CHAPTER II

CYTOSOLIC ISOFORMS OF ACTIVE OXYGEN SCAVENGING ENZYMES IN RICE

Introduction

In previous chapter, the author reports the gene cloning and characterization of SOD isoforms and the analysis of gene expression of SOD genes in rice. These results are, of course, invaluable to discuss the roles of SOD isoforms against environmental stresses. However these results are not enough to do the overall speculation at the view point of AOS-scavenging because SOD is only one of the AOS-scavenging enzymes. AOS-scavenging enzymes, such as SOD, APX, MDAR, DHAR, GR, PHGPX, are seemed to work coordinately under AOS-generating conditions, in other word, under stress conditions. To date, the overall speculation in the gene regulation of plant AOS-scavenging enzymes could not be obtained because the previous analyses were carried out only by focusing on one or several of AOS-scavenging enzymes.

In this chapter, the author's purpose is to clarify the molecular mechanisms in the gene regulation of AOS-scavenging enzymes. Prior to the analysis of the gene regulation, the author isolated and characterized cDNA and gene for GR (section 1), and two cDNAs for MDAR (section 2). Furthermore, to elucidate the regulatory mechanisms of rice AOS-scavenging enzyme genes under various stress conditions, the transcripts of these enzymes were examined in rice seedlings with the various stress treatments using the gene-specific probes to all AOS-scavenging enzymes. In particular, the author focused on the cytosolic isoforms of AOS-scavenging enzymes because the isolated cDNA for GR and MDAR encoded cytosolic isoforms, and the cDNAs for all AOS-scavenging enzymes were available only in the case of cytosolic isoforms. In rice, two cDNAs for cytosolic SOD and APX, and a cDNA for cytosolic DHAR have already been characterized. In the case of PHGPX, no PHGPX cDNAs have been characterized in rice. Therefore, a PCR-fragment, which was prepared as described in section 3, homologous to the putative cytosolic PHGPX isolated from *A. thaliana* was characterized in section 3 and used as a hybridization probe.

SECTION 1

Cloning of cDNA and genomic clone, and characterization of rice cytosolic glutathione reductase

Introduction

Glutathione reductase (GR; EC 1.6.4.2) is a flavoenzyme and has been found in all organisms examined. This enzyme catalyzes the reaction of oxidized-glutathione (GSSG) to GSH using NAD(P)H as an electron donor (Halliwell and Foyer, 1978). A highly reduced state at the intracellular level is maintained by this reaction. In plants, GR is localized not only in chloroplasts (Connell and Mullet, 1986) but also in cytosol (Drumm-Herrel et al., 1989; Edwards et al., 1990), and in mitochondria and peroxisomes (Jimenez et al., 1997). cDNAs for GR have been isolated from *A. thaliana* (Kubo et al., 1993) and soybean (Tang and Webb, 1994), and GR cDNAs and genes have been isolated from pea (Creissen et al., 1992; Mullineaux et al., 1996) and tobacco (Creissen and Mullineaux, 1995). Kubo et al. (1993) showed that *A. thaliana* GR cDNA encodes a chloroplastic isozyme by determining the N-terminal amino acid sequence of mature polypeptide, but Creissen et al. (1995) showed that the pea GR presequence has a targeting capability for chloroplasts and mitochondria. Recently, Stevens et al. (1997) reported the cloning and characterization of a putative cytosolic GR cDNA, although they did not clearly show that the protein encoded by this cDNA is actually localized in the cytosol. In rice, GR was purified from rice embryo (Ida and Morita, 1971).

