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Rapid Communication

Decrease in Activity of Glutathione Reductase Enhances Paraquat Sensitivity in Transgenic *Nicotiana tabacum*[1]

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Transgenic tobacco (*Nicotiana tabacum* L. cv SR1) with decreased activity of glutathione reductase exhibited enhanced sensitivity to paraquat in the light as evaluated by chlorophyll destruction and electrolyte leakage from leaf discs. This result indicates the involvement of glutathione reductase in the tolerance of plants to photooxidative stress caused by the herbicide.

Air pollutants and some herbicides are thought to cause photooxidative damage to plants via an increased generation of active oxygen species (Dodge, 1975; Shimazaki et al., 1980). GR (EC 1.6.4.2.) catalyzes the reduction of GSSG with the accompanying oxidation of NADPH. The enzyme is postulated to play an important role in plant protection against various forms of stress (Smith et al., 1990). Transgenic tobacco plants with high chloroplastic or cytosolic GR activity showed enhanced tolerance to photooxidative stress caused by the herbicide paraquat or the air pollutant sulfur dioxide (Aono et al., 1991, 1993). Paraquat is reduced by PSI within chloroplasts in the light and leads to generation of superoxide, hydroxyl radicals, and H_2O_2, which participate in the damage to plants (Dodge, 1975; Asada and Takahashi, 1987).

In the present study, transgenic tobacco (*Nicotiana tabacum* L.) plants with reduced GR activity were generated using a cDNA fragment of spinach chloroplastic GR as an antisense DNA. Paraquat sensitivity of leaves of transgenic plants was compared with that of leaves of control plants by analysis of electrolyte leakage and pigment content.

MATERIALS AND METHODS

A 1.8-kb cDNA fragment encoding chloroplastic GR was isolated from a spinach (*Spinacia oleracea* L. cv New Asia) cDNA library using antibody against this enzyme as a probe. The nucleotide sequence data will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence data bases with the accession number D37870. The extracts from *Escherichia coli* XL1-Blue expressing this cDNA in a plasmid Bluescript SK− (Stratagene) showed approximately 40 times higher GR activity than that from wild-type *E. coli* (data not shown). The deduced amino acid sequence was related to GR sequences from other organisms (K. Tanaka, T. Sano, K. Ishizuka, S. Shirai, K. Fujiyama, T. Beppu, T. Igarashi, J.J. Lee, M. Aono, and A. Kubo, unpublished data). The cDNA fragment, however, lacked an upstream part encoding the chloroplastic transit peptide. This cDNA fragment was cloned into pBI101 in the reverse direction downstream of the tomato Rubisco small subunit 3B gene promoter (Sugita et al., 1987). The resulting plasmid, pASGR1 (Fig. 1), was introduced into *Agrobacterium tumefaciens* C58C1 Rif^r harboring a Ti plasmid pGV2260 (Deblaere et al., 1985) by electroporation using *E. coli* Pulser (Bio-Rad Laboratories, Tokyo, Japan). This bacterium was used to transform leaf discs of *Nicotiana tabacum* SR1 (Horsh et al., 1985).

Transgenic tobacco plants were grown at 25°C under cycles of 14 h of light, 110 μmol m^{-2} s^{-1} PPFD, and 10 h of darkness as described previously (Aono et al., 1991). Crude extracts of leaves were obtained by homogenizing young expanded leaves in 5 mL g^{-1} fresh weight of 50 mM K-phosphate (pH 7.8) containing 5% PVP (w/v), 5 mM sodium ascorbate, 5 mM DTT, 5 mM EDTA, and 100 mM NaCl, with subsequent centrifugation at 6000*g* for 15 min. GR activity in the extracts was measured as described by Aono et al. (1991).

