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Gene Cloning and Expression of Cytosolic Glutathione Reductase in Rice (*Oryza Sativa* L.)

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We have isolated a cDNA (RGRC2) encoding glutathione reductase (GR) from rice (*Oryza sativa* L.). The comparison of deduced amino acid sequences from RGRC2 and other plant GR cDNAs indicated that RGRC2 encodes a putative cytosolic isoform. The recombinant RGRC2 protein had enzymatic properties comparable to those of GR from rice embryo. Subcellular fractionation showed that the *RGRC2* protein is localized primarily in cytosol. mRNA and protein of *RGRC2* were observed mainly in roots and calli but little in leaf tissues. Southern blot analysis showed that the *RGRC2* gene exists as a single copy gene. Here, we have also isolated a genomic clone completely corresponding to RGRC2. The *RGRC2* gene is split into 16 exons spread about 7.4 kb of chromosomal DNA, with coding sequence beginning in the 2nd exon and ending in the 16th exon. From the presence of two ABA-responsive elements in the 5′-flanking region of *RGRC2*, we examined the expression in rice seedlings treated with ABA and the ABA-related environmental stresses, chilling, drought and salinity. The expression of *RGRC2* was strongly induced by all these treatments. We suggest that the expression of the rice cytosolic GR gene is regulated via ABA-mediated signal transduction pathway under environmental stresses.

Key words: Abscisic acid — Cytosolic isoform — Gene structure — Glutathione reductase (EC 1.6.4.2) — *Oryza sativa* L. — Stress response.

Glutathione (GSH; gamma-glutamyl-L-cysteinyl glycine), a ubiquitous free thiol-containing tripeptide in most plants, is a major reductant and essential source for several cellular processes (Rennenberg 1982), and also plays a role in regulating the expression of plant defense genes (Wingate et al. 1988). In addition, GSH possesses antioxidant properties against oxidation by active oxygen species (Meister and Anderson 1983) and also acts as a donor of reducing equivalents to ascorbate in the active oxygen scavenging system (Foyer and Halliwell 1976). In all aerobic organisms, oxygen molecules can change to active oxygen species as a result of biochemical and physiological reactions at the cellular level. To protect themselves from the toxicity of such active oxygen species as superoxide anion radical (O_2^-) and hydroxyl radical (OH^-), every aerobic organism must have the ability to scavenge these molecules.

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 (Meister and Anderson 1983). A highly reduced state at the intracellular level is maintained by this reaction. In addition, GR has frequently been investigated as one of the key enzymes in the active oxygen scavenging system, involving superoxide dismutase (SOD; EC 1.15.1.1) and the enzymes of the ascorbate-glutathione cycle in higher plants (Asada 1994). 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 *Arabidopsis 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 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

Abbreviations: ABRE, abscisic acid-responsive element; Cu/Zn-SOD, copper/zinc-superoxide dismutase; GR, glutathione reductase; IPTG, isopropyl-β-D-thiogalactopyranoside; SOD, superoxide dismutase.

The nucleotide sequences in this paper have been submitted to the DDBJ, EMBL and GenBank under accession numbers D85751 (RGRC2) and AB009592 (gGRC-1).

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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.

To clarify the gene structures and the regulation of enzymes for the active oxygen scavenging system in rice, we previously isolated and characterized cDNAs or genes for SOD isozymes and cytosolic ascorbate peroxidases from rice (Sakamoto et al. 1992a, b, 1993, 1995a, Kaminaka et al. 1997, Morita et al. 1997a) and showed that cytosolic Cu/Zn-SOD genes are differentially expressed in response to the phytohormone (ABA) with promoter analysis in rice protoplasts (Sakamoto et al. 1995b). In this study, we have 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, we isolated and characterized a genomic clone (gGRC-1) for RGRC2 and examined the response of rice cytosolic GR to various environmental stresses.

Materials and Methods

Plant materials and growth conditions—Rice (*Oryza sativa* L. cv. Nipponbare) was used in all experiments. Seedlings were grown in a growth cabinet maintained at 28°C and a 16 h photoperiod at 220 μE m^{-2} s^{-1} for 10 d after germination. Etiolated seedlings were grown in darkness for the same period. Suspension cells derived from embryogenic calli were maintained by weekly subculture in MS liquid medium containing 1 mg $liter^{-1}$ of 2,4-D. The samples were immediately frozen in liquid N_2 and stored at −80°C until use.

Isolation and characterization of a cDNA for rice GR cDNA—Prior to the screening, an approximately 1.4 kbp DNA fragment was obtained using PCR with a pair of primers, ssGRF (5′-ATCGGTGCCGGAAGCGGCGGTGT-3′) and GRR-1 (5′-CTCAT[GT]GT[GC]ACAAACTC[CT]TC-3′), which were synthesized referring to the nucleotide sequences of spinach GR cDNA. This amplified product was subcloned into Bluescript SK(+) (Stratagene, La Jolla, CA) modified as T-vector in our laboratory according to Marchuk et al. (1991). 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, we screened a cDNA library, which was made from leaves of rice seedlings using lambda-ZAPII arms predigested with *Eco*RI (Stratagene) as described by Kaminaka et al. (1997). A putative full-length cDNA clone, designated as RGRC2, was then obtained and used for further analysis. The methods of screening and sequencing were followed as described previously (Kaminaka et al. 1997). The sequence data from RGRC2 were analyzed using GENETYX software (Software Development Co. Ltd., Tokyo, Japan).