In addition, GR has frequently been investigated as one of the key enzymes in the AOS-scavenging system. Therefore GR has been suggested to be regulated in response to various environmental stresses and to contribute to stress tolerance in GR-overexpressing plants, much like SOD (reviewed by Allen 1995). In studies at the protein level, GR activity has been shown to increase under various treatments or stresses (Cakmak and Marschner, 1992; Edwards et al., 1994; Gamble and Burke, 1984; Gogorcena et al., 1995; Madamanchi et al., 1992; Tanaka et al., 1988; 1990). The expression of chloroplastic GR in *Pinus sylvestris* is regulated by redox state and photooxidative condition, but not coordinately with chloroplastic and cytosolic Cu/Zn-SOD (Karpinski et al., 1993; Wingsle and Karpinski., 1996), while mRNAs of chloroplastic GR in pea and *A. thaliana* were not increased under oxidative stress (Edwards et al., 1994; Karpinski et al., 1997). Transgenic plants that overexpress genes for bacterial and plant GR have shown increased tolerance to methyl viologen (Foyer et al., 1991; Aono et al., 1991), air pollutants (Aono et al., 1993; Broadbent et al., 1995) and photoinhibition (Foyer et al., 1995).

In this section, the author has isolated a cDNA predicted to encode a cytosolic GR form from rice and used the antibody against the recombinant RGRC2 protein to demonstrate that it encodes the cytosolic isoform of rice GR by subcellular fractionation. In addition, a corresponding genomic clone (gGRC-1) to RGRC2 was isolated and characterized, and examined the response of rice cytosolic GR to various environmental stresses.

Materials and Methods

Plant materials and construction of cDNA library -

Rice (*Oryza sativa* L. cv Nipponbare) was used in all experiments, and seedlings and calli were prepared as described in CHAPTER I.

Total RNA of leaves from rice seedlings was prepared using GIT-CsCl method as described in CHAPTER I. Poly(A)$^+$ RNA was purified from the total RNA using Oligotex-dT30 (TAKARA Shuzo), and then used for the synthesis of the double-stranded cDNA using Time Saver cDNA synthesis kit (Amersham Pharmacia Biotech Japan). The *Eco*RI linker-ligated cDNA was ligated into λZAPII arms predigested with *Eco*RI (Stratagene), and then packaged into phage particles using GIGAPACK III GOLD (Stratagene). All procedures were carried out according to the manufacturers' instructions. Consequently, a cDNA library of approximately 2.0 x 10^5 independent recombinants was constructed and used for the isolation of rice GR cDNA.

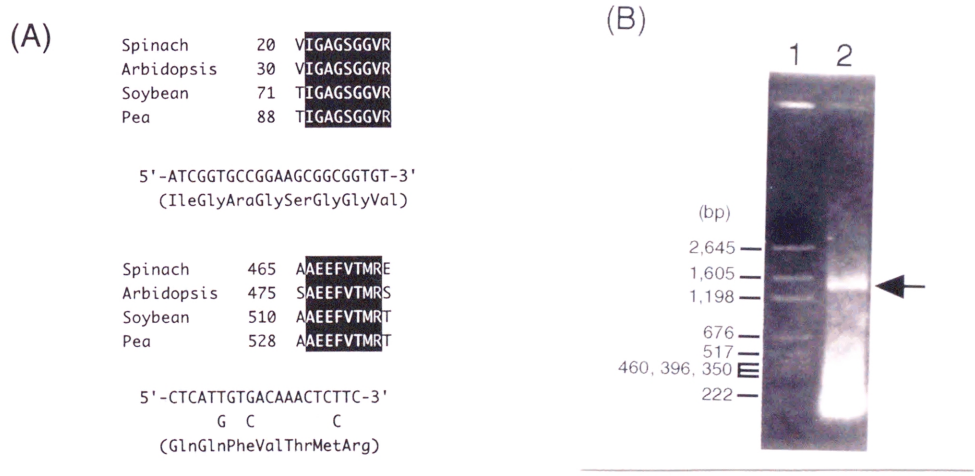


Fig. V-1. Preparation of a partial fragment of rice GR cDNA. (A) Alignment of the amino acid sequence from a part of the known plant GRs from spinach (accession number D37870), *A. thaliana* (Kubo et al., 1993), soybean (Tang and Webb, 1994), and pea (Creissen et al., 1992), and sequence of synthesized primers. (B) Electrophoresis analysis stained by ethidium bromide; lane 1: pGEM marker (Promega Japan), lane 2: PCR products amplified with a pair of primers as shown in previous Figures. Arrows indicated the recovered bands by electroelution for subcloning.