For paraquat treatment, leaf discs with a diameter of 7 mm were excised from young expanded leaves of 5- to 9-week-old transgenic and control plants. They were then supplied with 0.1% Tween 20 with or without 16 μM paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; Sigma) by vacuum infiltration. The remaining parts of leaves were stored at −80°C for measurement of GR activity. Ten leaf discs were preincubated in the dark for 1 h and then washed and transferred to 10 mL of deionized water in a Petri dish. They were exposed to light (130 μmol m^{-2} s^{-1} PPFD) at 25°C and conductivity of the bathing water was measured by a conductivity meter B-173 (Horiba Ltd, Kyoto, Japan) every 2 h. Total Chl contents of leaf discs were determined as described by Aono et al. (1993).

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Abbreviation: GR, glutathione reductase.

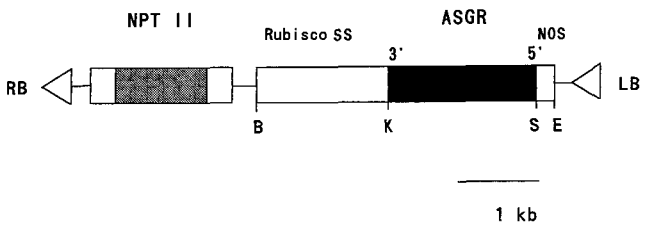

Figure 1. Construction of the T-DNA region of the plasmid pASGR1. RB, Right border; LB, left border; B, *Bam*HI; K, *Kpn*I; S, *Sac*I; E, *Eco*RI; NPT II, a chimeric gene for neomycin phosphotransferase that confers resistance to kanamycin on plants; Rubisco SS, the promoter of tomato Rubisco small subunit 3B gene; NOS, nopaline synthase terminator; ASGR, a cDNA for spinach chloroplastic GR connected in reverse direction to the promoter.

RESULTS

A DNA fragment that consisted of the promoter of tomato Rubisco small subunit 3B gene and the reverse-directed cDNA of spinach chloroplastic GR was introduced into tobacco cells for generating transgenic plants with reduced activity of GR (ASGR-transgenic plants, T_0 generation). Seventeen T_0 ASGR-transgenic plants were obtained and some of them showed foliar GR activities 50 to 70% lower than those of nontransgenic plants (data not shown).

Three T_0 ASGR-transgenic plants (1–3, 1–4, and 4–7) that had the lowest GR activities were chosen to obtain progeny (T_1 generation) by self-fertilization. It was estimated that T_0 ASGR-transgenic plant 1–3 had two copies of transgenes per genome, whereas 1–4 and 4–7 had one copy by the ratio of kanamycin-resistant to kanamycin-sensitive progenies. The kanamycin-resistant progenies were used for further analyses.

We treated leaf discs from the three lines of T_1 ASGR-transgenic plants and control nontransgenic plants with or without paraquat in the light to determine their sensitivity to the herbicide. Six plants of each T_1 ASGR-transgenic line were used for the experiments. Leaf discs were incubated in deionized water and the conductivity was determined (Fig. 2). When leaf discs of transgenic line 1–3 were exposed to paraquat, the net conductivity increased more rapidly than what was observed for control plants. Similar results were obtained from two other lines. Hence, the cellular membranes of leaves from transgenic plants are damaged to a greater extent after paraquat treatment than membranes of leaves from control plants.

The time elapsed before conductivity reached half of the maximum value (the half-maximum time) was used as a measure of the sensitivity of leaf discs to paraquat and was plotted against GR activity (Fig. 3). The two parameters showed a correlation coefficient (r = 0.503), and the half-maximum time decreased with decreasing GR activity. This result indicates that GR activity influences the tolerance of leaf tissues to paraquat under the conditions used.

