quantified and normalized by the amounts of 25S and 17S rRNAs. No cross-hybridization signals between the three isoforms were observed under high-stringency washing conditions (0.1× SSPE, 0.1% SDS, 65°C, 15 min).

When cells were transferred to fresh Murashige-Skoog medium after 5 d of precultivation, mRNA levels of *CysA* and *CysB* were temporarily decreased to approximately 50 to 60% during short-term (0–24 h) cultivation periods. However, the transcripts in the cells transferred to the sulfate-deficient medium retained approximately 70 to 90%, a 1.2- to 1.5-fold difference compared with those in the case of the control Murashige-Skoog medium (Figs. 6 and 7). During long-term (24–72 h) cultivation periods, mRNA levels of all three *Cys* isoforms in sulfate-starved cells showed no remarkable difference compared with those in the control group.

In nitrogen and nitrogen/sulfur double-deficient medium, more striking effects on the expression of *CysC* were observed (Fig. 8). The mRNA levels of *cysC* reached up to 500% of the original expression level at h 72 in the nitrogen-starved cells and at h 24 in the nitrogen-/sulfur-starved cells. On the contrary, *CysA* and *CysB* transcripts under the same conditions exhibited patterns similar to those of the control Murashige-Skoog medium throughout the stress cultivation periods (Figs. 6 and 7). These results suggest that expression of *CysC* is mainly governed by exogenous nitrogen conditions.

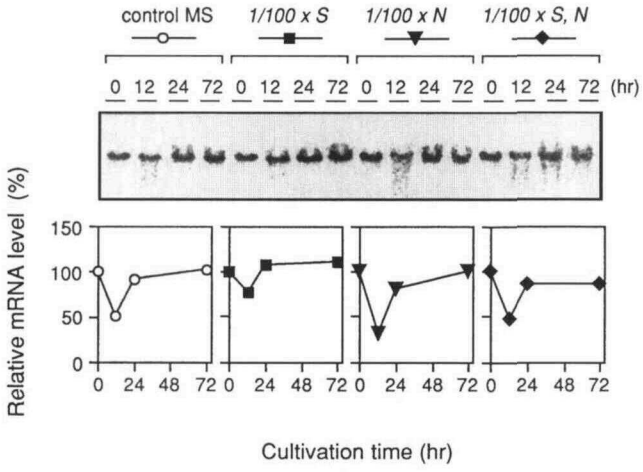

Figure 7. mRNA expression patterns of chloroplastic CS (*CysB*) in spinach cell cultures under sulfur and nitrogen starvation. All experimental conditions were the same as reported for Figure 6 except the membrane was hybridized with the ^{32}P-labeled *CysB* cDNA insert fragment. MS, Murashige-Skoog medium.

DISCUSSION

Biochemical studies have suggested that higher plants possess multiple CS isoforms in different subcellular locations (Lunn et al., 1990). We have recently isolated three distinct cDNAs, *CysA*, *CysB*, and *CysC*, representing the cytoplasmic, chloroplastic/plastidic, and putative mitochondrial CS isoforms, respectively, from spinach (Saito et al., 1992 1993, 1994). These isoforms were considered to be individually responsible for forming Cys for their own protein synthesis in their specific subcellular compartments. This speculation aroused our interest in confirming the subcellular localization of the three isoforms and investigating their physiologic roles.

We first demonstrated the exact subcellular localization of the *CysB* and *CysC* isoforms. The results shown here clearly indicate that the N-terminal leader sequences of 66 (CysB-TP) and 48 (CysC-TP) amino acids were sufficient for the recognition and processing of TP-GUS fusion proteins and for the subsequent accumulation of the enzymatically functional GUS in the specific organelles. These results provided us the in vivo evidence to confirm *CysB* and *CysC* as nuclear-encoded chloroplastic and mitochondrial isoforms, respectively. In transgenic tobaccos, a phenomenon called "dual-targeting" of foreign proteins to chloroplasts and mitochondria has been reported using leader sequences of a yeast mitochondrial Cyt *c* oxidase subunit (Huang et al., 1990) and pea chloroplastic glutathione reductase (Creissen et al., 1995). In our experiments, CysB-TP and CysC-TP showed their own characteristics as targeting TPs for chloroplastic stroma and mitochondrial matrix in transgenic tobaccos. The strict organelle and suborganelle specificity seemed to be caused by the conservation of secondary or tertiary structures of TPs. In fact, CysB-TP and CysC-TP possess typical structural features of chloroplastic and mitochondrial TPs, as we have discussed previously (Saito et al., 1993, 1994).