Isolation and characterization of a cDNA for rice GR cDNA -

Prior to the screening, an approximately 1.4 kb DNA fragment (Fig V-1B) was obtained using

PCR with a pair of primers, ssGRF (5'-ATCGGTGCCGGAAGCGGCGGTGT-3') and GRR-1 (5'-CTCAT[G/T]GT[G/C]ACAAACTC[C/T]TC-3'), which were synthesized referring to the nucleotide sequences of spinach GR cDNA (accession number D37870) (Fig. V-1A). This amplified product was subcloned into Bluescript SK(+) (Stratagene) modified as T-vector as described in CHAPTER I. The nucleotide sequence of this fragment was determined and it was recognized as a partial fragment of GR cDNA by the comparison with other known plant GR cDNAs. Using this PCR fragment, a putative full-length cDNA clone, designated as RGRC2, was isolated and used for further analysis. The methods of screening and sequencing were followed as described in CHAPTER I.

Expression of rice GR cDNA (RGRC2) in E. coli and preparation of anti-RGRC2 protein antibody -

A *Bam*HI-*Hin*dIII fragment of RGRC2, containing a full-length coding sequence, was inserted into plasmid, pQE30 (QIAGEN K.K.; Fig. V-5A) to produce a histidine-tagged recombinant protein in *E. coli* JM109. High expression levels of recombinant protein in *E. coli* induced by 1 mM IPTG were recognized by means of 12% SDS-PAGE and CBB staining (Fig. V-5B).

The recombinant protein expressed in *E. coli* was purified in a His Trap chelating column (Amersham Pharmacia Biotech Japan) according to the manufacturer's instructions but without addition of guanidine hydrochloride or urea as follows. The culture induced by 1 mM IPTG at 37 °C for 5 h was centrifuged at 4,000 x *g* for 20 min. The resulting pellet was resuspended in 5 ml/ g fresh weight cells with starting buffer (20 mM Na-phosphate buffer (pH 7.6), 0.5 M NaCl, 10 mM imidazole and 6 M guanidine hydrochloride) and then centrifuged at 10,000 x *g* for 15 min. The supernatant was filtered through a 0.45 mm filter and applied to a column pre-equilibrated with the starting buffer. The column was washed with 10 ml of the starting buffer, and histidine-tagged proteins were eluted by 5 ml of elution buffer (20 mM Na-phosphate buffer (pH 7.6), 0.5 M NaCl, 500 mM imidazole and 8 M urea). The purified protein was dialyzed four times by 50 mM Na-phosphate (pH 7.5) and fractionated by 12% SDS-PAGE.

4 mg of protein was used for the injection into two rabbits, and then the IgGs against the recombinant protein was purified in protein A Sepharose columns. All procedures for the production and purification of antibodies were performed by the Sawady Technology Co., Ltd (Tokyo, Japan).

Enzyme assay of GR -

GR activity was assayed by the decrease in absorbance at 340 nm due to the NADPH oxidation

(ε= 6.2 mM^{-1} cm^{-1}) as described previously (Foyer and Halliwell, 1976). For this experiment, recombinant proteins were induced by 0.1 mM IPTG at 28 °C for 7 h to avoid inclusion body formation. For the assay, the cytoplasmic fraction of *E. coli* was prepared according to the manufacturer's instructions (QIAGEN K.K.). The culture solution treated with IPTG was centrifuged at 4,000 x *g* for 10 min, and the pellet was resuspended in a sonication buffer (50 mM Na-phosphate (pH 7.8), 150 mM NaCl and 0.25% Tween-20). The resulting solution was sonicated and centrifuged at 10,000 x *g* for 20 min, and the supernatant used for the GR assay. In order to concentrate GR activity for the determination of kinetic parameters, the supernatant was passed through a His Trap chelating column (Amersham Pharmacia Biotech Japan) according to the manufacturer's instructions. The assay-mixture (1 ml) contained 0.1 M Tris (pH 7.8), 0.1 mM EDTA, 0.5 mM GSSG, 0.2 mM NADPH and 100 ml of the extract, and the activity was measured in at least triplicate by a BioSpec-1600 spectrometer (Shimadzu). The kinetic parameters were determined in triplicate assays using five different concentrations of NADPH (2-100 mM) and GSSG (10-200 mM). Protein concentrations were determined colorimetrically (Bradford, 1976) using BSA as a standard.

