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, 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.

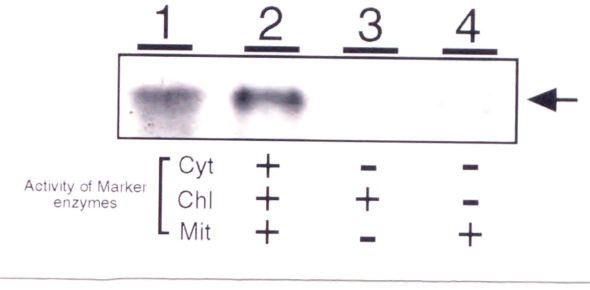


Fig. V-6. Immunoblot analysis of *RGRC2* protein in the total or subcellular fractions after native-PAGE. Total protein or subcellular fractions were separated by native-PAGE (10%), transferred to PVDF membrane and subjected to immunoblotting with the antibodies against recombinant RGRC2 protein. Subcellular 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).

Expression of RGRC2 gene in rice vegetative tissues -

To characterize the tissue specific expression of *RGRC2*, Northern blot analysis with the total RNAs isolated from different vegetative tissues of rice seedlings and from rice embryogenic calli was carried out using full-length RGRC2 as a probe (Fig. V-7A). 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 (Fig. V-7B). 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 kD) and a non-specific band (presumedly large subunit of Rubisco) are unseparatable (data not shown). The result showed that the detected band was found mainly in roots and callus, and less abundant in stems. 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 (data not shown) indicated that the protein for *RGRC2* corresponds to the major band (shown as a tailed arrow; data not shown). This result suggested that the gene product of *RGRC2* corresponds to the major band.

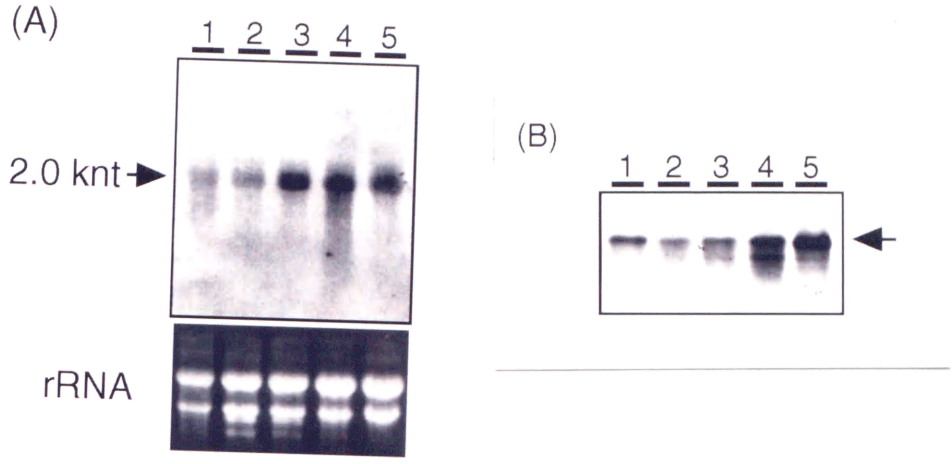


Fig. V-7. Expression of *RGRC2* in vegetative tissues. (A) Northern blot analysis of *RGRC2* gene expression. Transcripts were hybridized with ^{32}P-labelled *Eco*RI fragments of RGRC2. Each lane was loaded with 10 μg of total RNA. rRNA indicates the result of electrophoresis stained with ethidium bromide. Lane 1, etiolated leaves; lane 2, green leaves; lane 3, stems; lane 4, roots; lane 5, suspension culture of embryogenic-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.

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. V-8A). Two bands were observed by restriction enzyme fragments (except in the case of *Eco*RV). This result suggest that the *RGRC2* gene is a single copy gene in the rice genome, since only two bands were detected with *Hinc*II, 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 germ genomic library (2 x 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. V-8B). 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 15 introns in the coding region (Fig. V-9). 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. V-8B, 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 17 exons interrupted by 16 introns, containing the 5' non-coding exon and intron. The restriction map corresponds to the results of the genomic Southern blot analysis (Fig. V-8A).

