Cysteine Synthase Overexpression in Tobacco Confers Tolerance to Sulfur-Containing Environmental Pollutants[1]

Masaaki Noji, Maiko Saito, Michimi Nakamura, Mitsuko Aono, Hikaru Saji, and Kazuki Saito*

Graduate School of Pharmaceutical Sciences, Department of Molecular Biology and Biotechnology, Chiba University, Yayoi-cho 1–33, Inage-ku, Chiba 263–8522, Japan (M.N., M.S., M.N., K.S.); and Division of Environmental Biology, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–0053, Japan (M.A., H.S.)

Cysteine (Cys) synthase [*O*-acetyl-L-Ser(thiol)-lyase, EC 4.2.99.8; CSase] is responsible for the final step in biosynthesis of Cys. Transgenic tobacco (*Nicotiana tabacum*; F_1) plants with enhanced CSase activities in the cytosol and in the chloroplasts were generated by cross-fertilization of two transformants expressing cytosolic CSase or chloroplastic CSase. The F_1 transgenic plants were highly tolerant to toxic sulfur dioxide and sulfite. Upon fumigation with 0.1 μL L^{-1} sulfur dioxide, the Cys and glutathione contents in leaves of F_1 plants were increased significantly, but not in leaves of non-transformed control plants. Furthermore, the leaves of F_1 plants exhibited the increased resistance to paraquat, a herbicide generating active oxygen species.

Environmental pollution by sulfur-containing compounds, e.g. sulfur dioxide (SO_2), hydrogen sulfide (H_2S), sulfite (SO_3^{2-}), and sulfate ions (SO_4^{2-}), is a serious problem for the global environment. In particular, gaseous SO_2, which is emitted mainly by natural sources, i.e. microbial activities, volcanoes, etc. and by human activities, i.e. combustion of fossil fuels, industrial refining of sulfur-containing ores, etc., influences human health and the global ecological system of animals and plants (Wellburn, 1994; Murray, 1997). One of the most common types of visible injury to plants caused by SO_2 is foliar damage such as chlorosis and necrosis. Moreover, when the gaseous SO_2 encounters moisture, considerable amounts of SO_2 are converted into sulfite and sulfate. These are important components of acid rain and haze (Wellburn, 1994).

Although sulfur-containing compounds, e.g. SO_2 and SO_3^{2-}, are toxic for plants at higher concentrations, sulfur is also an essential nutrition for plants. The inorganic sulfur in the environment (e.g. SO_4^{2-} in the soil and SO_2 in the air) is assimilated into Cys mainly by the Cys biosynthetic pathway in plants (Saito, 1999, 2000). Cys is incorporated into proteins and glutathione (GSH) or serves as the sulfur donor of Met and sulfur-containing secondary products in plants. Thus, the engineering of this Cys biosynthetic pathway may be promising for development of the transgenic plant tolerant to sulfur-containing pollutants. The Cys biosynthetic pathway involves several enzymatic reactions (Brunold and Rennenberg, 1997; Leustek and Saito, 1999). The SO_4^{2-} is reduced to SO_3^{2-} and then sulfide (S^{2-}) through the sulfate reduction pathway. The final step of Cys biosynthesis is the incorporation of S^{2-} into Cys. The reaction is catalyzed by Cys synthase [*O*-acetyl-L-Ser(thiol)-lyase, EC 4.2.99.8; CSase], which uses S^{2-} and *O*-acetyl-L-Ser as the substrates. This final step of Cys biosynthesis seems to exist necessarily in three major compartments of plant cells, e.g. cytosol, chloroplasts, and mitochondria, since the presence of CSase has been demonstrated in these three compartments from several plants (Brunold and Suter, 1989; Lunn et al., 1990). In spinach leaves the major activity of CSase is localized in cytosol (44%) and chloroplasts (42%), and only 10% of the activity is present in mitochondria (Lunn et al., 1990).

We constructed transgenic tobacco (*Nicotiana tabacum*) carrying spinach cytosolic CSase A cDNA (Saito et al., 1992), designated 3F plants, or chimeric CSase A cDNA fused with the sequence for chloroplast-targeting transit peptide of pea RUBISCO, designated 4F plants (Saito et al., 1994). 3F and 4F showed enhanced CSase activity in the cytosol and in the chloroplasts, respectively. The leaf discs of these transgenic tobaccos showed partial tolerance to SO_3^{2-} (Saito et al., 1994).