The total Chl contents in leaf discs before and after the 21-h paraquat treatment in the light were used as an additional measure of paraquat sensitivity. Three plants of two T_1 ASGR-transgenic lines (1–3-3, 1–3-5, and 4–7) and a control plant were used for the experiment. The result shows lower Chl content in discs from transgenic plants than discs from a control plant after the paraquat treatment (Fig. 4, ■), providing further evidence that leaf tissues of T_1 ASGR-transgenic plants with low GR activities (30–60% of control activity) were more sensitive to paraquat than those of control plants. Furthermore, loss of Chl occurred after the illumination even without paraquat treatment in the discs from the transgenic plants (Fig. 4, ▨). This may suggest that leaf tissues of transgenic plants suffered from photooxidation in dim light during the treatment. Higher Chl content in discs from 4–7 T_1 ASGR-transgenic plant than in discs from two other transgenic plants after the treatment seems to be due to higher GR activity of 4–7 (about 60% of control activity) than that of the others.

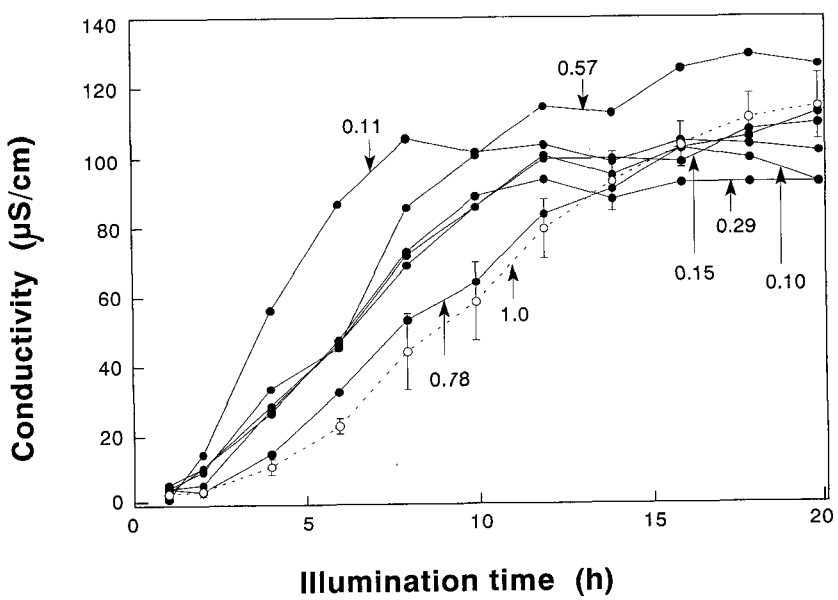

Figure 2. Electrolyte leakage from paraquat-treated leaf discs. Conductivity values for non-paraquat-treated leaf discs were subtracted from those for paraquat-treated discs, and the differences are shown. ●, The T_1 ASGR-transgenic plants of line 1–3; relative values of each GR activity against the mean value of controls are shown. ○, Control nontransgenic SR1. Values of controls are averages of three individuals. Error bars are SD.

DISCUSSION

We previously reported that transgenic tobacco plants with high GR activity inside or outside chloroplasts showed enhanced tolerance to photooxidative stress caused by paraquat or sulfur dioxide (Aono et al., 1991, 1993). Similarly, transgenic plants that overproduced superoxide dismutase that converts superoxide radicals ($O_2^{\cdot -}$) into H_2O_2 have been reported to have enhanced tolerance to photooxidative stress caused by paraquat or ozone (Bowler et al., 1991; Perl et al., 1993; Sen Gupta et al., 1993). We report here that suppression of the antioxidant enzyme GR with the antisense technique enhances sensitivity of plants to paraquat stress. The results of these different lines of research support the hypothesis that plant tolerance against photooxidative stress is influenced by the content of antioxidant enzymes such as GR.

There are several isozymes of GR in different compartments of plant cells (Edwards et al., 1990). Because the chloroplastic isozyme seems to be predominant (approximately 80%) in tobacco leaves (Aono et al., 1993) and GR activities of some ASGR-transgenic plants were as low as 5% of the control, it is assumed that the level of chloroplastic isozyme has decreased in these plants. However, the possibility of decreases in activities of other isozymes cannot be eliminated because the sequence relationship between chloroplastic and other isozymes of GR is not known.

