Journal of Plant Biology, June 2004, 47(2) : 111-116

Increased Tolerance to Methyl Viologen by Transgenic Tobacco Plants that Over-express the Cytosolic Glutathione Reductase Gene from *Brassica campestris*

Hyoshin Lee[1] and Jinki Jo[2]*

[1]Biotechnology Division, Korea Forest Research Institute, Suwon 441-350, Korea
[2]Department of Animal Science and Biotechnology, College of Agriculture and Life Science, Kyungpook National University, Daegu 702-701, Korea

We placed *BcGR1*, a Chinese cabbage (*Brassica campestris* var. *Pekinensis*) gene that encodes cytosolic glutathione reductase (GR), under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This gene was then introduced into tobacco plants via *Agrobacterium*-mediated transformation. Segregation and northern analyses of the transgenic plants revealed no relationship between the copy number of the introduced gene and levels of *BcGR1* transcript. Homozygous lines containing *BcGR1* were generated and tested for their acquisition of increased tolerance to oxidative stress. When 10-d-old transgenic tobacco seedlings were treated with 5 to 20 μM methyl viologen (MV), they showed significantly increased tolerance compared with the wild-type seedlings. The most drastic difference was observed at a concentration of 10 μM MV. In addition, when leaf discs were subjected to MV, the transgenic plants were less damaged than the wild types with regard to their electrical conductivity and chlorophyll content.

Keywords: *Brassica campestris*, glutathione reductase, methyl viologen, oxidative stress, transgenic tobacco plant

Environmental stresses such as ozone, drought, extreme temperatures, and heavy metals are the major limiting factors in plant productivity. Much of the injury caused by stress exposure is associated with oxidative damage because of the toxicity of reactive oxygen species (ROS), e.g., superoxide, H_2O_2, and hydroxyl radicals, at the cellular level (Allen, 1995). Aerobic organisms are constantly exposed to the toxic effects of ROS, which are a by-product of normal cell metabolism. Plants have developed cellular protective mechanisms against them, including ROS-scavenging enzymes and low-molecular-weight antioxidants such as ascorbate, glutathione (GSH), and α-tocopherol (Creissen et al., 1994). GSH, an essential component of these defense mechanisms, is involved in the storage and transport of sulfur, regulation of gene expression, detoxification of xenobiotics, and regeneration of ascorbate as a donor of reducing equivalents, in addition to its antioxidant function (Alscher, 1989; Creissen et al., 1994). Glutathione reductase (GR; E.C. 7.8.5.1) is widely distributed in all organisms and catalyzes the reduction of oxidized glutathione (GSSG) to GSH, with NADPH as an electron donor (Creissen et al., 1994). Thus, GR is important in maintaining the redox balance in cells.

GR activity can rise in response to a number of environmental or xenobiotic stresses, such as cold, drought, greening, MV, ozone, or the combination of magnesium deficiency with high light intensity (Gamble and Burke, 1984; Tanaka et al., 1988; Cakmak and Marschner, 1992; Madamanchi et al., 1992; Edwards et al., 1994). However, expression of the chloroplastic GR gene in pea and *Arabidopsis* is not increased by oxidative stress (Edwards et al., 1994; Karpinski et al., 1997).

In previous research to understand how changes in GR activity are related to this response, we reported that expression of the cytosolic GR gene *BcGR1* in *Brassica campestris* was induced rapidly by oxidative stress, e.g., ozone and MV (Lee et al., 1998, 2002). We also postulated that this increase was correlated with greater stress tolerance. To investigate the possible physiological functions of cytosolic GR *in vivo*, we have now produced transgenic tobacco plants that

*Corresponding author; fax +82-53-950-6750
e-mail jkjo@knu.ac.kr

Abbreviations: CaMV, cauliflower mosaic virus; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; MV, methyl viologen; ROS, reactive oxygen species

over-express *BcGR1*. In this report, we describe our evaluation of these plants, their inheritance of the *BcGR1* gene, and their enhanced tolerance to MV.