In the present study, to obtain transgenic plants highly tolerant to sulfur-containing pollutants, we crossed 3F plants with 4F plants to generate F_1 transgenic tobacco in which CSase activities were enhanced in the cytosol and in the chloroplasts. We found that the tolerance of F_1 plants to sulfur-containing pollutants was enhanced. These plants were also resistant to paraquat. We also discuss the mechanism of tolerance to SO_2 in transgenic plants.

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* Corresponding author; e-mail ksaito@p.chiba-u.ac.jp; fax 81–43–290–2905.

RESULTS

Expression of the CSase in Transgenic Tobacco

We previously constructed transgenic tobacco carrying spinach cytosolic CSase A cDNA (3F plants) or chimeric CSase A cDNA fused with the sequence for chloroplast-targeting transit peptide (4F plants) driven by the cauliflower mosaic virus 35S promoter (Saito et al., 1994). From these two classes of transgenic tobacco plants we selected two transgenic lines, 3F-24 and 4F-15 (Saito et al., 1994) as parents for mating because of their high levels of CSase activities. The homozygous plants of 3F-24 (abbreviated as 3F) and 4F-15 (abbreviated as 4F) were obtained by self-pollination. Then we crossed 3F with 4F to generate the plants of first filial generation, designated F_1 (Fig. 1A). Southern-blot analysis of total DNA isolated from leaves of 3F, 4F, and F_1 showed that 3F and 4F contained one copy and two copies of the introduced gene per haploid, respectively, and F_1 contained three copies of the introduced gene derived from 3F and 4F (data not shown).

To examine the expression of the CSase gene, total RNA isolated from leaves of transgenic tobacco was analyzed by northern hybridization analysis (Fig. 1B). Transcripts of 1.6 kb in length were detected in 3F. The 1.8-kb transcripts from the chimeric transit peptide/CSase gene were accumulated in 4F. F_1 expressed 1.6- and 1.8-kb transcripts descended from 3F and 4F, although the expression level of 1.6-kb transcripts was considerably lower than that of 1.8-kb transcripts.

Cell-free extracts, chloroplast fractions, and cytosol fractions isolated from transgenic plants (3F, 4F, and F_1) were analyzed by western blotting and assaying CSase activity. The accumulation of 34-kD CSase protein detected by western blotting was shown in the cell-free extracts of 3F, 4F, and F_1 (Fig. 2A). The molecular mass of the immunoreactive CSase protein of 4F was the same as that of 3F, suggesting that the pre-CSase with the pea transit peptide was correctly processed in the transgenic tobacco cells. In the extracts of 3F and 4F, the 34-kD band specific to spinach CSase A was detected in cytosolic or in chloroplast fraction, respectively, whereas in the extract of F_1, the 34-kD band was detected in cytosol and in chloroplasts. CSase activity in the cell-free extract of F_1 was about 5-fold higher than that of non-transformant (SR1; Fig. 2B). In the chloroplast fraction, CSase activity of F_1 was at the same level as that of 4F, and was about 6-fold higher than those of 3F and non-transformant. In the cytosol fraction, CSase activity of F_1 was at the same level as that of 3F, and was about 4-fold higher than those of 4F and non-transformant. These results indicated that F_1, in which CSase activities were enhanced in the chloroplasts and in the cytosol, had the highest activity of CSase in these transgenic tobacco plants.

Tolerance to Sulfur-Containing Pollutants

We examined the resistance of these transgenic plants to sulfur-containing pollutants. The transgenic plants were used for fumigation experiments with high levels of SO_2. We measured a quantum yield of photosynthetic activity of leaves of transgenic plants before SO_2 fumigation and after a 3-h fumigation with 1 $\mu L\ L^{-1}$ SO_2 (Fig. 3). Before fumigation, the

