Genomic Cloning and Characterization of Glutathione Reductase Gene from *Brassica campestris* var. *Pekinensis*

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We have isolated and characterized a gene encoding cytosolic glutathione reductase from *Brassica campestris* (*B. campestris*). The gene (*BcgGR1*) is presented as a single copy in the *B. campestris* genome and is composed of 17 exons and 16 introns in the transcribed region with coding sequence beginning in the 2nd exon and ending in the 17th exon. *BcgGR1* is expressed strongly in roots and calli, and moderately in stems and leaves. The transcription is strongly induced by various stress treatments including ozone, paraquat, salt, hydrogen peroxide, chilling or ABA but depressed by heat treatment. The transcript level of *BcgGR1* is increased significantly at 2 h after the onset of ozone (300 ppb), paraquat (10 µM) or salt (250 mM NaCl) treatments and reached a maximum level by 10−24 h. However, the maximum induction of *BcgGR1* is reached at 2−4 h after the onset of hydrogen peroxide (10 mM), chilling (10°C) or ABA (1 mM) treatments. The rapid reduction of *BcgGR1* transcripts after 4 h in ABA treatment is distinguished from hydrogen peroxide and chilling treatments.

**Keywords:** *Brassica campestris* (Chinese Cabbage); Gene Expression; Glutathione Reductase; Oxidative Stress; Reactive Oxygen Species.

**Introduction**

The tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinyl-glycine) is a predominant low molecular weight thiol compound in most plants having a central role in protecting plants from environmental oxidative stress such as reactive oxygen species (ROS), xenobiotics and some heavy metals (Meister and Anderson, 1983). Glutathione reductase (GR; EC 1.6.4.2) is widely distributed both in eukaryotes and prokaryotes and it catalyzes the reduction of oxidized glutathione disulfide (GSSG) to reduced glutathione (GSH) using the NADPH as an electron donor. It is important in maintaining high GSH/GSSG ratios in cells (Alschier, 1989; Meister and Anderson, 1983). Moreover, GR constitutes an ROS scavenging system in concert with superoxide dismutase (SOD) and the enzymes of the ascorbate-glutathione cycle in plants (Foyer and Halliwell, 1976).

GR has been purified and characterized on its properties from a variety of plants, and it has been reported that a number of GR isozymes are located in various subcellular compartments such as chloroplast, cytosol, mitochondrion and peroxisome (Edwards *et al*., 1990; Jimenez *et al*., 1997). Enhanced activities have also been demonstrated in response to oxidative stresses such as ozone fumigation, paraquat treatment, greening, chilling and the combination of magnesium deficiency with high light intensity (Cakmak and Marschner, 1992; Edwards *et al*., 1994; Guy and Carter, 1984; Mehlhorn *et al*., 1987; Tanaka *et al*., 1988).

So far, cDNAs or genes for GR from a variety of plants were cloned and they may be classified into two groups, that is, one is encoding chloroplastic/mitochondrial GRs (Creissen *et al*., 1992; Kubo *et al*., 1993; Mullineaux *et al*., 1992).
al., 1996; Tang and Webb, 1994) and the other is cytosolic one (Kaminaka et al., 1998; Lee et al., 1998; Stevens et al., 1997). The chloroplastic/mitochondrial GRs are known that the transcriptional or translational levels are not increased under oxidative stress due to constant expression in the plastids to detoxify ROS, the by-products of photosynthesis and respiration (Edwards et al., 1994; Stevens et al., 1997). Thus, to understand the relationship of the changes of GR activity and the expression of the corresponding gene in response to oxidative stress, we have previously reported that the transcriptional level of cytosolic GR gene in B. campestris is increased by oxidative stress and it was assumed that the cytosol is the first cellular compartment penetrated by oxidants and their reaction products after apoplastic and plasmalemma defenses are breached (Lee et al., 1998). In this study, we have studied the molecular characteristics of the gene encoding cytosolic GR of B. campestris and its expression patterns under various environmental or oxidative stresses.

Materials and Methods

Plant materials and growth conditions Seedlings of Brassica campestris var. Pekinensis were grown in a growth chamber on a 25°C day/20°C night cycle (day period being 16 h illumination at 350 µEm⁻²s⁻¹). The embryogenic calli were induced from seed on LS medium (Linsmaier and Skoog, 1965) supplemented with NAA (1 mg/L), BAP (5 mg/L), 2,4-D (0.1 mg/L) and casein hydrolysate (200 mg/L).