MATERIALS AND METHODS

Vector Construction and Tobacco Transformation

Full-length *BcGR1* cDNA (Lee et al., 1998) was subcloned into the *Sma*I site of the binary vector pBKS1-1, and introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Horsch et al., 1978). Leaf discs of tobacco (*Nicotiana tabacum* L. cv. Samsun) were transformed as described by Horsch et al. (1978). The resultant transgenic plants were regenerated on an MS medium supplemented with 0.1 mg L^{-1} NAA, 1 mg L^{-1} BAP, 100 mg L^{-1} kanamycin, and 250 mg L^{-1} cefotaxime, and were rooted on an MS medium containing kanamycin but no plant growth regulators. Thirty-two independent T_0 transformants were regenerated, then selfed to produce T_1 seeds. Afterward, seven T_1 plants were selfed to produce T_2 seeds. For our segregation analysis, the T_1 and T_2 seeds were surface-sterilized and sown in Petri plates containing an MS medium supplemented with 200 mg L^{-1} kanamycin. The number of green kanamycin-resistant seedlings and bleached kanamycin-sensitive plants were scored three weeks after sowing.

PCR Analysis

Genomic DNA was isolated from leaves as described by Murray and Thompson (1980), and 100 ng of each sample was subjected to PCR analysis. PCR was conducted with primers specific for either the 35S promoter or *BcGR1* cDNA. The four primers included: 35S sense-1 (5'-TTCAACAAAGGGTAATATCCGG-3'), 35S sense-2 (5'-CCCACCCACGAGGAGCATC-3'), 35S antisense-1 (5'-CGAACGATAGTGGGATTGTGC-3'), and GR antisense-1 (5'-CTAGCATCCTCAAGTTCACC-3'). The following settings were used with 1 U of Taq DNA polymerase (Takara, Japan): denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Thirty cycles of amplification were performed.

Southern and Northern Blot Analyses

Genomic DNA (10 μg) was digested with *Xba*I and *Pst*I, separated by electrophoresis on a 0.8% agarose gel, and blotted onto a nylon membrane (Nytran-Plus, Schleicher and Schuell, Germany) by the capillary transfer method (Southern, 1975). The membrane was hybridized with a ^{32}P-labeled full-length *BcGR1* cDNA probe, washed with 0.2×SSC and 0.1% SDS at 55°C for 1 h, and then autoradiographed.

For our northern blot analysis, total RNA was isolated from leaves according to the guanidine thiocyanate method (McGookin, 1984). RNA was quantified by measuring the A_{260}, and samples containing 10 μg of total RNA were fractionated on a 1.2% formaldehyde agarose gel. Gels were blotted onto Hybond-N nylon membranes (Amersham, UK) with 10×SSC. Hybridization was performed as above.

Immunoblot Analysis

Leaf samples (300 mg) were ground into fine powder in a mortar with liquid nitrogen, and proteins were extracted with a 1 mL extraction buffer containing 50 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone, 300 mM 2-mercaptoethanol, 2 mM PMSF, and 10% glycerol. After the extracted samples were centrifuged at 12,000*g* for 30 min at 4°C, the supernatants were removed and used for immunoblot analysis. Protein extracts equivalent to 20 μg protein were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Micron Separation, USA). Rabbit antiserum raised against the BcGR1 protein was used at a dilution of 1:2000. Bound antibodies were detected with an HRP-linked anti-rabbit IgG system (Cell Signaling, USA).

Identification of Oxidative Stress Tolerance

Seeds of the transgenic and wild-type tobacco plants were surface-sterilized and plated on MS media. The plates were then transferred to a growth chamber set at 28°C, with long-day conditions (16 h light) under white light illumination at 250 μmol $m^{-2}s^{-1}$. For our stress treatments, axenically grown 10-d-old seedlings of approximately equal size were submerged in 5, 10, 15, or 20 μM MV for 15 min, then incubated in the growth chamber.

Determination of Electrolyte Leakage

MV damage was assessed as described by Yun et al. (2000). Fifty leaf discs (6 mm diameter), prepared from the leaves of five plants, were floated on Petri plates containing 17 mL of 0, 5, 10, or 15 μM MV solutions. After incubating in the dark for 12 h at

25°C, the plates were illuminated at 250 μmol $m^{-2}s^{-1}$ until being analyzed for cell leakage. We used an Orion model 130 conductivity meter to measure initial conductivity of the decanted solution and total ionic conductivity after boiling the sample for 15 min. Percent leakage was determined by dividing initial conductivity by total conductivity.

Determination of Chlorophyll Content

Leaf discs were treated with MV, as above, for 10 h in the light. They were then ground in liquid nitrogen using a mortar and pestle, and samples were extracted with 95% (v/v) ethanol. Pigments were quantified according to the method of Lichtenthaler (1987).

