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Sequence Homologies of GTP-binding Domains of Rab and Rho between Plants and Yeast/Animals Suggest Structural and Functional Similarities

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Small GTP-binding proteins are divided into three major groups: Ras, Rho and Ypt/Rab. They have the conserved regions designated G1 to G5 that are critical in GDP/GTP exchange, GTP-induced conformational change and GTP hydrolysis. We isolated and characterized genomic DNA or cDNA fragments encoding G1 to G3 domains of small GTP-binding protein Rab and Rho from several plant species using two different PCR-based cloning strategies. Seven *rab* DNA fragments were isolated from 4 different plants, mung-bean, tobacco, rice and pepper using two degenerate primers corresponding to the GTP-binding domain G1 and G3 in small GTP-binding proteins. The amino acid sequences among these *rab* DNA fragments are very similar, having over 80% identity. Sequence comparison between these DNA fragments and other known small GTP-binding proteins shows that they belong to the Ypt/Rab family. Six *rho* DNA fragments were isolated from 5 different plants, mung-bean, rice, *Arabidopsis, Allium* and *Gonyaulax* using the nested PCR method that involves four degenerate primers corresponding to the GTP-binding domain G1, G3 and G4. The *rho* DNA fragments cloned show more than 90% homology to each other. Sequence comparison between plant and other known Rho family genes suggests that they are closely related (67 to 82% amino acid identity). Sequence analysis and southern blot analysis of *rab* and *rho* in mung-bean suggest that these genes are encoded by multigene family in mung-bean.

Keywords: small GTP-binding domains, Rab, Rho, multigene family

GTP-binding proteins play a diverse range of functions for cellular signal transduction and regulation (Gilman, 1987; Hepler and Gilman, 1992; Terryn *et al.*, 1993; Ma, 1994). They are divided into heterotrimeric G proteins and monomeric small GTP-binding proteins. In animals and yeast, a variety of small GTP-binding proteins have been described that are thought to regulate diverse cellular responses including cell growth, division, differentiation and vesicle trafficking (Balch, 1990; Hall, 1990; Novick and Brennwald, 1993).

Small GTP-binding proteins ranging in molecular mass from 20,000 to 30,000 referred to as the Ras superfamily share high amino acid sequence identity and overall structure, suggesting that they evolved from a common ancestral gene. These proteins also share a common mechanism to function as a molecular switch that can be turned on by binding to GTP and turned off by hydrolyzing GTP to GDP (Downward, 1990; Bourne *et al.*, 1991; Wittinghofer and Pai, 1991). Based on similarity in amino acid sequences and presumed function, small GTP-binding proteins are generally subdivided into three major groups: Ras, Rho and Ypt/Rab proteins. Sequence comparison of proteins belonging to the Ras superfamily reveals several regions of high homology. These regions, designated G1 to G5, are critical in GDP/GTP exchange, GTP-induced conformational change and GTP hydrolysis (Bourne, 1991). The amino acid sequence motif, $GX_4GK(S/T)$, of the G1 region is involved in binding to the phosphate moiety of GTP and appears with variations in many nucleotide triphosphate utilizing enzymes. Amino acid sequences corresponding to the G2 region are highly conserved within each GTPase family, but not bet-

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ween different families. Each pupative G2 sequence contains a conserved threonine residue and its side chain hydroxyl group is coordinated to Mg^{2+} ion that is essential for GTP hydrolysis. The DX_2G sequence motif of the G3 region is conserved in all GTPases and involved in determining guanine nucleotide specificity. And the sequence motif of the G4 region is characterized by four hydrophobic or apolar amino acids followed by (N/T)(K/Q)XD. These all conserved regions of the polypeptide chain associated with loops on one side of the protein, merit special attention.

In contrast to the extensive knowledge on small GTP-binding proteins in yeast and mammals, virtually no information is available on such proteins in plants. Currently, several small GTP-binding protein genes/cDNAs have been isolated from a variety of plant species, including *Arabidopsis thaliana* (Matsui *et al.*, 1989; Anai *et al.*, 1991; Terryn *et al.*, 1993; Yi and Guerinet, 1994), rice (Sano and Youssefian, 1991), tobacco (Dallmann *et al.*, 1992; Haizel *et al.*, 1995), maize (Palme *et al.*, 1992; Kang *et al.*, 1995), *Plumbaginifolia* (Terryn *et al.*, 1992), *Pisum sativum* (Sasaki *et al.*, 1991; Drew *et al.*, 1993; Nagano *et al.*, 1993), Brassica (Park *et al.*, 1994) and soybean (Cheon *et al.*, 1993). But, except for the pea Rho1Ps gene (encoding a Rho-like protein; Yang *et al.*, 1993), all other plant small GTP-binding protein genes isolated are Ypt/Rab genes. Moreover, the precise physiological functions of the small GTP-binding proteins are not fully understood in plants.

In this study, to understand the functions of the small GTP-binding proteins in plants, we isolated and characterized genomic DNA or cDNA fragments encoding G1 to G3 domains of small GTP-binding protein Rab and Rho from several plant species using two different PCR-based cloning strategies.