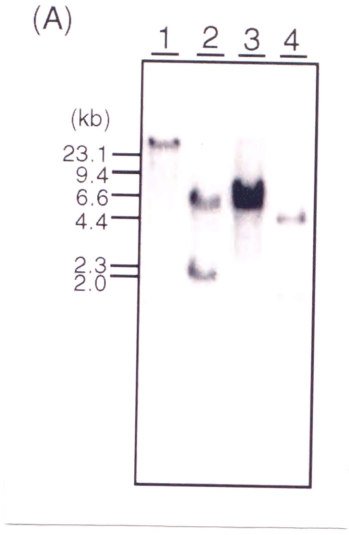
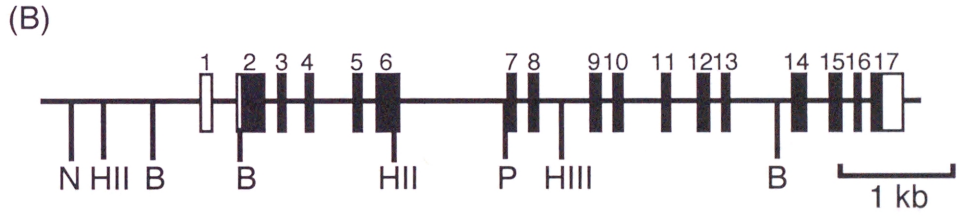
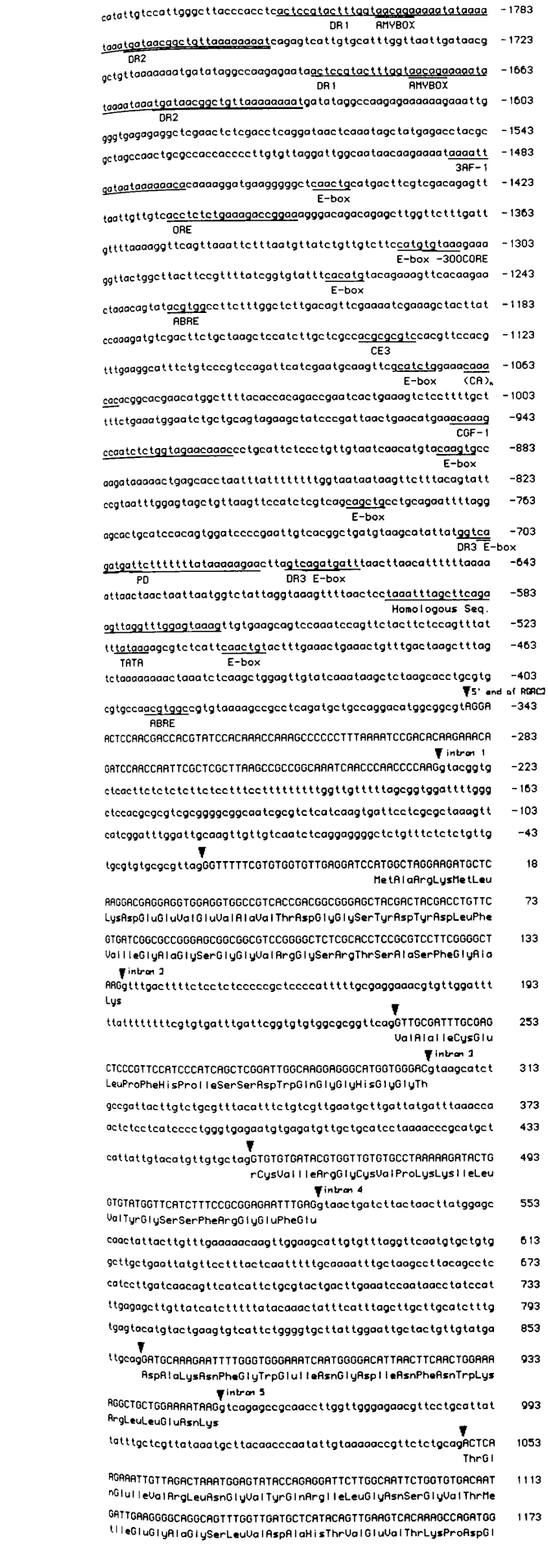


Fig. V-8. Analysis of a gene for rice cytosolic GR gene (*RGRC2*). (A) Southern blot analysis of rice genomic DNA. Rice genomic DNA was digested with *Eco*RV (1), *Hin*cII (2), *Hin*dIII (3), and *Pst*I (4), transferred onto Hybond-N membrane, probed with a ^{32}P-labelled full-length cDNA (RGRC2) and washed at the stringency (1 x SSC, 42 °C). The molecular marker (λ/*Hin*dIII) is indicated at left. (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.