Subcellular fractionation and assay of marker enzymes -

Leaf tissues (30 g fresh weight) from rice seedlings were chopped into small pieces and homogenized on ice with 3 volumes of frozen grinding buffer (330 mM sorbitol, 50 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 25 mM cystine and 0.1% bovine serum albumin) using a Polytron homogenizer PT3100 (KINEMATICA AG, Switzerland). The homogenate was filtered through two layers of MIRACLOTH (CALBIOCHEM, CA) and then centrifuged at 10,000 rpm for 10 s at 4 °C. The pellet (crude chloroplast fraction) was resuspended with a small volume of the washing buffer (grinding buffer without cystine), layered on a Percoll (Amersham Pharmacia Biotech Japan) gradient (70% and 40%) in washing buffer and then centrifuged at 7,700 x *g* for 15 min at 4 °C. Intact chloroplasts were obtained at the boundary between the 70% and 40% Percoll layers.

The supernatant from the first centrifugation (10,000 rpm, 10 s) was recentrifuged at 10,000 x *g* for 20 min at 4 °C. Then the supernatant was used as a crude cytosol fraction for the following experiments. The supernatant was centrifuged at 12,000 x *g* for 20 min. To obtain purified mitochondria, the pellet was resuspended with a small volume of the washing buffer and layered on a Percoll gradient (60%, 45%, 28% and 5%) in washing buffer and then centrifuged at 30,000 x *g* for 30 min at 4 °C according to Yamaya et al. (1984). Intact mitochondria were obtained at the boundary between the 45% and 28% Percoll layers.

These intact organelles (chloroplasts and mitochondria) were collected from the Percoll gradient, resuspended with 10 volumes of the washing buffer and then centrifuged at 10,000 x *g* for 10 min twice. The washed pellet was resuspended with 200 ml of 50 mM K-phosphate (pH 7.8), 0.1 mM EDTA and 0.1% Triton-X 100, and the supernatant was obtained as the proteins of purified chloroplasts or mitochondria fractions by the centrifugation (10,000 x *g*, 20 min). These proteins were subjected to immunoblotting after native-PAGE as described above.

To evaluate the purity of these fractions, the enzyme activity specific to each organelle was assayed. The cytosol, chloroplast and mitochondria fractions activities were assayed for phospho*enol*pyruvate (PEP) carboxylase (Wong and Davies 1973) or glucose-6-phosphate dehydrogenase (Doehlert et al., 1988), NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase (Wu and Racker, 1959) and cytochrome C oxidase (Tolbert, 1974), respectively.

Genomic Southern blot analysis, screening and sequencing of the RGRC2 gene -

Genomic Southern blot analysis was performed as described in CHAPTER I, with ^{32}P-labelled *Eco*RI-fragment of RGRC2 cDNA (full-length cDNA) and washing at high-stringency conditions (1 x SSC, 42 °C). The restriction endonucleases of *Eco*RV, *Hin*cII, *Hin*dIII and *Pst*I were used for the digestion of rice genomic DNA.

The isolation of genomic clone for rice GR (gGRC-1) from a rice genomic library (2 x 10^5 plaques), λphage DNA preparation, the recombinant DNA techniques, and sequencing were performed as described in CHAPTER I, with the same probe as described above and washing at the stringency (2 x SSC, 42 °C).

Northern blot and immunoblot analysis -

The experimental samples of the rice vegetative tissues and stress-treated seedlings for Northern blot and immunoblot analyses were prepared as described in CHAPTER I-4, with minor changes as follows. For chilling stress, the seedlings were exposed to 10 °C under continuous light (170 μmol quanta m^{-2} s^{-1}). All stress-treated samples were collected at 0, 3, 6, 12, 24 and 48 h after the start of each treatment.