Construction and screening of genomic DNA library Genomic DNA was obtained from leaves of 3-week-old plants according to the method of Murray and Thompson (1980). A genomic DNA library was constructed in the λBlueSTAR vector (Novagen, USA) according to the manufacturer’s instructions. The full-length cytosolic GR cDNA of B. campestris (BcGR1; Lee et al., 1998) was labeled with [α⁻³²P] dCTP by the Multi-prime DNA labeling system (Amersham, UK) and used as a probe for screening the genomic DNA library. The genomic GR clones were screened from B. campestris genomic DNA library on 150 mm plates at a density of 50,000 pfu/plate as described by Sambrook et al. (1989). Phage clones were converted to phagemid vector (pBlueSTAR-1) according to the manufacturer’s instructions (Novagen, USA).

DNA sequencing and sequence analysis Plasmids were isolated according to the standard protocol (Sambrook et al., 1989). The genomic insert DNA was sequenced of both strands by the dideoxy chain termination method (Sanger et al., 1977) using an ALFExpress Auto Cycle Sequencing Kit and an automated ALFExpress DNA sequencer (Pharmacia, Uppsala). Nucleotide and deduced amino acid sequences were analyzed using Genetyx software (SDC Software Development, Tokyo).

Southern and Northern blot analyses Genomic DNA (10 µg) from leaves of B. campestris was digested with Clal, EcoRI, or XbaI, separated by electrophoresis on a 0.8% agarose gel and blotted onto Nytan-Plus nylon membrane (Schleicher & Schuell, Germany) by an alkaline transfer method. The membrane was hybridized with a 32P-labeled BcGR1 cDNA probe, washed with 0.2× SSC and 0.1% SDS at 55°C for 1 h and then autoradiographed. For Northern blot analysis, total RNA was isolated by the guanidine thiocyanate method (McGookin, 1984). Total RNA (15 µg) was fractionated on 1.2% formaldehyde agarose gel and blotted onto Hybond-N nylon membrane (Amersham, UK) with 10× SSC. Hybridization was performed as described above.

Stress treatments Three-week-old plants grown in a growth chamber were used for chemical or stress treatment. All treated plant materials were immediately frozen in liquid nitrogen and stored at −80°C until use. For heat stress treatment, the leaves were detached and incubated at designated temperatures in a shaking water bath for 1 h under white light illumination at 200 µEm⁻²s⁻¹. For chilling stress treatment, plants were exposed to 10°C under white light illumination at 200 µEm⁻²s⁻¹. For ozone fumigation, plants were transferred to ozone fumigation open-top chambers (300 ppb) under similar growth conditions. Ozone levels were continuously monitored and maintained within 50 ppb of the set limit (model IN-2000 UV absorption analyzer). For paraquat and H2O2 treatments, plants were misted with an aqueous solution containing either paraquat (10 µM) or H2O2 (10 mM) and 0.01% Tween 20. Plants were then maintained in a growth chamber at 25°C and kept under illumination at 350 µEm⁻²s⁻¹. For salt and ABA treatment, the leaves were detached and incubated on 250 mM NaCl and 1 mM ABA solutions under white light illumination at 200 µEm⁻²s⁻¹.

Results and Discussion

Isolation and characterization of the BcgGR1 gene By two round screening of the genomic DNA library using the full-length cytosolic GR cDNA as a probe we obtained two positive clones. One of these clones spanned the entire cytosolic GR gene of B. campestris which was designated as BcgGR1 (GenBank accession No. AF 255651). Restriction fragments overlapping the BcgGR1 were subcloned into pBluescript II vector and the entire nucleotide sequence was determined. As shown in Fig. 1A, BcgGR1 is 6,416 bp long and has 2,265 bp of 5’ upstream region, 3,886 bp of cDNA coding region, and 265 bp of 3’ downstream region. Structural alignment of the genomic and cDNA sequences revealed the presence of 17 exons and 16 introns in the transcribed region (Fig. 1B). The introns were found to contain a high A + T content (average of 65.8%) which is common in the genes of dicotyledonous species (Santos et al., 1996). All introns are bordered by the nucleotides GT-AG which conforms...
The first intron is located upstream of the ATG initiation codon (which is placed at the second exon) and it is thus designated as the 5′ non-coding intron. The 5′ non-coding intron seems to be involved in regulating transcription of the corresponding gene presumably because it contains intronic enhancer sequences (Gadea et al., 1999). In plants, there are many reports that the 5′ non-coding intron acts functionally in correct and increased expression of a variety of genes including APX20 which participates in ascorbate-glutathione cycle with GR gene (Gadea et al., 1999). At present, there is no direct evidence that the 5′ non-coding intron affects the regulation of GR gene, but it is possible that it may affect the expression of cytosolic GR gene in response to oxidative stress at the transcriptional or post-transcriptional level considering that cytosolic GR gene is strongly induced by various stresses whereas chloroplastic/mitochondrial counterpart genes are not (Edwards et al., 1994; Lee et al., 1998).