Expression of rice GR cDNA (RGRC2) in Escherichia 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., Tokyo, Japan), to produce a histidine-tagged recombinant protein in *E. coli* JM109. High expression levels of recombinant protein in *E. coli* induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were recognized by means of 12% SDS-PAGE and Coomassie brilliant blue staining.

The recombinant protein expressed in *E. coli* was purified in a His Trap chelating column (Amersham Pharmacia Biotech Japan, Tokyo, Japan) according to the manufacturer's instructions but without addition of guanidine hydrochloride or urea. The culture induced by 1 mM IPTG at 37°C for 5 h was centrifuged at 4,000 × *g* for 20 min. The resulting pellet was resuspended in 5 ml per one gram 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 × *g* for 15 min. The supernatant was filtered through a 0.45 μm 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.

Four 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 A_{340} due to the NADPH oxidation ($\varepsilon = 6.2$ 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 × *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 × *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 μl of the extract, and the activity was measured in at least triplicate by a BioSpec-1600 spectrometer (Shimadzu, Kyoto, Japan). The kinetic parameters were determined in triplicate assays using five different concentrations of NADPH (2–100 μM) and GSSG (10–200 μM). Protein concentrations were determined colorimetrically (Bradford 1976).

Subcellular fractionation and assay of marker enzymes—Leaf tissues (30 g FW) 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 gradient (70% and 40%) in washing buffer and then centrifuged at 7,700 × *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 × *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 × *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 × *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 × *g* for 10 min twice. The washed pellet was resuspended with 200 μl 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 × *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—Total genomic DNA was isolated from rice etiolated seedlings using the cetyltrimethylammonium bromide method according to the standard procedures (Sambrook et al. 1989). The genomic DNA (10 μg) was digested with 50 units of each restriction enzyme, then electrophoresed in a 0.7% agarose gel. The transferred membrane was hybridized with an *Eco*RI-fragment of RGRC2 cDNA (full-length cDNA) as a ^{32}P-labelled probe using a BcaBest labeling kit (Takara Shuzo, Kyoto, Japan) at high-stringency conditions (1 × SSC, 42°C).

The genomic clone (gGRC-1) was isolated from a rice genomic library constructed with λEMBL3 arms (Sakamoto et al. 1992b). This library (2 × 10^5 plaques) was screened with the probe as described above at the stringency (2 × SSC, 42°C). Lambda phage DNA preparation and the recombinant DNA techniques were as described by Sambrook et al. (1989), and sequencing were performed as described by Kaminaka et al. (1997).

Stress treatments, Northern blotting and immunoblotting—Young rice seedlings grown as described in Materials and Methods were used for chemical and stress treatments. For ABA (Sigma, St. Louis, MO) and salt stress treatments, the seedling roots were placed in ABA (1 mM) and NaCl (250 mM) solutions. For chilling stress, the seedlings were exposed to 10°C. Drought stress was induced by withholding water. These stress treatments were performed under continuous light (220 $\mu E\,m^{-2}\,s^{-1}$). Samples were collected at 0, 3, 6, 12, 24 and 48 h after the start of each treatment, immediately frozen in liquid N2, and stored at −80°C.

Extraction of total RNA and Northern blotting were performed as described previously (Kaminaka et al. 1997). An *Eco*RI-fragment of RGRC2 cDNA (full-length cDNA) was used as the hybridization probe.

Samples for the estimation of expression in tissues and rice seedlings treated as described above 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 × *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 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 (data not shown). Consequently, the longest cDNA of 21 positive clones, designated as RGRC2, was obtained from 4.5 × 10^5 plaques, and both strands of the nucleotide sequence were determined and used for further analysis. RGRC2 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. 1). 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 procaryotes (data not shown). However, because of the difference of primary structure, containing N-terminal or C-terminal length, and the difference in homology among plant GRs, we suggest that these plant GRs are distinguished into two types. AT-2 (*A. thaliana* chloroplastic GR; Kubo et al. 1993) and Pea-2 (*P. sativum GOR1*; Creissen et al. 1992, 1995) 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. Recently, cloning and characterization of a putative cytosolic GR cDNA (*GOR2*) was reported by Stevens et al. (1997). However these authors did not demonstrate that the protein encoded by the cDNA was actually localized in the cytosol.