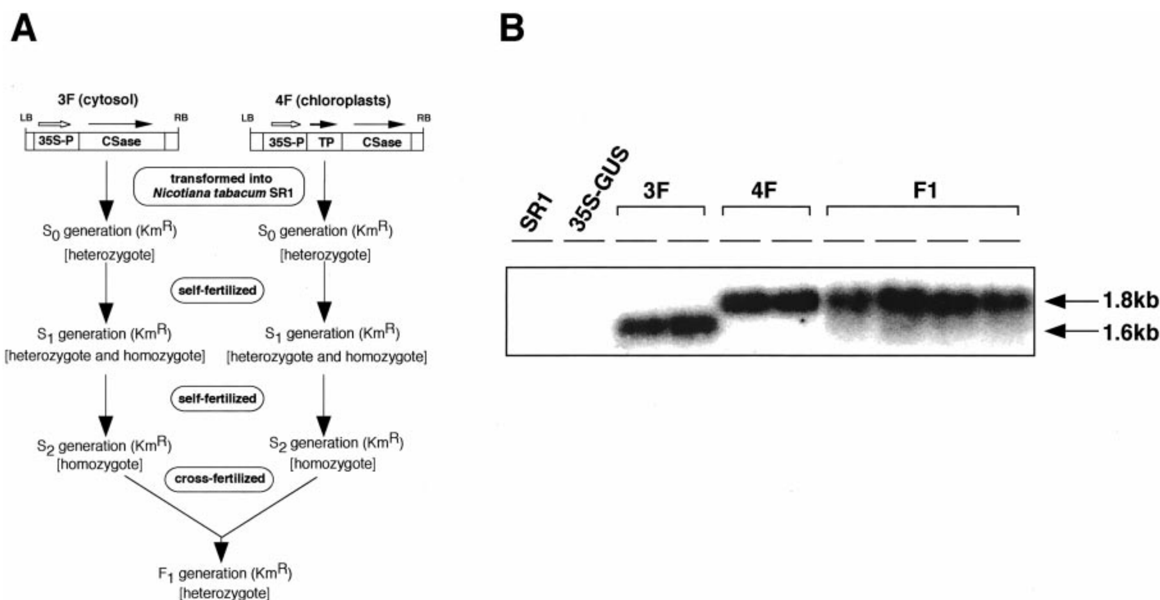


Figure 1. A, Construction of transgenic plants overexpressing CSase. 35S-P, Cauliflower mosaic virus 35S RNA promoter; CSase, CSase transgene from spinach; TP, the chloroplast-targeting transit peptide of pea RUBISCO. B, Northern-blot analysis of total RNA of transgenic plants. SR1, Non-transformed tobacco (cv SR1); 35S-β-glucuronidase (GUS), tobacco plants transformed with a bacterial *UidA* gene encoding GUS using as a negative control.

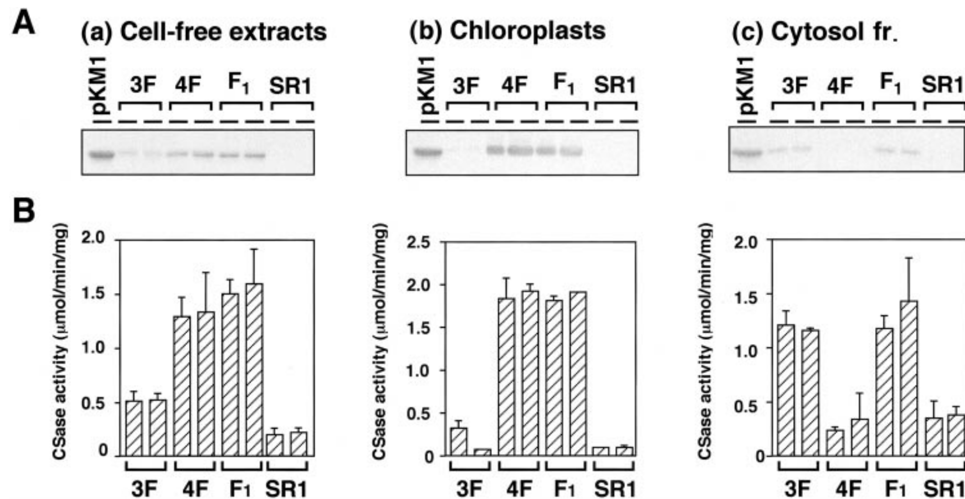


Figure 2. Expression analysis of spinach CSase in subcellular fractions of transgenic plants. A, For western blotting, 16 μg of protein were separated by 12% (w/v) SDS-PAGE, transferred onto an Immobilon P membrane, and then localized by immunostaining using rabbit anti-spinach CSase A serum. B, For the enzyme assay, the activities of CSase were determined in each extract from fully expanded leaves of transgenic plants. SR1, Non-transformed tobacco (cv SR1); pKM1/NK3, bacterial extract expressing Spinach CSase A as a positive control (CSase activity was not determined). Data are the means of triplicate analyses ± SD.

quantum yields of the leaves were at the same level for all transgenic plants. After a 3-h fumigation of SO_2, the quantum yields of F_1 leaves were significantly higher than those of control ($P < 0.005$) and 3F ($P < 0.05$) plants. The quantum yield of F_1 exhibited the higher trend than that of 4F plant, although the difference was not statistically significant.