Comparison of the BcgGR1 with GR genes from other plants revealed structural differences between cytosolic and chloroplastic/mitochondrial GR genes. The cytosolic GR genes show more comprehensive structures in the genome than its chloroplastic/mitochondrial counterparts, that is, the cytosolic GR genes of B. campestris and Oryza sativa (O. sativa; Kaminaka et al., 1998) are composed of 17 exons interrupted by 16 introns in the transcribed region but chloroplastic/mitochondrial GR genes of Pisum sativum (P. sativum; Mullineaux et al., 1996) and Arabidopsis thaliana (A. thaliana; Kubo et al., 1998) are composed of 10/9 and 11/10 exons/introns, respectively. Moreover, the length and nucleotide sequence of exons in the genes of the same isoform is very similar but introns are not except for high A + T content. A 5′ non-coding intron and exon is found in the genes for cytosolic GR genes of the same isoform is very similar but introns are not except for high A + T content. A 5′ non-coding intron and exon is found in the genes for cytosolic GR genes but not in that for chloroplastic/mitochondrial GR genes. The sizes of open reading frame of the genes in the genome varies from 2,744 bp of A. thaliana to 5,733 bp of O. sativa.

Genomic organization of BcgGR1 gene To determine the copy number of the BcgGR1 in the genome of the B. campestris, we performed Southern blot hybridization using a full-length BcgGR1 cDNA as a probe. The signals obtained corresponded to fragments predicted by the restriction map deduced for the BcgGR1 (Fig. 2). Thus, we have concluded the genome of B. campestris has a single copy of the cytosolic GR gene. Cytosolic GR genes from P. sativum (Stevens et al., 1997) or O. sativa (Kaminaka et al., 1998) and chloroplastic/mitochondrial GR genes from A. thaliana (Kubo et al., 1993) or P. sativum (Mullineaux et al., 1996) are also reported to be a single copy. It is suggested, however, chloroplastic/mitochondrial GR gene from root nodule of Glycine max is composed of a multigene family (Tang and Webb, 1994).

Expression of BcgGR1 gene in various tissues To evaluate the tissue specific expression of BcgGR1, total RNAs

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**Fig. 1.** Characterization of the BcgGR1 gene. **A.** Nucleotide sequence of the exons and flanking regions of BcgGR1 (GenBank accession No. AF 255651). The deduced amino acid sequence is shown below the nucleotide sequence in the single-letter code. Exon sequences are shown in upper case letters and nontranscribed sequences are shown in lower case letters. Sites of introns in the genomic sequence are marked by asterisks. Nucleotide number 1 is the first nucleotide identified in the BcGR1 cDNA. The 5′ leader untranslated exon is shown in stippled bar. Possible TATA box and polyadenylation signals are underlined. Closed box indicates an ABRE-like sequences. **B.** Restriction map and genomic organization of the BcgGR1 gene. The structure of BcgGR1 gene is shown, with exons and nontranslated regions are represented by open and solid boxes, respectively, separated by introns represented as stippled bars. Exon sizes are indicated in bp above the diagram, intron sizes are given below the scheme. Sizes of the putative promoter and the 3′ nontranscribed region are given on the stippled bar.
from different tissues of \textit{B. campestris} are subjected to Northern blot analysis. As shown in Fig. 3A, the \textit{BcgGR1} is expressed in calli, leaves, stems, and roots of \textit{B. campestris}. However, the transcription levels are different among tissue types, with the highest levels in roots and calli and moderate levels in leaves and stems. The strong induction in roots or calli without any artificial stress treatment suggests the formation of oxidative environment in those tissues.

**Expression of \textit{BcgGR1} gene under extreme temperatures**
Expression of \textit{BcgGR1} under extreme temperatures was investigated by Northern blot analysis. As shown in Figs. 3B and 3C, the \textit{BcgGR1} responds differently to heat and chilling stress treatments. The \textit{BcgGR1} transcript level was decreased remarkably by heat treatment at temperatures of 38°C and higher (Fig. 3B). By contrast, the transcript level was significantly increased by chilling treatment: the induction shows a peak at 2 h after treatment at 10°C (Fig. 3C) and this is the most rapid induction of the gene among the various stresses examined.