RESULTS AND DISCUSSION

Expression of *BcGR1* in Transgenic Plants

We subcloned full-length cytosolic GR cDNA from *B. campestris* (*BcGR1*; Lee et al., 1998) into the plant expression vector pBKS1-1 under the control of the CaMV 35S promoter. This was then used for *Agrobacterium*-mediated transformation of *N. tabacum*. Transgenic plants were selected with kanamycin, and 32 independent tobacco plants were regenerated. These plants did not differ conspicuously in phenotype from the untransformed wild types. To detect the presence of the 35S promoter and *BcGR1* cDNA, the transgenic plants were amplified by PCR, as performed with internal primers (Fig. 1). Southern blot hybridization of the genomic DNA was also conducted, using native *BcGR1* cDNA as a probe (data not shown). Both analyses confirmed that all 32 independent tobacco plants selected had been transformed as expected.

Total RNAs from both transgenic and wild-type plants were subjected to northern blot analysis. *BcGR1* was expressed, although at varying levels, in all transgenic (T_0) plants (Fig. 2A). In contrast, neither the Southern nor the northern blot analyses of the wild types revealed any hybridization bands with *BcGR1*, a result of having as little as 40 to 50% homology between that gene and the indigenous tobacco GR genes (Creissen and Mullineaux, 1995). We obtained the same results from our immunoblot analysis of the transgenic and wild-type tobacco plants when antiserum was raised against the BcGR1 protein (Fig. 2D).

To establish the homozygous lines, 32 independent

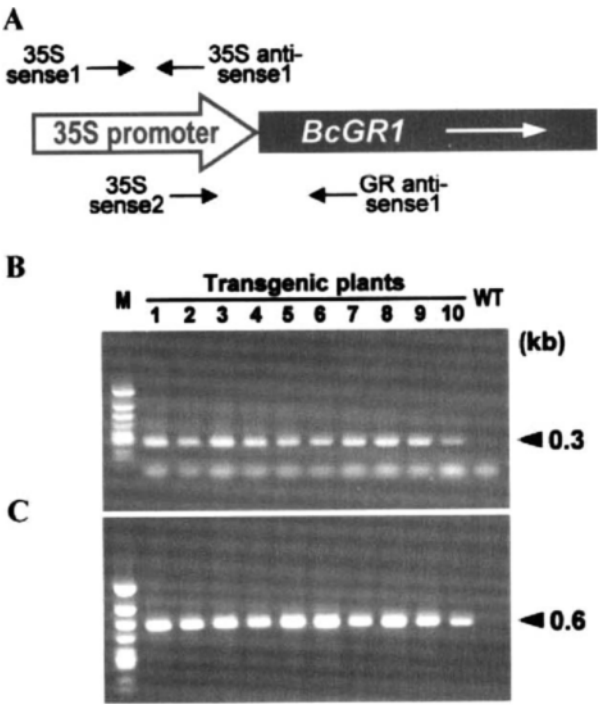

Figure 1. PCR analysis of transgenic tobacco plants. **A.** Schematic diagram for PCR amplification of 35S promoter and *BcGR1* cDNA fragment for identification of transformation. **B.** PCR amplification with 35S sense1 and 35S antisense1 primers. **C.** PCR amplification with 35S sense2 and GR antisense1 primers. M, DNA size marker (100-bp ladder); WT, wild-type. Numbers indicate independent transgenic lines.

T_0 plants were selfed to produce T_1 seeds and 7 T_1 plants were selfed to produce T_2 seeds. Kanamycin susceptibility of the T_1 seeds demonstrated that one to three copies of the transgene had been inserted into the genomes of the 32 independent plants (data not shown). Comparing the results of the kanamycin susceptibility assessment and those of our northern blot analysis (Fig. 2A), we found no relationship between copy number of the introduced gene and strength of the RNA transcript. This indicated that other factors also contribute to a lack of correlation between steady-state mRNA levels and copy number. Broadbent et al. (1995) have suggested that these variations in expression among transgenic lines may be caused by positioning effects, co-suppression, T-DNA insertion mutations, gene dosage effects (homo- versus heterozygous individuals), or somaclonal variation. Our PCR and northern analyses of the T_1 and T_2 lines showed that *BcGR1* is inherited as a dominant Mendelian trait, and was expressed normally in both T_1 and T_2 plants (Fig. 2B and C). In the T_1 generation, however, its level of expression differed greatly among