Since SO_2 absorbed by plants changes into SO_3^{2-} rapidly, the toxicity of SO_2 can be reproduced by SO_3^{2-}. Therefore, leaf discs of transgenic tobacco were cultured with 20 mM SO_3^{2-} under constant illumination. After cultivation for 54 h, the leaf discs of F_1 showed a tolerance to 20 mM SO_3^{2-}, whereas the leaf discs of non-transformed tobacco (cv SR1) were seriously damaged, and those of 3F and 4F were partially damaged (Fig. 4A). Then we determined the remaining chlorophyll of each leaf disc after this treatment (Fig. 4B). The F_1 plant showed significantly higher resistance to SO_3^{2-} (higher percentage of residual chlorophyll) as compared with the non-transformed control and the parents (3F and 4F). Taken together, these results may suggest that F_1 plants obtain the high tolerance to SO_3^{2-} and SO_2 by overexpressing CSase in cytosol and in chloroplasts.

Analysis of Cys and GSH Levels in Transgenic Plants

We exposed the transgenic plants to lower level of SO_2, namely 0.1 μL L^{-1} for 4 d, and then measured the contents of Cys and GSH in leaves. After the fumigation with 0.1 μL L^{-1} SO_2 for 4 d, there was no visible damage on the leaves of control or transgenic plants (data not shown). However, Cys contents in F_1 plants after a 4-d fumigation was increased significantly (Fig. 5A). GSH contents in F_1 and 3F plants were also increased significantly after a 2-d fumigation (Fig. 5B). Cys and GSH contents were not changed in control plants (Fig. 5, A and B). An unexpected finding was that Cys and GSH contents in 4F plants were not increased during the fumigation. These results suggest that transgenic plants overexpressing CSase, especially overexpressing in cytosol, can fix the atmospheric SO_2 into Cys and GSH more efficiently than the control plant, since the contents of Cys and GSH in the control plant were unchanged during the fumigation with 0.1 μL L^{-1} SO_2.

Effect of Photooxidative Stress on Transgenic Plants

The toxicity of SO_2 is thought to result from generation of active oxygen species (Shimazaki et al., 1980). To investigate the enhanced tolerance of transgenic plants to active oxygen species we treated the leaf discs of transgenic plants with paraquat. Paraquat is a reagent that generates active oxygen species in chloroplasts under constant illumination (Dodge, 1975). After a 40-h cultivation with 2 μM paraquat, the leaf discs of F_1 again showed the highest resistance to paraquat (Fig. 6A). We determined the remaining chlorophyll of each leaf disc after the paraquat treatment (Fig. 6B). The F_1 plant showed significantly higher resistance to paraquat (higher percentage of residual chlorophyll) as compared with the control and the parents (3F and 4F). The activities of enzymes involved in scavenging active oxygen species in plants (superoxide dismutase, catalase, and ascorbate peroxidase) were at the same level among control and CSase overexpressing plants;

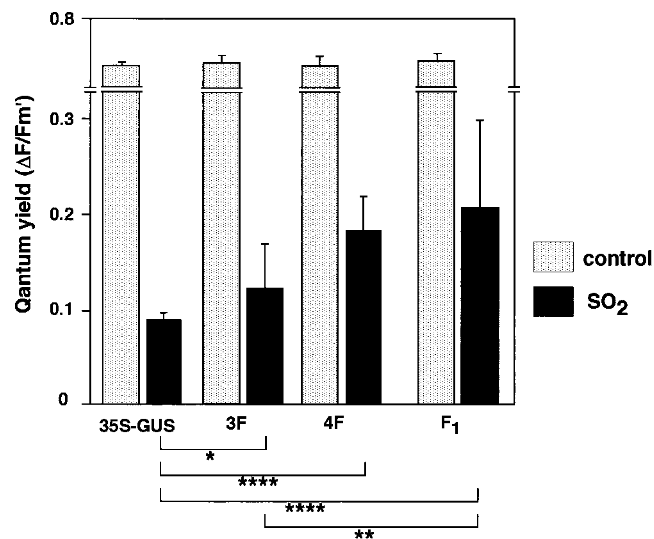


Figure 3. Photosynthetic quantum yield of transgenic plants exposed to SO_2. Six transgenic plants from each transgenic line were exposed to 1 μL L^{-1} SO_2 for 3 h. The third leaves from the top of transgenic plants were assayed for quantum yield. The differences in the quantum yield among transgenic plants were statistically significant by Student's *t* test (*, $P < 0.1$; **, $P < 0.05$; ****, $P < 0.005$). Data are the means of analyses of six plants ± SD. 35S-GUS, Tobacco plants transformed with a bacterial *UidA* gene encoding GUS using as a negative control.