GR is postulated to supply GSH to the ascorbate-glutathione cycle for scavenging active oxygen species (Foyer and Halliwell, 1976). On the other hand, GSH may have other protective roles, including maintenance of thiol groups, like Cys, homocysteine, and sulfhydryl proteins, in the reduced form (Rennenberg and Brunold, 1994). These roles of GSH could be important for the tolerance of plants to photooxidative stress.

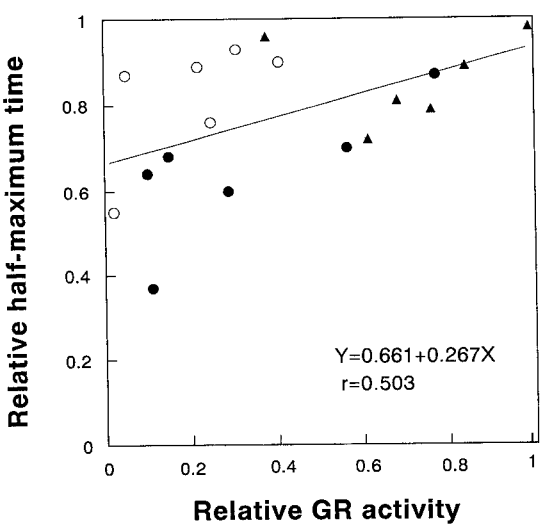

Figure 3. Correlation of GR activity and tolerance to paraquat of T_1 ASGR-transgenic plants. Relative values of both GR activity and half-maximum time against those of control nontransgenic SR1 are shown. ●, Line 1–3; ○, line 1–4; ▲, line 4–7. Values for 100% relative GR activity and 100% relative half-maximum time correspond to 76.7 ± 64.0 nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$ and 8.2 ± 1.8 h (mean ± SD, n = 6), respectively. A regression line, a regression equation, and correlation coefficient (r) are shown.

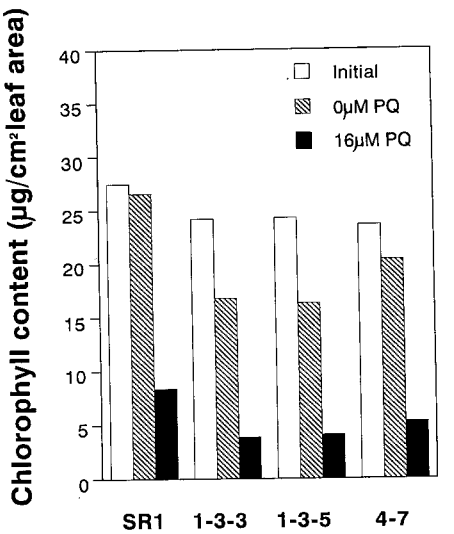

Figure 4. Total Chl contents in leaf discs from T_1 ASGR-transgenic and control plants before and after 21-h treatment in the light. SR1, Control; 1–3-3, 1–3-5, and 4–7, T_1 ASGR-transgenic plants; Initial, total Chl content in leaf discs before the treatment; 0 μM PQ, total Chl content in discs treated with distilled water containing 0.1% Tween 20; 16 μM PQ, total Chl content in discs treated with 16 μM paraquat in 0.1% Tween 20. GR activities of SR1, 1–3-3, 1–3-5, and 4–7 were 25.0, 8.3, 8.9, and 15.7 nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$, respectively.

ASGR-transgenic plants with extremely low GR activities showed no significant differences from control plants in growth under normal growing conditions, probably because there might be sufficient residual GR activity in the ASGR-transgenic plants; the ASGR-transgenic plants had a Chl content similar to that of the control plant before the treatment (Fig. 4, □). However, the enzyme activity would become limiting under stress conditions, resulting in damage to plants.

In future studies we will investigate whether ASGR-transgenic plants also show enhanced sensitivity to other environmental stresses, such as low temperature or desiccation, since active oxygen species seem to be involved in the damage of plants caused by these stresses (Elstner, 1982; Smirnoff, 1993).

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