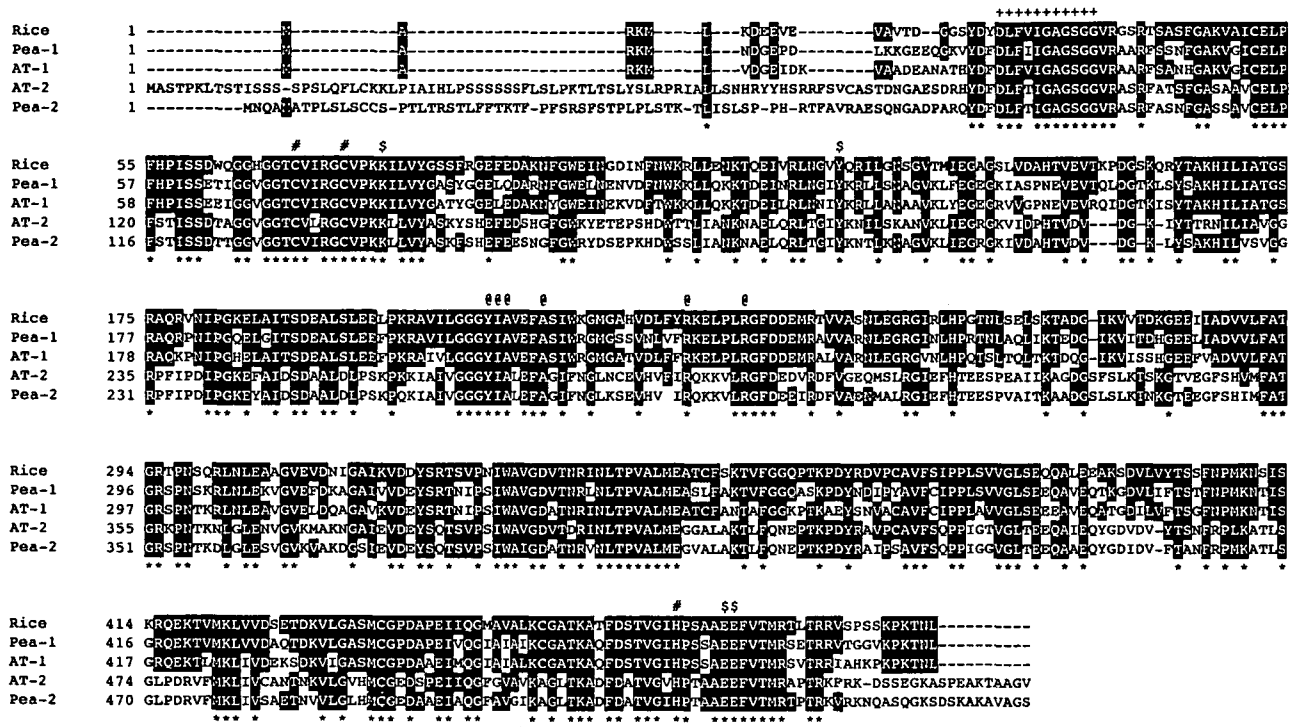

Fig. 1 Multiple alignment of a rice glutathione reductase (GR) and the other plant GRs. Rice, *Oryza sativa* GR (RGRC2, in this study); Pea-1, *Pisum sativum* putative cytosolic GR (*GOR2*, Stevens et al. 1997); AT-1, *Arabidopsis thaliana* GR (accession number U37697); AT-2, *Arabidopsis thaliana* chloroplastic GR (Kubo et al. 1993); Pea-2, *Pisum 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., Tokyo, Japan). 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.

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 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. 2A, 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. 2B). We demonstrated the reaction of the anti-recombinant RGRC2 protein antibody with the purified recombinant RGRC2 proteins by immunoblot analysis (Fig. 2A, 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. 2A, lane 6). The kinetic properties of this purified protein are shown in Table 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).

Subcellular location of RGRC2 protein—To determine whether the protein encoded by *RGRC2* was actually localized in the cytosol, we examined the immunoblotting results of the subcellular fractionation prepared as described in Materials and Methods (Fig. 3). 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 mitochondria fractions (lane 4) showed no detectable activity of NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase (chloroplast marker). The activities of PEP carboxylase and glucose-6-phosphate dehydrogenase (cytosol marker) were clearly detected only in the crude cytosolic fraction (lane 2), which showed all enzyme activities examined because this fraction contained not only cytosolic proteins but also the proteins from broken organelles. A band corresponding to *RGRC2* protein was not detected in the chloroplast fraction but slightly apparent in the mitochondria fraction,

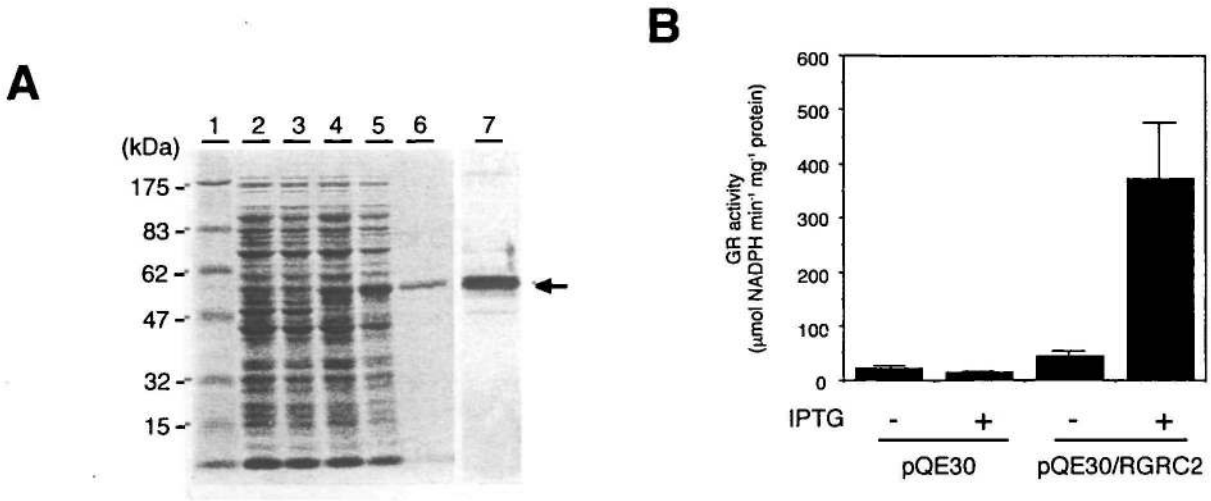

Fig. 2 Expression in *E. coli* and purification of recombinant RGRC2 proteins. (A) SDS-PAGE gel (12%) was stained with Coomassie brilliant blue (lane 1–6) or subjected to immunoblotting with the antibodies against recombinant RGRC2 protein (lane 7). Molecular marker (lane 1; Prestained protein marker, New England Biolabs, MA, U.S.A.); 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 μg of purified recombinant RGRC2 protein (lane 6); lane 6 subjected to immunoblot (lane 7). Molecular size is indicated in the left margin. (B) 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.

whereas this band was strongly detectable in the crude cytosol fraction compared with that in the total protein fraction from leaves of rice seedlings (lane 1). These results indicated that most of the protein encoded by *RGRC2* was localized in the cytosol.