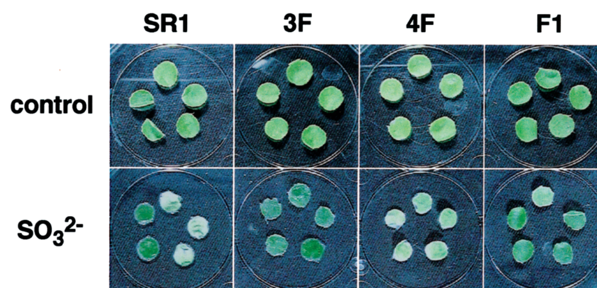
moreover, the activities of these enzymes never changed after a 12-h cultivation with 1 μM paraquat (data not shown).

DISCUSSION

We generated transgenic tobacco plants (F_1) in which CSase activities were enhanced in the chloroplasts and in the cytosol. The F_1 transgenic tobacco showed higher tolerance to sulfur-containing pollutants, i.e. SO_3^{2-}, than the parent plants (3F and 4F) and the control tobacco.

To reveal the mechanism of resistance to SO_2 in transgenic plants we exposed the plants to 0.1 μL L^{-1} SO_2 and then measured the contents of Cys and GSH in the leaves. During the SO_2 fumigation, Cys and GSH contents were increased significantly in F_1 plants, but not in control plants (Fig. 5), suggesting that the F_1 transgenic plants may fix the atmospheric SO_2 into Cys and GSH more efficiently than the control plants. Thus, the tolerance to SO_2 may be caused by the efficient assimilation of sulfur into Cys and GSH. After a fumigation time of 4 d, there is no difference in the GSH content between 3F, 4F, and F_1. A 4-d fumigation of 0.1 μL L^{-1} SO_2 may cause some physiological damage to these transgenic plants even though no visible damage was not observed on the leaves of transgenic plants after a 4-d-fumigation. Since the level of SO_2 normally found in the atmosphere is less than 0.04 μL L^{-1}, 0.1 μL L^{-1} SO_2 may be sufficient to cause the physiological damage to the plants despite no visible damage observed.

Since SO_2 is highly soluble in water, SO_2 absorbed by plants changes into SO_3^{2-} rapidly. Some part of SO_3^{2-} is used for the biosynthesis of Cys, and the rest is changed into SO_4^{2-} by oxidation in a light-dependent manner. Produced SO_4^{2-} are stored in vacuoles and are subsequently used for the biosynthesis of Cys (Rennenberg, 1984). During oxidation of SO_3^{2-} to SO_4^{2-} in plant cells, active oxygen species are produced as secondary toxic substances (Cohen et al., 1973). Therefore, the toxicity of SO_2 may be due to the generation of the active oxygen species. In fact, transgenic tobacco in which the ability to scavenge the active oxygen species was enhanced by express-



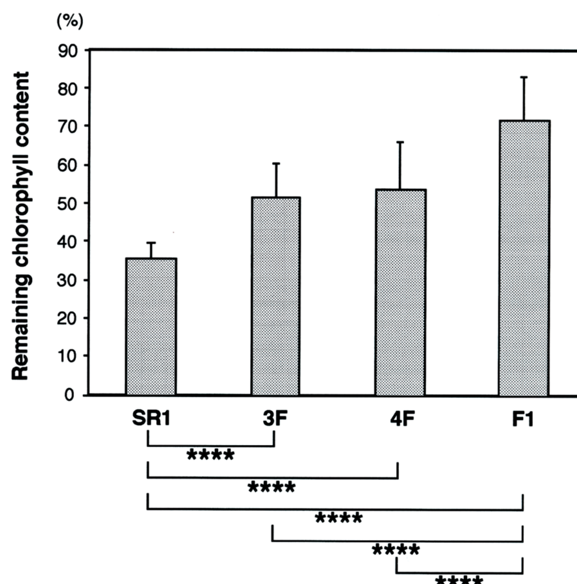
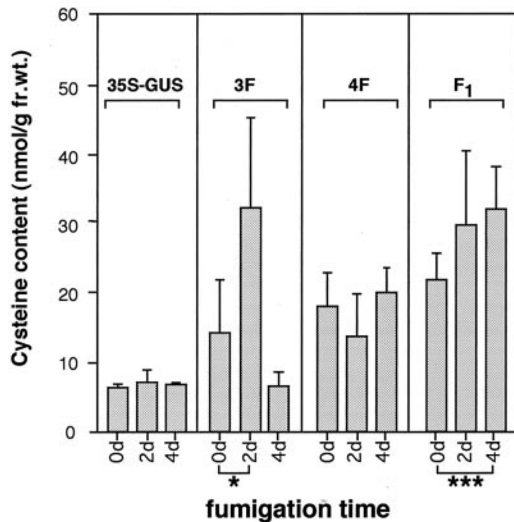