Northern blot analysis was carried out as described in CHAPTER I, with ^{32}P-labelled *Eco*RI-fragment of RGRC2 cDNA (full-length cDNA) and washing at the stringency (1 x SSC, 42 °C).

Samples for immunoblotting were immediately ground with 2 ml of 50 mM K-phosphate (pH 7.8), 0.1 mM EDTA and 0.1% Triton-X 100 and then centrifuged at 10,000 x *g* for 20 min at 4 °C, and the supernatant was used for the immunoblot analysis. Proteins of these homogenates (30 μg)

were separated on 10% native-PAGE and transferred to a PVDF membrane (Trans-Blot transfer medium; BIO-RAD, Hercules, CA) by electroblotting (100 mA, 30 min). For immunoblotting, rice GR on the membrane was incubated with an anti-recombinant RGRC2 protein antibody (1:1000 dilution) with the alkaline phosphatase conjugated goat anti-rabbit IgG antibodies (Promega Japan, Tokyo, Japan), and the band of rice GR was stained with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (BIO-RAD) according to the manufacturer's instructions.

Results

Isolation and characterization of a cDNA encoding rice GR -

The library from rice green leaves was screened with an approximately 1.4 kb DNA fragment, which was amplified using the synthesized primer pairs with the reference of the nucleotide sequences of spinach GR (accession number D37870) and identified as a partial cDNA of GR (Fig. V-1). Consequently, the longest cDNA of 21 positive clones, designated as RGRC2, was obtained from 4.5 x 10^5 plaques, and both strands of the nucleotide sequence were determined and used for further analysis. RGRC2 (accession number D85751) is 1,822 bp in length and has an open reading frame encoding a polypeptide of 496 amino acids with a predicted molecular weight of 53,506 (Fig. V-2). The deduced amino acid sequences showed a high preservation of some binding sites and active residues (Krauth-Siegel et al., 1982; Kubo et al., 1993) by comparison with previously published GR sequences from other plants, animals and prokaryotes (data not shown). However the difference of primary structure, containing N-terminal or C-terminal length, and the difference in homology among plant GRs were observed by the comparison of rice GR with the known plant GRs (Fig. V-3). AT-2 (*A. thaliana* chloroplastic GR; Kubo et al., 1993) and Pea-2 (*P. sativum GOR1*; Creissen et al., 1992) have already been identified as chloroplastic and/or mitochondrial isoforms, while the other types, including Rice (RGRC2), Pea-1 (*P. sativum GOR2*; Stevens et al., 1997) and AT-1 (*A. thaliana* GR; accession number U37697), are putatively cytosolic isoforms because of the lack of the typical transit sequences expected in the N-terminal regions and peroxisome targeting signal (SKL) in C-termini. Furthermore the phylogenic relationships among rice GR and the known following GRs were also analyzed (Fig. V-4): previously shown plant GRs, *Braccica rapa* (accession number AF008441), *N. tabacum* (accession number X76533), *G. max* (accession number L11632), *Onchocerca volvulus* (Muller et al., 1997), *Plasmodium falciparum* (Farber et al., 1996), *Mus musculus* (accession number X76341), *H. sapiens* (accession number X15722), *S. cerevisiae* (accession number D37871), and *E. coli* (accession number M13141). These results suggest that these plant GRs are distinguished into two types, cytosolic isoform and

Fig. V-2. Nucleotide and deduced amino acid sequences of a cDNA for rice cytosolic GR, RGRC2. The nucleotide (upper line) and amino acid residues (lower line) are numbered on the right. The putative polyadenylation signal is - underlined and asterisks indicate a termination codon. The nucleotide sequence data in this figure (RGRC2) have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D85751.