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 V-9. This flanking region was searched 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. 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), 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, a 42 bp homologous sequence (shown as Homologous seq. in Fig. V-9) was found


DR1
AMYBOX
DR2
DR1
AMYBOX
DR2
E-box
ORE
E-box -300CORE
E-box
ABRE
CE3
E-box
(CA)n
CGF-1
E-box
DR3 E-box
PD
DR3 E-box
Homologous Seq.
TATA
E-box
5' end of RGA1
ABRE
intron 1
intron 2
intron 3
intron 4
intron 5
intron 6
intron 7
intron 8
intron 9
intron 10
intron 11
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intron 13

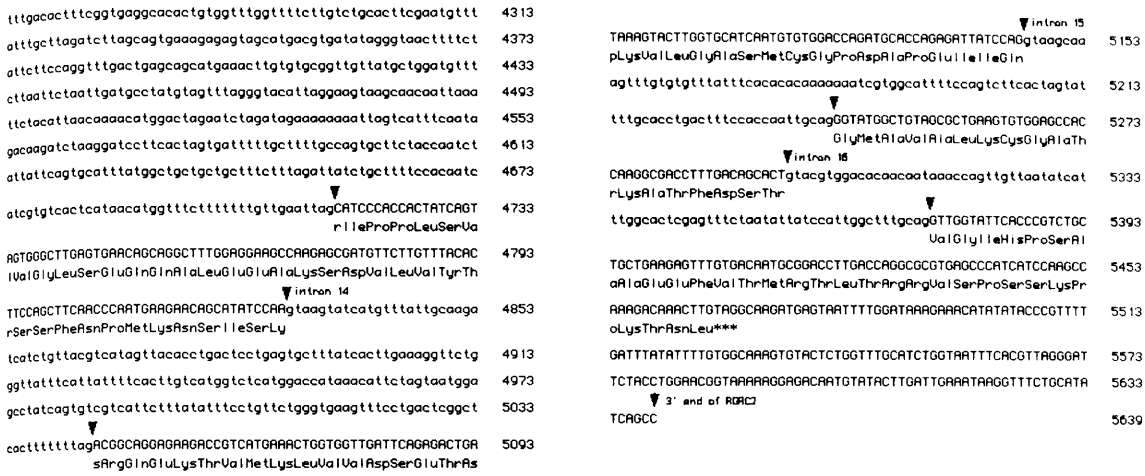

Fig. V-9. Nucleotide sequence of the *RGRC2* gene. The translational start site is shown as +1, and the deduced amino acids in the exon are represented using the triple-letter amino acid code. The 5'-flanking region and introns are in lower case, while the exons are represented in upper case. A putative 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. Homologous Sequence represents a 42 bp homologous sequence among the 5'-flanking regions of *RGRC2*, *RCg2* (Xu et al., 1995) and *RTrxh* (Ishiwatari et al., 1995). The nucleotide sequence data in this figure (gGRC-1) have been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB009592.

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, any homologous sequences between the sequences in the promoter regions of RGRC2 gene and pea chloroplastic/mitochondrial GR gene (Mullineaux et al., 1996) could not be found.

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, it is 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, the Northern blot analysis and immunoblot analysis were performed in rice seedlings treated with ABA (1 mM), drought, salt stress (NaCl, 250 mM) and chilling (10 °C) for 48 h (Fig. V-10). 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. V-10A). 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. V-10B), 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. V-10C). Chilling induced a continuous increase in the mRNA level throughout the treatment period, and protein accumulation was significantly increased at 48 h (Fig. V-10D).