Figure 4. Resistance to SO_3^{2-} toxicity of leaf discs of transgenic plants. A, Photographs of the leaf discs cultivated on A1 medium with 20 mM sodium sulfite (bottom layer) and on A1 medium (top layer) for 54 h at 25°C under light (25 μE m^{-2} s^{-1}). SR1, Non-transformed tobacco (cv SR1). B, Resistance is shown as a percentage of remaining chlorophyll content in a leaf disc after the cultivation in the presence of 20 mM sodium sulfite. The differences in the remaining chlorophyll content among control and transgenic plants were statistically significant by Student's *t* test (****, $P < 0.005$). Data are the means of analyses of 10 leaf discs ± SD.



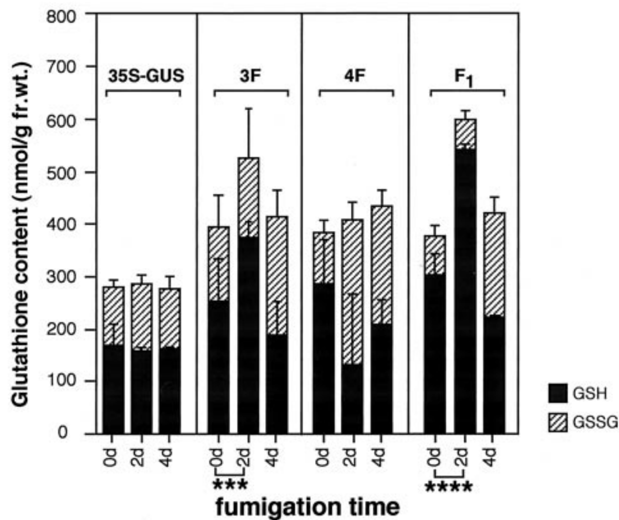
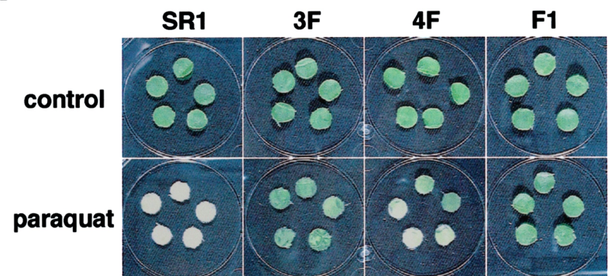


Figure 5. Contents of Cys and GSH in transgenic plants exposed to 0.1 μL L^{-1} SO_2. Six transgenic plants from each transgenic line were exposed to 0.1 μL L^{-1} SO_2 for 4 d. The third leaves from the top of the transgenic plants were prepared and immediately subjected to quantification of the sulfhydryl compounds. The differences of the thiol content between 0- and 2- or 4-d treatment were analyzed statistically using Student's *t* test. (*, $P < 0.1$; ***, $P < 0.025$; ****, $P < 0.005$). Data are the means of triplicate analyses ± SD. 35S-GUS, Tobacco plants transformed with a bacterial *UidA* gene encoding GUS using as a negative control.

ing a bacterial GSH reductase gene in chloroplasts showed tolerance to SO_2 (Aono et al., 1993). GSH reductase is involved in the ascorbate-GSH cycle. This cycle is an enzymatic-scavenging mechanism for the removal of active oxygen species (De Kok and Stulen, 1993).

F_1 transgenic tobacco also showed high tolerance to paraquat, which generates active oxygen species. The activities of enzymes involved in scavenging active oxygen species, however, were not enhanced in F_1 plant. GSH levels respond to the availability of Cys (Strohm et al., 1995), therefore, F_1 possessing high activity of CSase was expected to have high ability to synthesize GSH. The increased GSH is most likely to be involved in the detoxification of active oxygen; accordingly, F_1 showed a high tolerance to active oxygen species. Taken together, these results suggest that the tolerance of F_1 plants to sulfur-containing pollutants may be caused not only by the efficient assimilation of sulfur into Cys, but also by the detoxification of active oxygen species by GSH.