Expression of RGRC2 gene in rice vegetative tissues— To characterize the tissue specific expression of *RGRC2*, we analyzed the total RNAs isolated from different vegetative tissues of rice seedlings and from rice embryogenic calli by Northern blot analysis using full-length RGRC2 as a probe (Fig. 4A). A single but broad mRNA band of approximately 1,800–2,000 nucleotides was detected in all tissues examined. mRNA of *RGRC2* was strongly expressed in roots, stems and callus but little was expressed in leaf tissues. The *RGRC2* protein was analyzed by immunoblot analysis using the anti-RGRC2 protein antibody in the same tissues as used for the Northern blotting. In this study, a native-PAGE was used for the separation of proteins to avoid overlapping with a non-specific band, since the molecular masses of the GR specific band (53 kDa) and a non-specific band (presumedly large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) are nearly identical (data not shown). The result showed that the detected band was found mainly in roots and callus, and less abundant in stems (Fig. 4B). This result obtained by native-PAGE was identical to that by SDS-PAGE (data not shown). However additional bands were also detected in roots and calli. The same experiment using transgenic tobacco overexpressing RGRC2 (unpublished) indicated that the protein for *RGRC2* corresponds to the major band

Table 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)

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

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

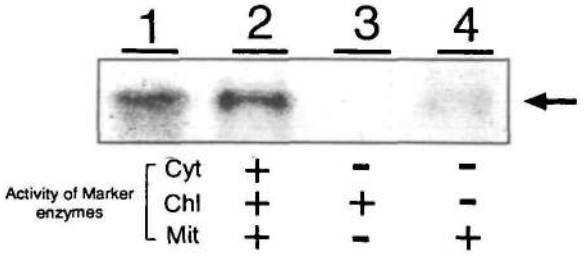

Fig. 3 Immunoblot analysis of *RGRC2* protein in the total or subcellular fractions after native-PAGE. Total protein or subcellular fractions were separated by native-PAGE (10%), transferred to PVDF membrane and subjected to immunoblotting with the antibodies against recombinant RGRC2 protein. Subcellular fractionation from rice leaves was performed as described in Materials and Methods. The presence or absence of marker enzyme activity (Cyt, cytosol marker; Chl, chloroplast marker; Mit, mitochondria marker) in each subcellular fraction is indicated as plus or minus, respectively. Lane 1, total proteins from rice leaves (30 μg); lane 2, proteins from crude cytosolic fraction (100 μg); lane 3, proteins from purified chloroplast fraction (60 μg); lane 4, proteins from purified mitochondrial fraction (60 μg).

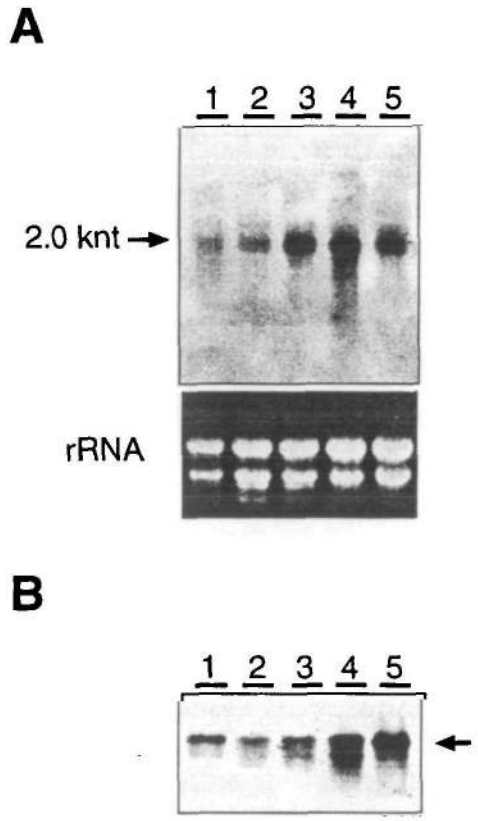

Fig. 4 Expression of *RGRC2* in various tissues. (A) Northern blot analysis of *RGRC2* gene expression. Transcripts were hybridized with ^{32}P-labelled *Eco*RI fragments of RGRC2. Each lane was loaded with 10 μg of total RNA. rRNA indicates the result of electrophoresis stained with ethidium bromide. Lane 1, etiolated leaves; lane 2, green leaves; lane 3, stems; lane 4, roots; lane 5, calli. (B) Immunoblot analysis of *RGRC2* protein. Each total protein (30 μg) of the same tissue as used in Northern blotting was separated by native-PAGE (10%), transferred to PVDF membrane and subjected to immunoblotting with the antibodies against recombinant RGRC2 protein. The band corresponding to gene product of *RGRC2* is indicated as a tailed arrow.

(shown as a tailed arrow; data not shown). This result suggested that the gene product of *RGRC2* corresponds to the major band.

Gene structure of the RGRC2 for rice GR—Prior to the isolation of the genomic clone corresponding to RGRC2, the copy number of this gene in the rice genome was analyzed by Southern blotting with a full-length cDNA fragment (Fig. 5A). Two bands were observed by restriction enzyme fragments (except in the case of *Eco*RV). We suggest that the *RGRC2* gene is a single copy gene in the rice genome, since only two bands were detected with *Hin*cII, and these bands were expected by the restriction site residing in the coding region of RGRC2 cDNA.