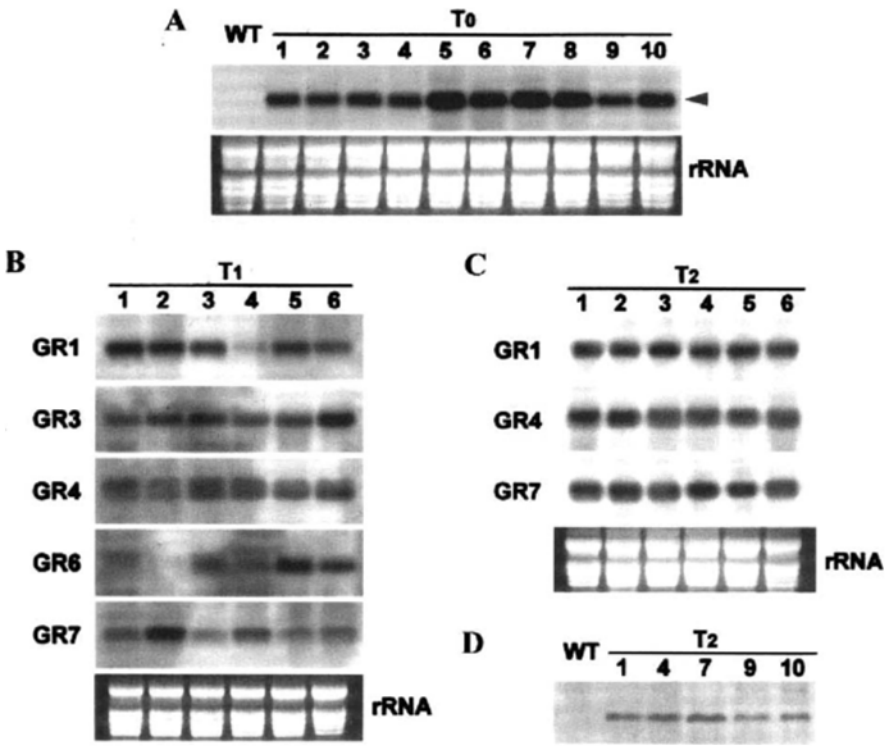

Figure 2. Expression of *BcGR1*. **A to C.** Northern blot analyses of T_0 (**A**), T_1 (**B**), and T_2 (**C**) transgenics. Total RNA was isolated from the leaves of wild-type (WT) and transgenic tobacco. Numbers indicate independent transgenic lines (**A**) and individual plants in the same line (**B** and **C**). Ethidium bromide-stained ribosomal RNA (rRNA) served as a loading control. **(D)** Immunoblot analysis of T_2 transgenic plants. Total proteins (20 μg) were extracted from leaves of wild-type (WT) and transgenic tobacco plants and subjected to immunoblotting. Numbers indicate independent transgenic lines.

plants in the same line (Fig. 2B). This may be a result of gene-dosage effects by segregation. In contrast, fluctuations in expression among homozygous T_2 plants of the same line were very small (Fig. 2C). Furthermore, overproduction of the BcGR1 protein in the leaves of T_2 transgenic plants was verified by immunoblot analysis (Fig. 2D).

Improved Tolerance to Methyl Viologen by Transgenic Plants

To ascertain the development of tolerance to methyl viologen, we conducted two types of experiments, using either seedlings or leaf discs from mature plants. First, seeds of transgenic and wild-type tobacco plants were sown on MS agar media; 10-d-old seedlings were then treated with 5, 10, 15, or 20 μM MV. This herbicide was used to generate active oxygen species and to quantify the oxidative-stress tolerance of the transgenic plants. At all treatment levels, the transgenic seedlings showed much higher tolerance than the wild types. The most drastic difference