A part of SO_2 absorbed by plants is emitted as H_2S (Stulen and De Kok, 1993); however, strictly speaking, this system may not be the detoxification mechanism of SO_2 because H_2S itself is a toxic substance to



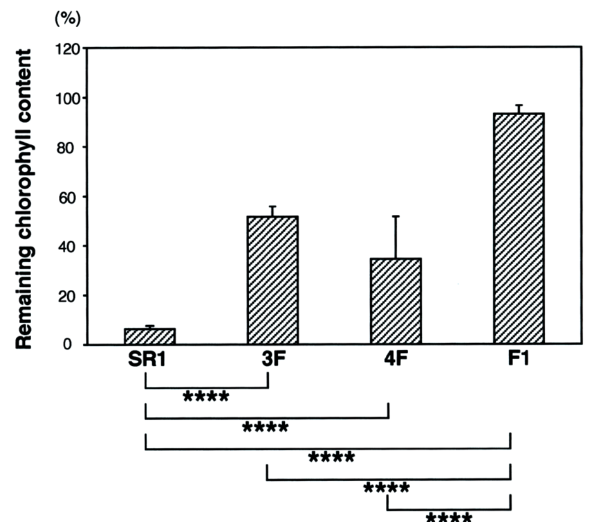


Figure 6. Effects of paraquat on leaf discs of transgenic tobacco. A, Photographs of the leaf discs cultivated on solutions with 2 μM paraquat (bottom layer) and without paraquat (top layer) for 40 h at 25°C under light (25 μE m^{-2} s^{-1}). SR1, Non-transformed tobacco (cv SR1). B, Resistance is shown as a percentage of remaining chlorophyll content in a leaf disc after the treatment of 2 μM paraquat. The differences in the remaining chlorophyll content among control and transgenic plants were statistically significant by Student's *t* test (****, $P < 0.005$). Data are the means of analyses of five leaf discs ± SD.

plants and animals (Wellburn, 1994). There is a report that the enhanced activity of cytosolic CSase resulted in the increased resistance to H_2S (Youssefian et al., 1993). Therefore, the Cys biosynthetic pathway may be considered as the sole detoxification mechanism of SO_2, and the enhancement of the ability of Cys synthesis in plants may be the best way to produce the transgenic plant tolerant to SO_2. Our observations suggest that the transgenic plants in which the ability of Cys synthesis has been enhanced in the cytosol and in the chloroplasts by overexpressing CSase may be applied to produce the transgenic plants resistant to oxidative stress caused by the photochemical oxidant such as ozone.

MATERIALS AND METHODS

Plant Growth and Cross Fertilization

The transgenic plants were grown on A1 agar medium (one-half-strength Murashige and Skoog salts [Murashige and Skoog, 1962], 1% [w/v] Suc, and 0.8% [w/v] agar, pH5.7) containing kanamycin (100 mg/L) under 16-h light (25 μE m^{-2} s^{-1}) and 8-h dark cycles at 25°C. For fertilization and fumigation of SO_2, transgenic plants were transferred to vermiculite after a 4-week cultivation on A1 agar medium, and they were then watered with 1,000-fold diluted Hyponex 5-10-5 (Hyponex-Japan, Osaka). Cross-fertilization was performed with the pollen of homozygous plants of 3F-24 and the pistils of homozygous plants of 4F-15.

Northern-Blot Analysis

Isolation of total RNA from the leaves of 8-week-old plants and northern-blot analysis were performed as described previously (Saito et al., 1991). Twenty micrograms of total RNA was separated under denaturing conditions on a 1.2% (w/v) agarose gel containing formaldehyde, transferred to a nylon membrane, and then hybridized with a ^{32}P-labeled DNA fragment of spinach CSase A coding region. The final washing was performed in 0.1× SSPE (1× SSPE = 0.18 M NaCl, 0.01 M sodium phosphate, pH 7.7, and 1 mM Na_2-EDTA) and 0.1% (w/v) SDS at 65°C for 15 min (Sambrook et al., 1989).