The genomic clone (gGRC-1) was isolated from a rice genomic library (2×10^5 plaques) as described in Materials and Methods. About 7.4 kb of the nucleotide sequence (accession number AB009592) from gGRC-1 was completely determined and its structure analyzed (Fig. 5B). This sequence contains 1.5 kb of promoter region and coding region spanning about 6 kb of genomic DNA. Structural alignment provided not only perfect agreement between the sequences analyzed for the cDNA and the putative exons in this gene, but also the existence of 14 introns in the coding region (data not shown). All introns showed GT-AG intron border sequences. The analysis also revealed that the 5′ transcribed but untranslated region is interrupted by an additional intron (5′ non-coding intron) at 26 bp upstream from the translation start site (Fig. 5, white box). The 5′ non-coding intron and exon were not found in a gene for pea chloroplastic/mitochondrial GR, which is only a characterized GR gene in plants (Mullineaux et al. 1996). In summary, this gene (*RGRC2*) is composed of 16 exons interrupted by 15 introns, containing the 5′ non-coding exon and intron. The restriction map corresponds to the results of the genomic Southern blot analysis (Fig. 5A).

Characterization of the 5′-flanking region in the RGRC2 gene—The nucleotide sequence of the 5′-flanking region, that includes the first exon and ending in second exon of the *RGRC2* gene, is shown in Figure 6. We searched this flanking region for any known motifs or regulatory elements of other plant genes. The consensus sequence of a putative TATA box (5′-TATAA-3′) was found at position −520 (Fig. 6, underlined). A 60 bp direct repeat sequence (DR1) was found at positions −1688 and −1813 and contained a consensus sequence motif among plant amylase genes (AMYBOX, 5′-TAACAAGA-3′; Huang et al. 1990). DR1 also contained a 23 bp direct repeat sequence (DR2),

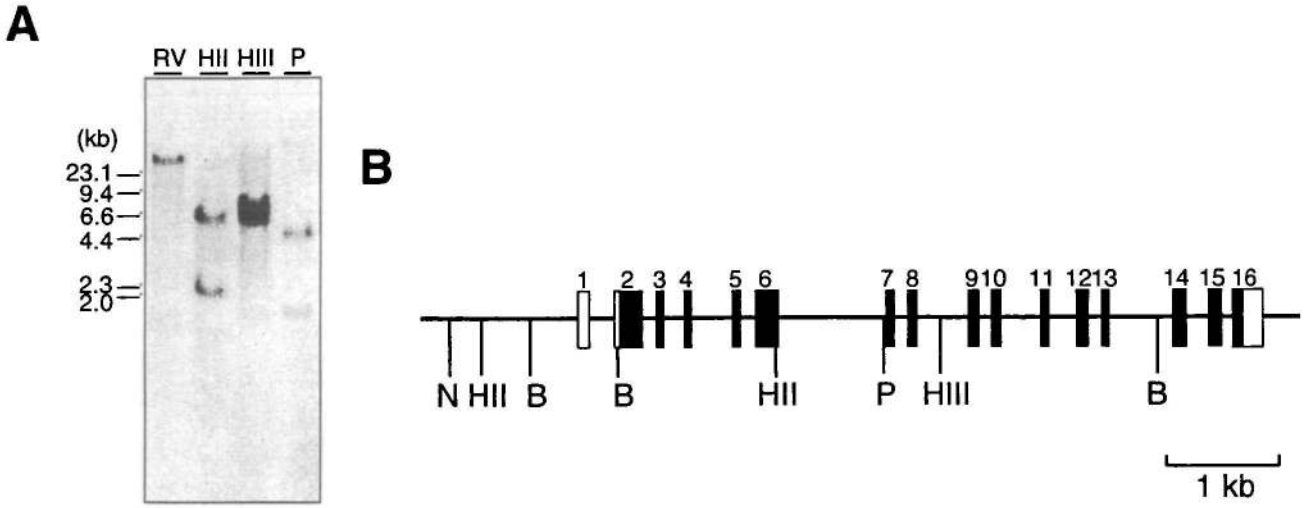

Fig. 5 Analysis of a gene for RGRC2. (A) Southern blot analysis of rice genomic DNA. Rice genomic DNA was digested with *Eco*RV, *Hin*cII, *Hin*dIII and *Pst*I, transferred onto Hybond-N membrane, probed with a ^{32}P-labelled full-length cDNA (RGRC2) and washed at the stringency (1 × SSC, 42°C). The molecular marker (λ/*Hin*dIII) is indicated at left. RV, *Eco*RV; HII, *Hin*cII; HIII, *Hin*dIII; P, *Pst*I. (B) Structure and restriction maps of the *RGRC2* gene. The boxes correspond to the exons; translational and untranslational regions are indicated as dark boxes and light boxes, respectively. B, *Bam*HI; HII, *Hin*cII; HIII, *Hin*dIII; N, *Nhe*I; P, *Pst*I.

which was found at −1729. An unknown 20 bp palindrome sequence (PD) was found between direct repeat sequences (DR3), which contained an E-box consensus sequence (5′-CANNTG-3′). Seven other E-boxes were also found in this promoter region. Two ABA-responsive element (ABRE; Mundy et al. 1990, Guiltinan et al. 1990) core sequences (5′-ACGTGGC-3′) were found at positions −394 and −1230. The regulatory elements −300 element (5′-TGTAAAG-3′; Thompson et al. 1990) and (CA)n element (5′-CAAACAC-3′; Ellerstrom et al. 1996) were found at positions −1311 and −1066, respectively. Through a search of DNA databases, we found a 42 bp homologous sequence (shown as HS in Fig. 6) among the promoter regions of *RGRC2* gene, rice root-specific protein gene (*RCg2*; Xu et al. 1995) and rice thioredoxin *h* gene (*RTrxh*; Ishiwatari et al. 1995) at position −604. The homologous region showed a perfect match between *RGRC2* and *RCg2* and between *RGRC2* and *RTrxh*, 20 bp and 15 bp, respectively. However, we could not find any homologous sequences between the sequences in the promoter regions of RGRC2 gene and pea chloroplastic/mitochondrial GR gene (Mullineaux et al. 1996).