Expression of \textit{BcgGR1} gene under oxidative stresses
To investigate the response of the \textit{BcgGR1} to oxidative stress, plants were treated with ozone, paraquat, or H2O2 (Fig. 4). In ozone fumigation, the \textit{BcgGR1} transcript level was increased gradually after 1 h of treatment and reached a maximum level at 10 h (Fig. 4A). In the case of paraquat treatment, the transcript level was increased after 2 h of treatment, reached a maximum level at 12 h, and then decreased during the next 12 h (Fig. 4B). It is known that ozone fumigation and paraquat treatment of leaves with illumination lead to the generation of superoxide anions and H2O2 in the cell (Asada, 1994; Pell \textit{et al.}, 1994). We, therefore, examined the effect of H2O2 that was the direct substrate of ascorbate-glutathione cycle (Fig. 4C). The transcript level increased after 1 h of H2O2 treatment, reached a maximum level at 4 h, and then decreased. It was reported that the level of mRNAs for cyto-
solic antioxidant enzymes such as SOD, ascorbate peroxidase, or glutathione S-transferase were elevated by ozone, drought, or UV-B treatments (Conklin and Last, 1995; Mittler and Zilinskas, 1994). Thus, considering the levels of mRNAs of chloroplastic antioxidant enzymes are depressed or not affected by the oxidative stresses (Conklin and Last, 1995; Stevens et al., 1997; Willekens et al., 1995), the cytosolic antioxidative defences including GR are important for the protection of plants from environmental stresses.

Expression of BcgGR1 gene under ABA and NaCl treatments The leaves were also treated with NaCl and ABA. The BcgGR1 responded differently to NaCl and ABA treatment. In the case of NaCl, the BcgGR1 transcript level increased gradually and reached a maximum level at 24 h (Fig. 5A). By contrast, the transcript level was rapidly induced by ABA, that is, the induction peaked at 4 h, and then decreased rapidly (Fig. 5B).

Recently, it was reported that two ABA-responsive elements (ABREs) core sequences (5′-ACGTGGC-3′) existed in the 5′-flanking region of the RGRC2 gene encoding rice cytosolic GR (Kaminaka et al., 1998). The expression of RGRC2 was strongly induced by ABA treatment or drought stress, but was induced weakly by salt or chilling treatments. Thus, they suggested that the expression of rice cytosolic GR gene was regulated via ABA-mediated signal transduction under environmental stresses (Kaminaka et al., 1998). However, there is no ABRE core sequence but only an ABRE-like sequence (5′-ACGT-CGG-3′) in the 5′-flanking region of the BcgGR1 in this experiment. In general all genes regulated by ABA appear to have a common ABRE containing a G-box motif (5′-CACGTG-3′), associated with hormone response (Mundy et al., 1990; Pla et al., 1993; Quatrano et al., 1992). Modification of the conserved bases in the G-box element resulted in the loss of responsiveness of the gene to ABA (Guiltinan et al., 1990). Whether the ABRE-like sequence of BcgGR1 which has a base mismatch, that is, G → C in the G-box motif can act as an ABRE is not clear yet. Other than ABA, ethylene, ginseng saponin, salicylic acid or H2O2 is a candidate for a signal molecule in antioxidant defense genes (Foyer et al., 1997). As H2O2 is a relatively stable metabolite and it can diffuse readily from the site of production it may act as a messenger to induce antioxidant defense genes such as glutathione S-transferase and glutathione peroxidase (Levine et al., 1994). At present, accordingly, it is not appropriate to say definitely that the expression of BcgGR1 by oxidative stress follows by a certain signal transduction pathway including ABA. Thus, we are on the way to study the signal transduction pathway that regulates the expression of genes for ROI scavenging enzymes including GR by oxidative stress.

Fig. 4. Effect of ozone, paraquat or H2O2 on the expression of BcgGR1 gene in B. campestris. The 3-week-old plants were exposed to 300 ppb ozone (A) and were misted once with 10 µM paraquat (B) or 10 mM H2O2 (C). RNAs were prepared from the treated seedlings at indicated times. Transcripts were hybridized with 32P-labeled BcGR1 cDNA. Each lane was loaded with 15 µg of total RNA. Ethidium bromide-stained RNA served as a loading control.

Fig. 5. Effect of NaCl or ABA on the expression of BcgGR1 gene in B. campestris. For NaCl (A) or ABA (B) treatment, detached leaves of 3-week-old plants were placed in 250 mM NaCl solution and 1 mM ABA solution, respectively. RNAs were prepared from the treated seedlings at indicated times. Transcripts were hybridized with 32P-labeled BcGR1 cDNA. Each lane was loaded with 15 µg of total RNA. Ethidium bromide-stained RNA served as a loading control.
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References