Protein Extraction, Fractionation of Crude Chloroplasts and Crude Cytosol, and Expression Analysis

Leaves from 10-week-old plants were lightly homogenized in an extraction buffer (330 mM sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 2 mM sodium isoascorbic acid, and 50 mM MES [2-(*N*-morpholino)-ethanesulfonic acid], pH 6.1), and then the resulting extracts were filtered with nylon mesh (508 mesh). The filtrate was used as cell-free extracts. Crude cytosol fraction was obtained from the cell-free extracts by centrifugation at 1,000*g* for 100 s. The resulting supernatant was used as the crude cytosol fraction, and the resulting precipitate was resuspended in a resuspension buffer (330 mM sorbitol, 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.9). After the centrifugation at 1,000*g* for 100 s, the precipitate was resuspended in 200 mM K-Pi buffer, which was used as the crude chloroplast fraction. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Proteins (16 μg) in these extracts were used for western blotting. Western-blot and immunostaining analyses were carried out on an Immobilon P membrane (Millipore, Bedford, MA) as reported (Saito et al., 1991). The rabbit anti-spinach CSase A serum (Saito et al., 1992) was used at a 1:400 dilution as a primary antibody. The immunoreactive protein was visualized using phosphatase-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine/nitroblue tetrazolium chloride (Gibco-BRL, Cleveland) as substrates. The enzymatic activity of CSase was determined in the reaction mixture (0.5 mL) containing 50 mM potassium phosphate, pH 8.0, 4 mM Na_2S, 12.5 mM *O*-acetyl-L-Ser, and the enzyme solution. The incubation was performed at 30°C for 10 min and was terminated by the addition of 7.5% (w/v) trichloroacetic acid. The amount of Cys in the resulting mixture was spectrophotometrically determined by the acid-ninhydrin method at 560 nm (Gaitonde, 1967).

Exposure of Plants to SO_2

Six-week-old transgenic plants were exposed to 1.0 μL L^{-1} SO_2 for 7 h or 0.1 μL L^{-1} SO_2 for 4 d under light (400 μE m^{-2} s^{-1}) at 25°C with a relative humidity of 70%. The quantum yield was determined by PAM 2000 (Walz, Effeltrich, Germany).

Exposure of Leaf Discs to SO_3^{2-}

Leaf discs (7 mm in diameter) from 16-week-old transgenic plants were cultured in A1 medium with 20 mM sodium sulfite for 54 h under constant illumination (25 μE m^{-2} s^{-1}).

Determination of Chlorophyll

The contents of chlorophyll in the leaf discs were determined as described previously (Saito et al., 1989). Leaf disc was homogenized and extracted with 80% (w/v) ethanol. After centrifugation at 3,000 rpm for 5 min, the chlorophyll in the supernatant was quantified fluorophotometrically at an excitation wavelength of 413 nm and an emission wavelength of 672 nm. The values of remaining chlorophyll contents after the stress treatment were determined as follows: the amount of chlorophyll per leaf disc (7 mm in diameter) treated with SO_3^{2-} or paraquat was divided by the amount of chlorophyll per leaf disc treated without sulfite or paraquat and was expressed as a percentage.

Determination of Cys and GSH

Quantitative analyses of monobromobimane derivatives of reduced forms of Cys and GSH were performed by

HPLC (Anderson, 1985; Fahey and Newton, 1987). The frozen leaf was ground in a mortar. Then, 2 volumes of extraction buffer (0.1 N HCl containing 4 μM *N*-acetyl-Cys as an internal standard) were added and grinding was continued. Twenty microliters of extract was reacted with 2 μL of 1 mM monobromobimane in acetonitrile and 10 μL of 1 M *N*-ethylmorpholine for 20 min at 37°C in the dark. The labeling reaction was terminated by the addition of 8 μL of acetic acid, and then the resulting solution was subjected to HPLC analysis. HPLC was carried out as previously described (Saito et al., 1994). The oxidized form of GSH were determined using the following procedure. After the assay of the reduced form of GSH, the rest of extract from the leaves was reduced by dithiothreitol (1 mM final concentration), and was then labeled with monobromobimane and analyzed by HPLC. The difference between the quantity of GSH from this assay and the quantity of GSH that had been determined previously was considered as the quantity of oxidized form of GSH.

Treatment of Leaf Discs with Paraquat

Discs with a diameter of 7 mm excised from leaves of 16-week-old transgenic plants were soaked in the solution that contained 2 μM paraquat (methyl viologen, 1, 1′-dimethyl-4, 4′-bipyridinium dichloride; Sigma, St. Louis) and 0.1% (w/v) Tween 20. They were placed under light (25 $\mu E\ m^{-2}\ s^{-1}$) at 25°C for 24 h, and they were then examined visually for damage.

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