Expression of RGRC2 under ABA and stress treatments—Based on the observation of two ABREs and several regulatory motifs in the 5′-flanking region of the *RGRC2* gene, we hypothesized that the gene expression of *RGRC2* was regulated under environmental stresses via ABA-mediated signal transduction pathway, in addition to being induced by ABA treatment. To evaluate the expression of *RGRC2* under these conditions, we examined the results of the Northern blot analysis and immunoblot analysis of rice seedlings treated with ABA (1 mM), drought, salt stress (NaCl, 250 mM) and chilling (10°C) for 48 h (Fig. 7). The mRNA level of *RGRC2* was increased significantly at 6 h after the onset of ABA treatment, and reached a maximum level by 12 h (Fig. 7A). In accordance with the accumulation of mRNA, an increase of RGRC2 protein was observed from 12 to 24 h. Similarly, the expression of *RGRC2* gene was induced strongly by drought treatment (Fig. 7B), the maximum level of mRNA being observed apparently at 12 h. Coordinately, accumulation of protein was observed at 24 h. However, degradation of the protein and decrease in mRNA levels were apparently observed at 48 h. This decrease was probably due to the serious damage of the seedlings caused by the stress treatment. The changes in mRNA and protein levels induced by salt stress were similar to those induced by ABA treatment (Fig. 7C). Chilling induced a continuous increase in the mRNA level throughout the treatment period, and protein accumulation was significantly increased at 48 h (Fig. 7D).

Discussion

We isolated and characterized a GR cDNA (RGRC2) from rice. Although GR has previously been purified from rice embryo (Ida and Morita 1971), the molecular biological characterization of the GR in rice has not been reported. By comparing the protein properties of recombinant RGRC2 and purified protein from rice embryo, we determined that the molecular size and amino acid composition of RGRC2 protein (data not shown), as well as the K_m value for NADPH and GSSG of recombinant RGRC2 pro-