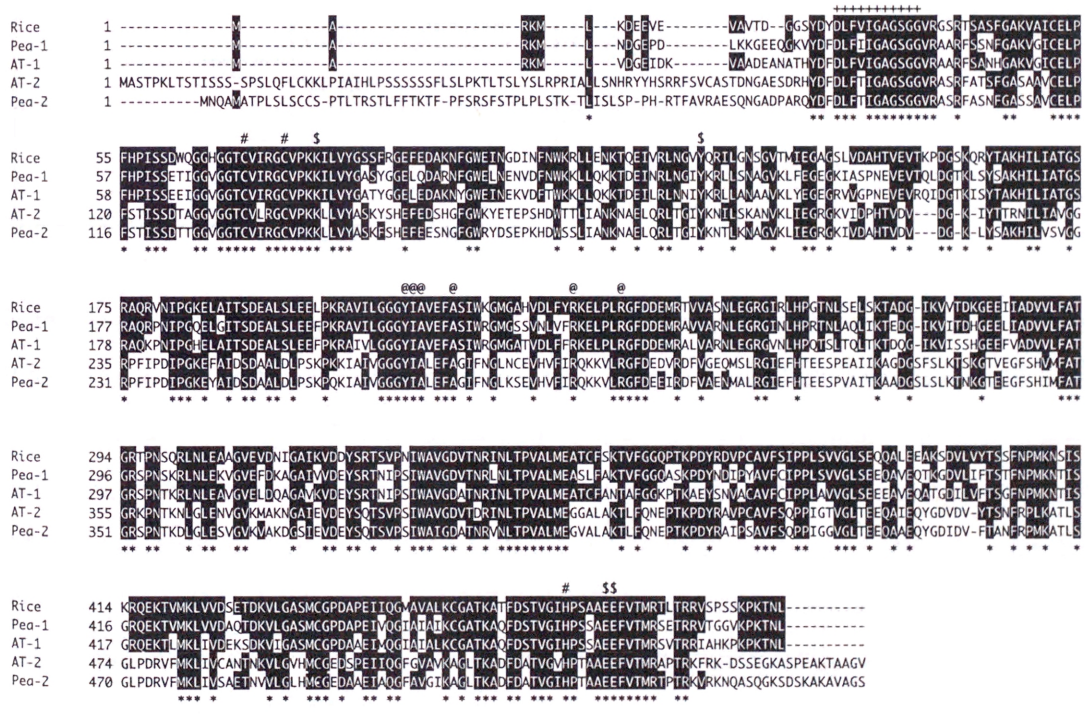


Fig. V-3. Multiple alignment of a rice GR and the other plant GRs. Rice, Rice GR (RGRC2, in this study); Pea-1, *P. sativum* putative cytosolic GR (*GOR2*, Stevens et al., 1997); AT-1, *A. thaliana* GR (accession number U37697); AT-2, *A. thaliana* chloroplastic GR (Kubo et al.. 1993); Pea-2, *P. sativum* chloroplastic/mitochondrial GR (*GOR1*, Creissen et al., 1992). Alignment of the deduced amino acid sequences from these plant GR cDNAs was obtained by the program MAlign in GENETYX (Software Development Co., Ltd.). Identical residues with rice cytosolic GR are shown as black boxes. Asterisks indicate identical residues among all five sequences. Binding sites of FAD, GSSG and NAD(P)H and active residues (Krauth-Siegel et al., 1982, Kubo et al., 1993) are indicated by the following marks above each amino acid: +, binding site of FAD; #, binding site of GSSG; $, binding site of NAD(P)H; @, active residue.

chloroplastic/mitochondrial isoform, and distinct group from other GRs, including mammalians and microorganisms.