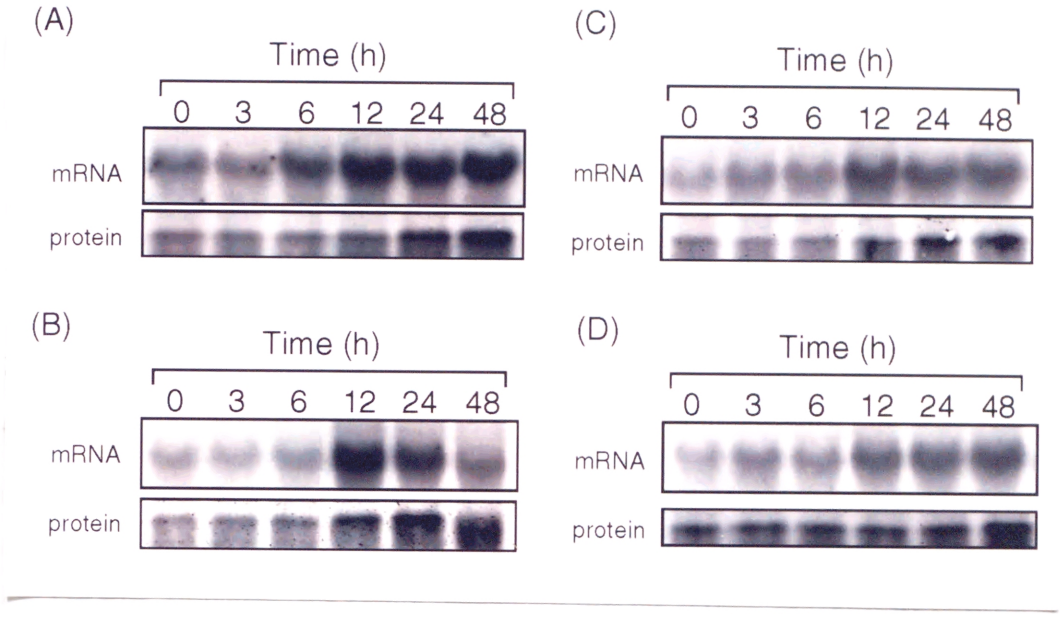

Fig. V-10. The effect of ABA, salt stress, chilling and drought on the mRNA and protein abundance of *RGRC2* in rice seedlings. RNA and protein samples were prepared from treated seedlings, which were sampled at indicated times after each stress treatment. Total RNAs (20 μg) were fractionated by electrophoresis 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.

Discussion

In this section, the author isolated and characterized a GR cDNA (RGRC2) from rice (Kaminaka et al., 1998b). 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, the molecular size and amino acid composition of RGRC2 protein were determined (data not shown), as well as the K_m value for NADPH and GSSG of recombinant RGRC2 proteins, were similar to those of GR isolated from rice embryo (Table V-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. V-7).

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. V-3). 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, the immunoblot analysis was carried out in subcellular fraction with anti-RGRC2 protein antibody (Fig. V-6). 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. These problems could not be resolved because mitochondrial GR has not been characterized in rice. However, the author 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. V-3).

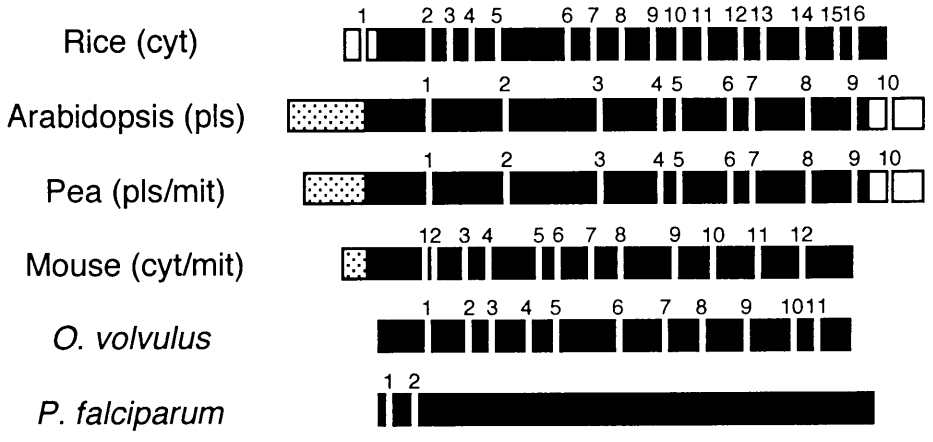

Fig. V-11. Comparison of exon/intron location in the amino acid sequences of GRs. Genomic sequences of GR gene from pea (Mullineaux et al., 1996), *A. thaliana* (Kubo et al., 1998), mouse (Tamura et al., 1997), *O. volvulus* (Muller et al., 1997) and *P. falciparum* (Farber et al., 1996) were used for comparison. Each intron number is indicated above the amino acid sequences.

A gene (*RGRC2*) corresponding to RGRC2 from rice have been isolated and characterized. The GR genes in eukaryotes have been reported with pea (Mullineaux et al., 1996), *A. thaliana* (Kubo et al., 1998), mouse (Tamura et al., 1997), *O. volvulus* (Muller et al., 1997) and *P. falciparum* (Farber et al., 1996) (Fig. V-11). *RGRC2* gene contains so many introns (16 introns) compared with other GR genes, pea and *A. thaliana* GR gene is split into 10 exons, mouse into 13 exons, *O. volvulus* into 12 exons and *P. falciparum* into 3 exons. There is not any correlations among the intron numbers of the GR genes. The phylogenic analysis (Fig. V-4) indicated plant GRs and other GRs are divided into two groups in the more early step of evolution pathway than the step of the division of plant GRs. Even between plant GR genes, there are the differences of intron number and the inserted positions (Fig. V-11; 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, several known motifs or regulatory elements were observed (Fig. V-9). 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, 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), was found near the E-box sequence motifs. Therefore actually these direct repeats and motifs may play a role as regulatory elements of *RGRC2* gene. While a 42 bp sequence (Fig. V-9) homologous with those of rice root-specific protein gene (*RCg2*; Xu et al., 1995) and rice thioredoxin *h* gene (*RTrxh*; Ishiwatari et al., 1995) was obtained. 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. V-9). 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. V-10A,B), but weakly by salt stress and chilling treatment (Fig. V-10C,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. V-10). These results suggested that the expression of the rice cytosolic GR gene is regulated via ABA-mediated signal transduction under environmental stresses.