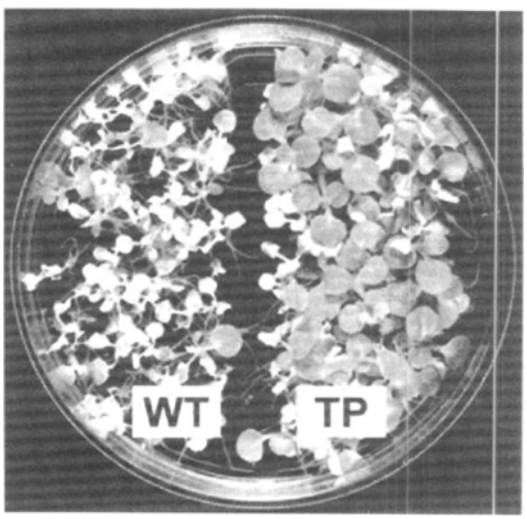

Figure 3. Tolerance of transgenic tobacco plants to 10 μM MV. Wild-type (WT) and transgenic (TP) plantlets, grown for 10 d on MS medium, were first subjected to MV treatment in the light (as described in Materials and Methods), then incubated under normal growth conditions. Photograph was taken 5 d after MV treatment.

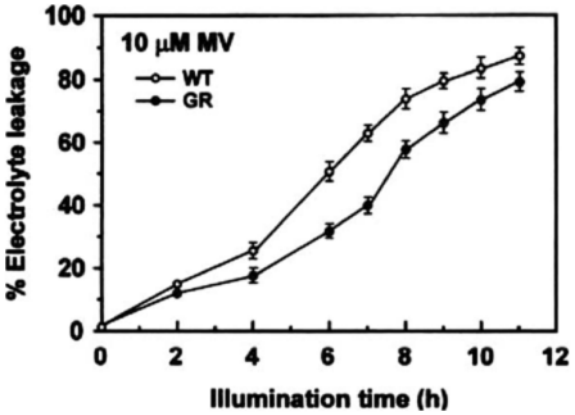

Figure 4. Analysis of cell damage in MV-treated leaf discs from transgenic and wild-type tobacco plants, as measurement of electrolyte leakage over time. Electrical conductivity of the MV solution was determined and compared with total conductivity of the solution following MV treatment. Means (± SE) are for three replicates per treatment.

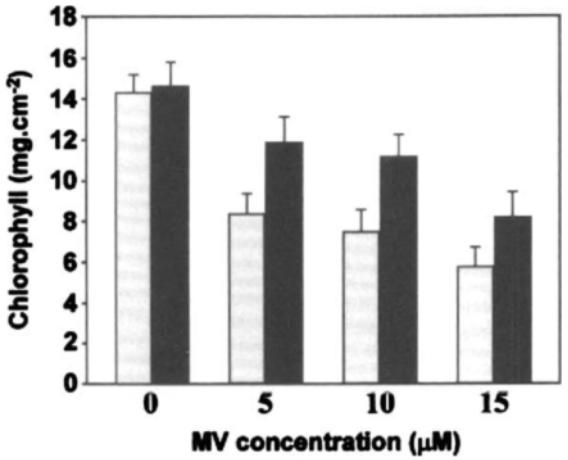

Figure 5. Effect of MV concentration on chlorophyll loss in leaf discs from wild-type (gray bars) and transgenic (black bars) tobacco plants. Means (± SE) are for three replicates per treatment.

was at 10 μM MV (Fig. 3), where >60% of the transgenics grew normally while all the wild-type seedlings withered.

In the second experiment, we treated leaf discs from six-week-old transgenic and wild-type tobacco plants with MV in the light. MV-dependent oxygen radicals cause the destruction of membrane lipids; this damage is quantified by measuring solute leakage (Slooten et al., 1995). Electrical conductivity of discs from the transgenic plants was lower than for the wild types (Fig. 4). For example, when discs were subjected to 10 μM MV for 7 h, electrical conductivity in the transgenics was reduced by >24% compared with that of the wild-type plants. Values increased with length of treatment period and MV concentration. Total chlorophyll contents in the leaf discs were also measured as an index of membrane lipid degradation after treatment with 0, 5, 10, or 15 μM MV for 10 h. As seen with the changes in electrical conductivity, chlorophyll loss was lower for the transgenics than for the wild-type plants at all concentrations of MV (Fig. 5).