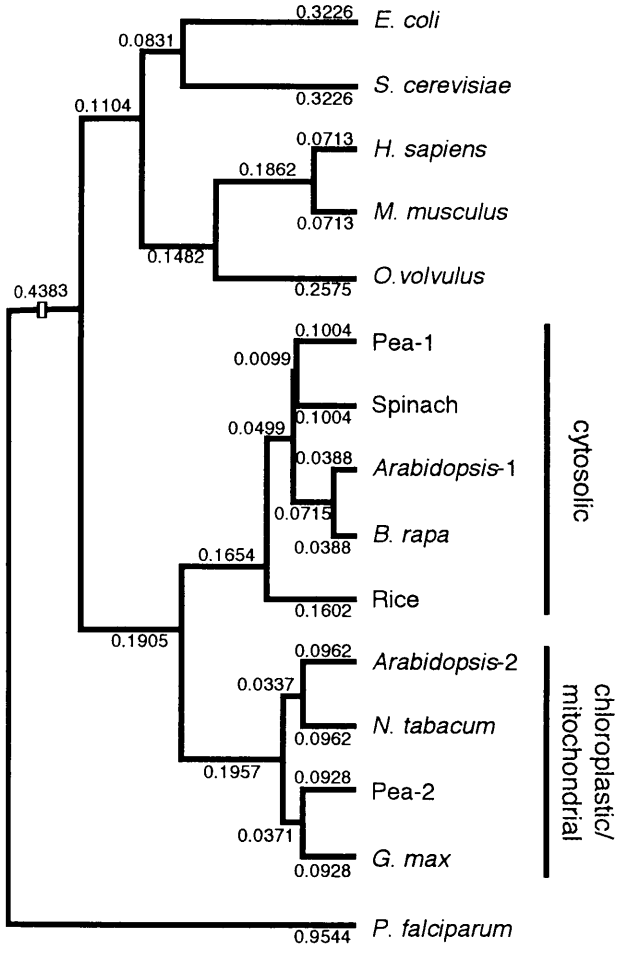
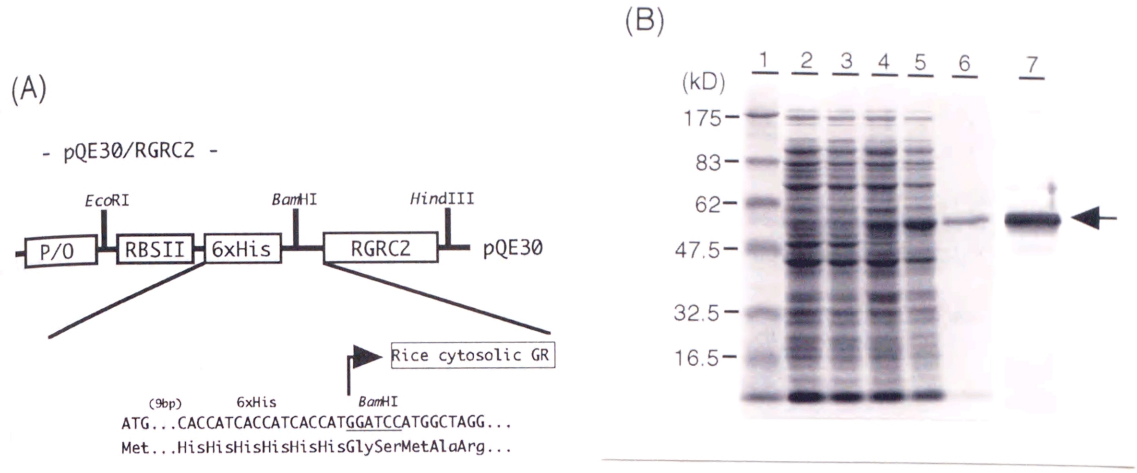


Fig. V-4. Molecular phylogenic tree of GR. The phylogenic tree was constructed with GeneWorks software (IntelliGenetics). Numbers indicate the branch length as proportional genetic divergence. The sequences of GRs are shown in Fig. V-3, and from *Braccica rapa* (accession number AF008441), *N. tabacum* (accession number X76533), *G. max* (accession number L11632), *Onchocerca volvulus* (Muller et al., 1997), *Plasmodium falciparum* (Farber et al., 1996), *M. musculus* (accession number X76341), *H. sapiens* (accession number X15722), *S. cerevisiae* (accession number D37871), and *E. coli* (accession number M13141).