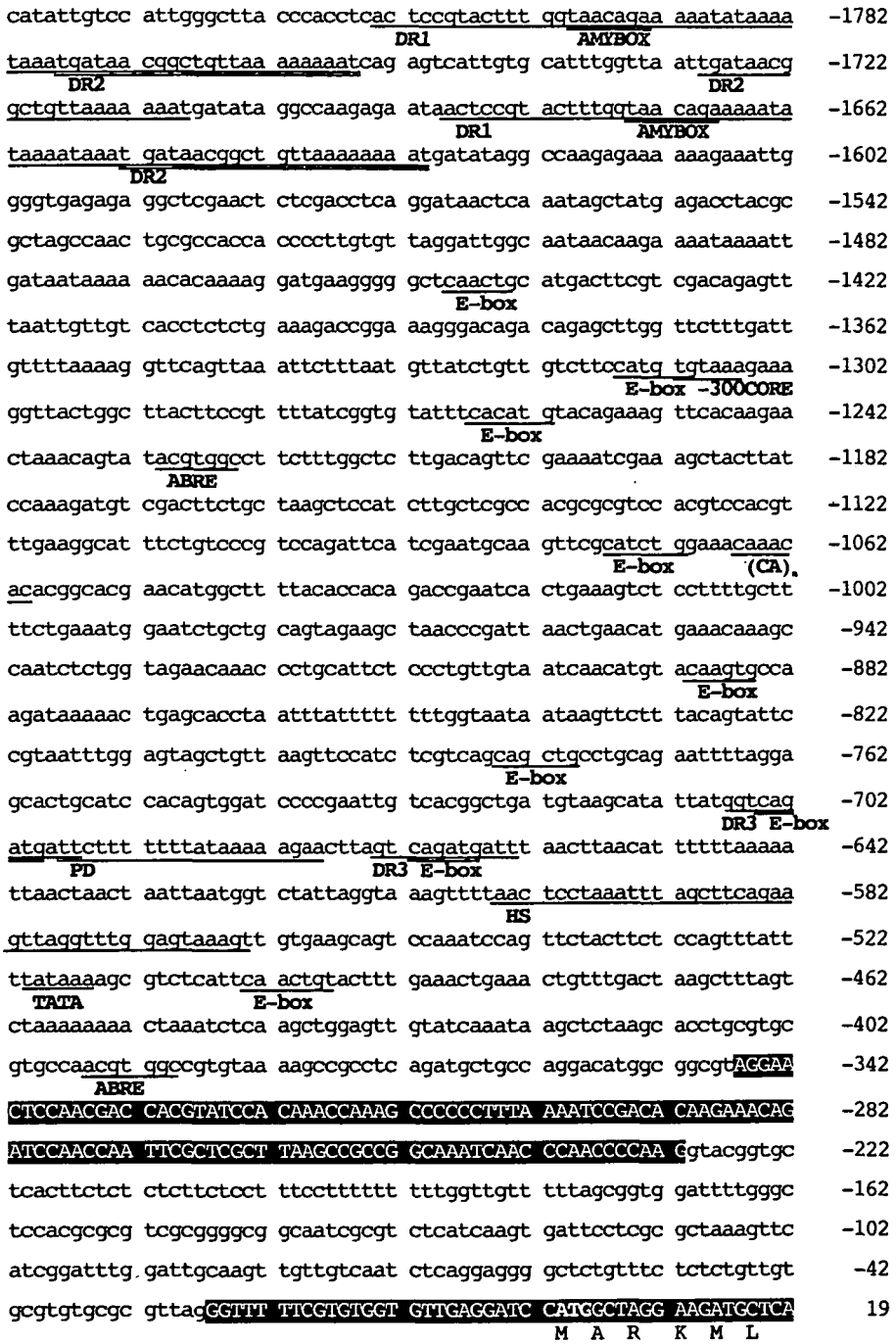

Fig. 6 *Nucleotide sequence in the 5′-flanking region of the RGRC2 gene.* The translational start site is shown as +1, and the deduced amino acids in 2nd exon are represented using the single-letter amino acid code. The 5′-flanking region and introns are in lower case, while the exons are represented with black boxes and in upper case. A putative TATA box, ABREs, E-boxes, Amylase box-I (AMYBOX), (CA)n element, −300 bp core sequences and palindrome sequence (PD) are labeled. Three pairs of direct repeat sequences are indicated as DR1,DR2 and DR3. A 42 bp homologous sequence among the 5′-flanking regions of *RGRC2, RCg2* (Xu et al. 1995) and *RTrxh* (Ishiwatari et al. 1995) is indicated as HS.

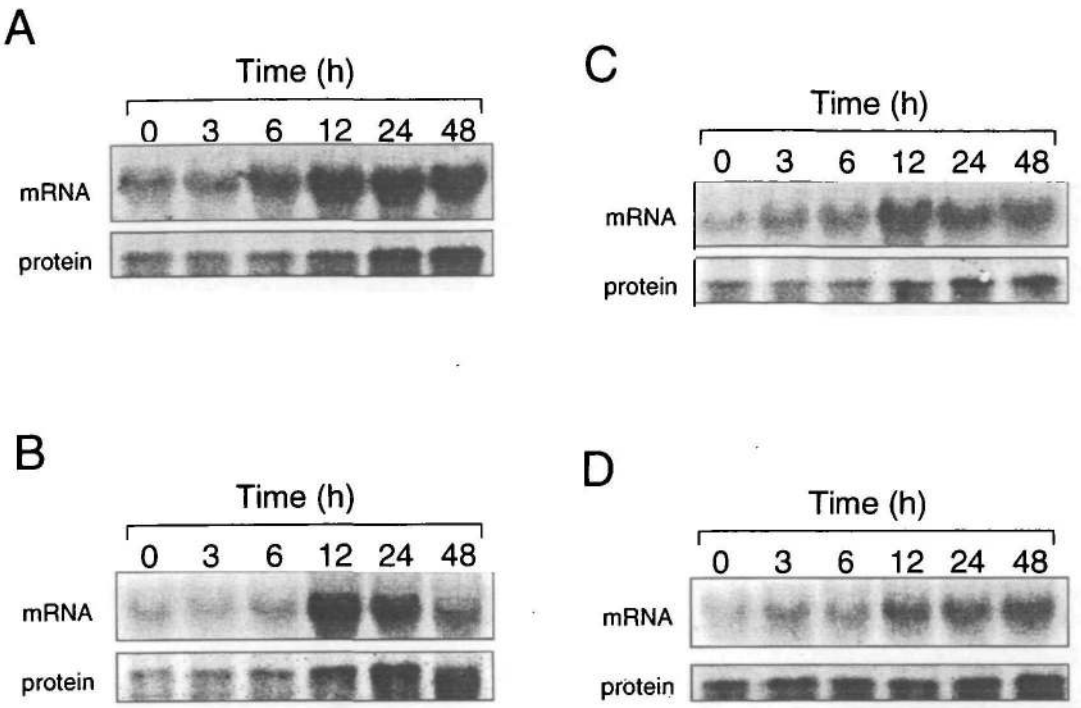

Fig. 7 The effect of ABA, salt stress, chilling and drought on the mRNA and protein abundance of *RGRC2* in rice seedlings. RNA and protein samples were prepared from treated seedlings, which were sampled at indicated times after each stress treatment. Total RNAs (20 μg) were fractionated by electrophoresis on a 1.2% formamide-containing agarose gel. Total protein samples (30 μg) were fractionated by 10% native-PAGE. Blotting and detection were performed as described in Materials and Methods. A, ABA (1 mM); B, drought; C, salt stress (250 mM NaCl); D, chilling (10°C). The results of Northern blotting and immunoblotting are indicated as mRNA and protein, respectively.

teins, were similar to those of GR isolated from rice embryo (Table 1). These results suggested that the protein encoded by RGRC2 corresponds to the purified GR from rice embryo, and this assumption is supported by the strong expression of the gene for RGRC2 in embryogenic-calli (Fig. 4).