We were interested to note that our transgenic tobacco plants did indeed acquire tolerance to MV because the GR gene we used for transformation was targeted, not to the chloroplasts, but to the cytosol. This suggests that GR produced by the introduced gene was catalyzed only in the cytosol. MV is known to inhibit electron transport by intercepting electrons between ferredoxin to $NADP^+$ during photosynthesis in the chloroplast. The intercepted electrons then reduce O_2 to a superoxide anion, O_2^- (Kleczkowski, 1993). These superoxide anions are readily dismutated to H_2O_2 by the action of superoxide dismutases inside the chloroplast (Asada, 1994). Considering that oxidant production was induced in the chloroplasts by MV treatment, but that their toxicity was alleviated in our transgenic plants, we postulate that the cytosolic GSH/GSSG ratio, increased by over-expression of *BcGR1*, affects the detoxification of those oxidants. We previously reported that *BcGR1* in *B. campestris* is strongly induced by MV treatment at the onset of stress (Lee et al., 1998, 2002). This means that the ROS produced in the chloroplast are propagated out to the cytosol and influence its redox state. Accordingly, we believe that GSH pools in both the cytosol and the chloroplasts do not act separately but, rather, they cooperate with each other in responding to external stresses and maintaining the equilibrium of the redox state in cells.

ACKNOWLEDGEMENT

This work was supported by the Korea Research Foundation (2000-005-G00003).

Received January 28, 2004; accepted March 12, 2004.

LITERATURE CITED

Allen RD (1995) Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol 107: 1049-1054

Alscher RG (1989) Biosynthesis and antioxidant function of glutathione in plants. Physiol Plant 77: 457-464

Asada K (1994) Production and action of active oxygen species in photosynthetic tissues, *In* CH Foyer, PM Mullineaux, eds, Cause of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, FL, pp 77-104

Broadbent P, Creissen GP, Kular B, Wellburn AR, Mullineaux PM (1995) Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. Plant J 8: 247-255

Cakmak I, Marschner H (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol 98: 1222-1227

Creissen GP, Edwards EA, Mullineaux PM (1994) Glutathione reductase and ascorbate peroxidase, *In* CH Foyer, PM Mullineaux, eds, Cause of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, FL, pp 343-364

Creissen GP, Mullineaux PM (1995) Cloning and characterization of glutathione reductase cDNAs and identification of two genes encoding the tobacco enzyme. Planta 197: 422-425

Edwards EA, Enard C, Creissen GP, Mullineaux PM (1994) Synthesis and properties of glutathione reductase in stressed peas. Planta 192: 137-143

Gamble PE, Burke JJ (1984) Effect of water stress on the chloroplast antioxidant system: Alterations in glutathione reductase activities. Plant Physiol 76: 615-621

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1978) Leaf disc transformation, *In* SB Gelvin, ed, Plant Molecular Biology Manual. Kluwer Academic Publishers, The Netherlands, pp 1-9

Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. Plant Cell 9: 627-640

Kleczkowski LA (1993) Inhibitors of photosynthetic enzymes/carriers and metabolism. Annu Rev Plant Physiol Plant Mol Biol 45: 339-367

Lee H, Jo J, Son DY (1998) Molecular cloning and characterization of the gene encoding glutathione reductase in *Brassica campestris*. Biochim Biophys Acta 1395: 309-314

Lee H, Won S, Lee B, Park H, Chung W, Jo J (2002) Genomic cloning and characterization of glutathione reductase gene from *Brassica campestris* var. *Pekinensis*. Mol Cells 13: 245-251

Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol 148: 350-382

Madamanchi NR, Anderson JV, Alscher RG, Cramer CL, Hess JL (1992) Purification of multiple forms of glutathione reductase from pea (*Pisum sativum* L.) seedlings and enzyme levels in ozone-fumigated pea leaves. Plant Physiol 100: 138-145

McGookin R (1984) RNA extraction by the guanidine thiocyanate procedure, *In* JM Walker, ed, Methods in Molecular Biology. Humana Press, New Jersey, pp 113-116

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8: 4321-4325

Slooten L, Capiau K, van Camp W, van Montagu M, Sybesma C, Inze D (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. Plant Physiol 107: 737-750

Southern EM (1975) Detection of specific sequences among DNA fragments. J Mol Biol 98: 503-517

Tanaka K, Saji H, Kondo N (1988) Immunological properties of spinach glutathione reductase and inductive biosynthesis of the enzyme with ozone. Plant Cell Physiol 29: 637-642

Yun BW, Huh GH, Lee HS, Kwon SY, Jo JK, Kim JS, Cho KY, Kwak SS (2000) Differential resistance to methyl viologen in transgenic tobacco plants that express sweet potato peroxidases. J Plant Physiol 156: 504-509