As mentioned above, specificity of pre-protein transport presumably depends on the characteristics of the individual TPs. Aside from these posttranslational controls of pre-protein transport, accumulation of the functional enzymes required for the development of chloroplasts and mitochondria is presumably controlled mainly by gene expression. Therefore, we next examined the expression of the three *Cys* genes to characterize their physiologic roles and functions in the three specific subcellular compartments. These compartments where CS enzymes localize contain individual sulfur and nitrogen metabolic pathways, which may coordinately regulate the appropriate balance of amino acid and sulfur-containing amino acid production. Since CS is found at the junction of these two metabolic pathways, sulfur and nitrogen nutritional conditions could both have some influence on their gene expression, reflecting the demands of sulfur-containing amino acids in each subcellular compartment. We have characterized the expression patterns of the three genes in sulfur- and nitrogen-starved spinach cell cultures.

Since de novo biosynthesis of Cys is supposed to be done in chloroplasts containing the general sulfate reduction pathway, striking effects of sulfate starvation on the expression of *CysB* were first expected. We found only slight differences (1.2- to 1.5-fold) in the mRNA level of *CysA* and *CysB* in sulfate-starved cells during the starvation periods of 0 to 24 h compared with cells grown in the normal Murashige-Skoog medium. Hell et al. (1994) have demonstrated a 1.5- to 2.0-fold up-regulation of these two *Cys* isoforms in sulfate-starved Arabidopsis seedlings. However, it is questionable that *Cys* genes respond directly to the exogenous sulfate supply, reflecting the demands of Cys production for protein synthesis in these subcellular compartments to maintain cell activities.

The expression pattern of the minor constituent *CysC* was quite different from that of the other two isoforms when cells were subjected to nitrogen-deficient stress. The

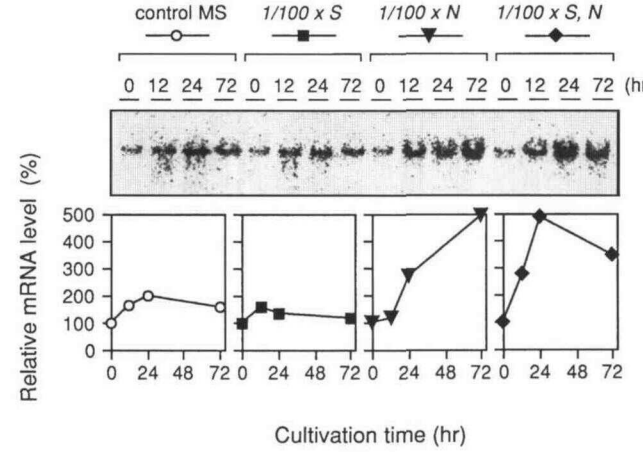

Figure 8. mRNA expression patterns of mitochondrial CS (*CysC*) in spinach cell cultures under sulfur and nitrogen starvation. All experimental conditions were the same as reported for Figure 6 except the membrane was hybridized with the ^{32}P-labeled *CysC* cDNA insert fragment.

mRNA level of *CysC* increased up to 5.0-fold the original level in cells deprived of a nitrogen source and in those deprived of both nitrogen and sulfur, whereas *CysA* and *CysB* transcripts showed no marked changes. Since there is no evidence for the existence of a sulfate reduction pathway in mitochondria, the *CysC* enzyme may utilize sulfide released from catabolism of sulfur-containing storage compounds. Although the changes in *Cys* mRNA levels may not necessarily be translated into changes in the CS enzyme activities in the specific subcellular compartments, it is likely that the accumulation of *CysC* transcripts under nitrogen-deficient growing conditions is caused by the requirement for detoxification of excess amounts of sulfide, presumably released by the breakdown of sulfur-containing storage compounds to supply nitrogen sources.

In conclusion, the present investigation confirmed the exact subcellular location of the three CS isoforms and characterized their functional participation related to sulfur and nitrogen assimilation pathways. We have provided a clue for the investigation of the regulatory mechanism of nitrogen/sulfur assimilation and Cys biosynthesis in plants from the molecular biologic perspective.

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