In plants, GR cDNAs that are clearly characterized by protein analysis, are only of chloroplastic or chloroplastic/mitochondrial type (Kubo et al. 1993, Creissen et al. 1995). RGRC2 has a high homology and similar primary structure to recently reported pea cDNA encoding a putative cytosolic GR (*GOR2*; Stevens et al. 1997) but not to chloroplastic types (Fig. 1). However, characterization of the pea cDNA as a cytosolic type was deduced only from the feature of the primary structure. Therefore, to clarify the location of a putative cytosolic isoform of GR, we examined the immunoblotting results of subcellular fractionation with anti-RGRC2 protein antibody (Fig. 3). An immunoreaction band was observed in cytosol fractions but not in chloroplast fractions. However a weak signal was also observed in the purified mitochondria fraction. This was probably due to the cross-reaction of the used antibody with cytosolic and mitochondrial GRs. Similar results have also been reported in rat liver (Taniguchi et al. 1986). The mitochondrial and cytosolic GRs were immunologically indistinguishable, and the enzyme properties were very similar between these isoforms. Recombinant protein of pea cytosolic GR cDNA expressed in *E. coli* cross-reacted against the chloroplastic/mitochondrial GR antibody (Stevens et al. 1997). The difference in cross-reactivity between the antibodies of rice cytosolic GR and pea chloroplastic/mitochondrial GR is probably due to the difference of used antigens and epitopes of antibodies. We could not resolve these problems because mitochondrial GR has not been characterized in rice. However, we concluded that the protein encoded by *RGRC2* was localized almost exclusively in cytosol because the band detected in the cytosolic fraction was much more abundant than that detected in mitochondrial fractions, as if mitochondrial GR can be detected immunologically using the anti-RGRC2 protein antibody, and no transit peptide or targeting motifs to organelles were observed in the primary structure of RGRC2 (Fig. 1).

We have isolated and characterized a gene (*RGRC2*) corresponding to RGRC2 from rice. The GR genes in eukaryotes have been reported with pea (Mullineaux et al. 1996), mouse (Tamura et al. 1997), *Onchocerca volvulus*

(Muller et al. 1997) and *Plasmodium falciparum* (Farber et al. 1996). *RGRC2* gene contains so many introns (15 introns) compared with other GR genes, pea GR gene is split into 10 exons, mouse into 13 exons, *O. volvulus* into 12 exons and *P. falciparum* into 3 exons. Even between plant GR genes, there are the differences of intron number and the inserted positions (several inserted positions are similar but most are not identical). This is probably due to the difference between chloroplastic/mitochondrial isoform and cytosolic isoform. These findings suggested that the distribution of many introns in *RGRC2* gene is the result of more recent insertion event in the evolution pathway. In the plant GR gene, the presence of the 5′ non-coding intron is unique in *RGRC2* gene. The functional significance for 5′ non-coding intron sequence in gene expression has been revealed by gene transfer analyses in monocotyledonous plant cells (McElroy et al. 1991). Therefore, the 5′ non-coding exon and intron in the *RGRC2* gene may have some functional role in controlling gene expression at the transcriptional or post-transcriptional level.

By analysis of the promoter region (about 1.5 kb) in the *RGRC2* gene, we observed several known motifs or regulatory elements (Fig. 6). Interestingly two direct repeat sequences, DR1 and DR3, contained the known regulatory motifs, consensus sequence motif among plant amylase genes (AMYBOX; Huang et al. 1990) and E-box core motif. E-box is known to be identical to a core sequence for a class of transcription factors basic helix-loop-helix proteins (bHLH) and can form homo- and hetero-dimers to exert regulatory functions (Pabo 1992). Furthermore, we found a −300 element and (CA)n element, which are found in the promoter regions of storage proteins and exist as regulatory elements (Thompson et al. 1990, Ellerstrom et al. 1996), near the E-box sequence motifs. Therefore actually these direct repeats and motifs may play a role as regulatory elements of *RGRC2* gene. While we obtained a 42 bp sequence (shown as HS in Fig. 6) homologous with those of rice root-specific protein gene (*RCg2*; Xu et al. 1995) and rice thioredoxin *h* gene (*RTrxh*; Ishiwatari et al. 1995). The root-specific protein is an unknown protein, but an in situ hybridization experiment in maize roots suggested this protein has the function of transporting molecules to and/or from the vasculature (John et al. 1992). Similarly, rice thioredoxin *h* has been identified as one of the major proteins in phloem sap (Ishiwatari et al. 1995). The *RGRC2* protein was localized in the phloem vessels of rice roots (data not shown). Therefore, this homologous sequence among the three genes may be concerned with the specific-expression in phloem cells in rice.

Furthermore, two ABA-responsive elements (ABREs) were observed in the 5′-flanking region of the *RGRC2* gene (Fig. 6). The ABA content increases in plant tissues under stress conditions such as dehydration, high concentration of salts, and low temperature (Skriver and Mundy 1990). GR activity has also been reported to increase under various stress treatments, including drought (Gamble and Burke 1984, Tanaka et al. 1990, Gogorcena et al. 1995), chilling (Edwards et al. 1994), magnesium deficiency and high light intensity (Cakmak and Marschner 1992) and exposure to air pollutants (Tanaka et al. 1988, Madamanchi et al. 1992). Expression of the *RGRC2* gene was strongly induced by ABA treatment and drought stress (Fig. 7A, B), but weakly by salt stress and chilling treatment (Fig. 7C, D). Gene expression of pea cytosolic GR has been reported to be induced by chilling and during recovery from drought stress, but not in parallel with GR activity (Stevens et al. 1997), whereas in this study a parallel change was observed between the amounts of mRNA and protein of *RGRC2* (Fig. 7). We have previously indicated that the expression of cytosolic Cu/Zn-SOD gene (*sodCc2*) was strongly induced in response to ABA on the basis of promoter analysis in rice protoplasts (Sakamoto et al. 1995b), and that one of the rice cytosolic ascorbate peroxidase (APX) genes (*apxa*) was induced by ABA treatment (Morita et al. 1997b). The transcripts of pea cytosolic APX were also increased by ABA and drought stress (Mittler and Zilinskas 1992, 1994). We suggested that the expression of the rice cytosolic GR gene is regulated via ABA-mediated signal transduction under environmental stresses.

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