Expression in E. coli and enzymatic characterization of the recombinant rice GR -

Histidine-tagged recombinant rice GR protein was expressed in *E. coli* strain JM109 harboring plasmid pQE30/RGRC2 (Fig. V-5A) as described in Materials and Methods. The molecular size of the recombinant protein estimated by SDS-PAGE is approximately 53,000, nearly identical to that of the deduced amino acid sequence from RGRC2 (Fig. V-5B, lanes 4-6). Furthermore, the cytoplasmic fraction of *E. coli* harboring pQE30/RGRC2 induced by IPTG showed about 17.3-fold GR activity in the comparison to that of the control plasmid, pQE30 (Fig. V-5C). Furthermore the reaction of the anti-recombinant RGRC2 protein antibody with the purified recombinant RGRC2 proteins by immunoblot analysis was demonstrated (Fig. V-5B, lane 7).

For enzymatic characterization of recombinant RGRC2 proteins, the recombinant protein was purified as described in Materials and Methods. It was detected as a single band on SDS-PAGE Fig. V-5B, lane 6). The kinetic properties of this purified protein are shown in Table V-1. The ratio and value of K_m for NADPH and GSSG are comparable to those of the purified GR protein from rice embryo (Ida and Morita, 1971).



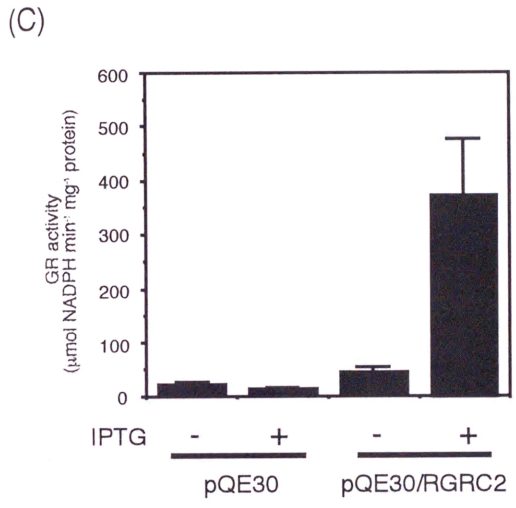


Fig. V-5. Expression in *E. coli* and purification of recombinant RGRC2 proteins. (A) Fusion construction in frame of pQE30/RGRC2. (B) SDS-PAGE gel (12%) was stained with CBB (lane 1-6) or subjected to immunoblotting with the antibodies against recombinant RGRC2 protein (lane 7). Molecular marker (lane 1; Prestained protein marker, New England Biolabs); Total protein extract of *E. coli* strain JM105 harboring pQE30 (non-induction, lane 2; 4 h after induction by IPTG, lane 3); that of *E. coli* strain JM109 harboring pQE30/RGRC2 (non-induction, lane 4; 4 h after induction by IPTG, lane 5); 2 mg of purified recombinant RGRC2 protein (lane 6); lane 6 subjected to immunoblot (lane 7). Molecular size is indicated in the left margin. (C) Assay of GR activity in the total extract of RGRC2 proteins expressed in *E. coli*. The cytoplasmic fraction of *E. coli*, harboring pQE30 or pQE30/RGRC2 and with or without induction by IPTG, were assayed for GR activity as described in detail in Materials and Methods. GR enzymatic estimations were done in triplicate for five extracts each.

Table V-1. Comparison of K_m values for NADPH and GSSG of purified recombinant RGRC2 protein and a purified GR from rice embryo (Ida and Morita, 1971).

The kinetic parameters were determined in triplicate assays using five different concentrations of NADPH (2-100 mM) and GSSG (10-200 mM). For details of this measurement see Materials and Methods.

Substrates	K_m (M)		Ratio (A/B)
	A) Rice GR (recombinant)	B) GR from rice embryo	
NADPH	3.9×10^{-5}	1.3×10^{-5}	3.0
GSSG	9.2×10^{-5}	3.4×10^{-5}	2.7

Subcellular location of RGRC2 protein -

To determine whether the protein encoded by *RGRC2* was actually localized in the cytosol, the immunoblotting results of the subcellular fractionation, which was prepared as described in Materials and Methods, were examined (Fig. V-6). Each subcellular fraction was analyzed by assay of the activities of marker enzymes (data not shown). Purified chloroplast fraction (lane 3) had no detectable activity of cytochrome C oxidase (mitochondria marker), and purified