MATERIALS AND METHODS

Experimental plants and cDNA library

Seven different plants, mung-bean, tobacco, rice, pepper, *Arabidopsis, Allium sativum* and *Gonyaulax* were used in this study. Genomic DNA from mung-bean, tobacco, rice, pepper and *Allium sativum* and cDNA libraries of mung-bean (auxin-treated hypocotyl, obtained from Dr. Woo-Taek Kim of Yonsei University), *Arabidopsis* (leaf, obtained from Dr. Seong-Ryong Kim of Seogang University), rice (leaf and sheath, obtained from Dr. Seong-Ryong Kim of Seogang University) and *Gonyaulax* (Lee *et al.*, 1993) were used.

Chemicals

The oligonucleotides for PCR were synthesized by Korea Biotech. Inc. A sequenase version 2.0 DNA sequencing kit was from United States Biochemical, [^{35}S]dATP was Amersham and [^{32}P]UTP for riboprobe was purchased from Dupont NEN. Nylon memebrane, Hybond-N, for blotting was from Amersham. General nucleic acid modifying enzymes were from Promega, KOSCO, and Amersham. All other chemicals were from Sigma, which were usually molecular biology grade or high purity reagent grade.

Preparation of genomic DNA and cDNA from cDNA libraries

For the isolation of genomic DNA, the method described by Rogers and Bendich (1988), utilizing cetyltrimethylammonium bromide (CTAB) to remove polysaccharides, was employed with minor modifications. Frozen cells were ground with liquid nitrogen in a precooled mortar, the powdered material was then transferred to a 50 ml screw-cap centrifuge tube. One volume of hot (65°C) 2x CTAB buffer (pH 8.0) (2%(w/v) CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, and 1% polyvinylpyrrolidone (*Mr* 40,000)) was then added, gently mixed with 1 volume of chloroform/isoamyl alcohol (24 : 1), and centrifuged at 11,000×g for 30 s and the top phase was transferred to a new tube. After adding 0.1 volume of 10% CTAB (10% CTAB and 0.7% NaCl), another extraction with chloroform/isoamyl alcohol was performed. To separate nucleic acids from polysaccharides, 1 volume of CTAB precipitation buffer (pH 8.0) (10 mM Tris, 1 mM EDTA and 1 M NaCl) and then reprecipitated with 2 volumes of 95% ethanol. The nucleic acid was recovered with a hooked glass rod, vaccum-dried, and resuspended in TE buffer. To assure the purity of the DNA, the preparation was treated with RNase A and extracted successively with phenol, phenol/ chloroform, and chloroform, followed by ethanol precipitation (Sambrook *et al.*, 1989). The pellet was resuspended in TE buffer and stored at - 20°C.

Preparation of cDNA from cDNA libraries was

performed according to Sambrook *et al.* (1989).

Isolation of *rab* gene fragments

Two degenerate oligonucleotides, OLE 1005; 5'-GTN GGN GA[C,T] GGN GCN GTN GGN AA[A, G] AC-3', and OLE 1006; 5'-TA[A,G] TC[C,T] TC [C,T] TGN CCN GCN GT[A,G] TC-3', encoding VGDGAVGKT and DTAGQEDY, respectively, were used as primers for PCR amplification. The reaction condition used for amplification of genomic DNA was 40 cycles with denaturation for 30 s at 94°C, annealing for 1 min at 52~58°C and extension for 1 min at 72°C. Each reaction, typically in a 20 μL volume, contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.9 mM $MgCl_2$, 200 μM each dNTP, 10~50 pmole of appropriate primers and variable amounts of template DNA. The reaction was initiated by addition of 1 unit of *Taq* DNA polymerase. From 50 ng to 200 ng of mung-bean, tobacco, rice and pepper genomic DNA were used as a template and two degenerate oligonucleotides, OLE 1005 and OLE 1006 were used as primers. The product of the PCR amplification was analysed on a 1.4% agarose gel.

Isolation of *rho* gene fragments

Two oligonucleotides, OLE 1007; 5'-GAT GGC GCC GTT GGA AAG ACT CGC [C,T]T[T,G] [C,T] T[T,G] AT-3' and OLE 1009; 5'-CTT ATC ATC TCT [A,C]A[G,A] ATC [A,C]A[G,A] CTT AGT TCC AAC-3', encoding DGAVGKTCLLI and VGTKLDLRDDK, respectively, in addition to OLE 1005 and OLE 1006, were utilized. These amino acid sequences are located within the nucleotide-binding and GTPase domains conserved in all known Rho proteins. To screen *rho* gene of several representative plant species, a nested PCR strategy was employed using four different primers, OLE 1005, OLE 1006, OLE 1007 and OLE 1009. Mung-bean, rice, *Arabidopsis* and *Gonyaulax* cDNA prepared from cDNA libraries were used as templates. Primary PCR using OLE 1005 and 1009 was performed under the following conditions; 30 s at 94°C for denaturation, 1 min at 36~52°C for annealing and 1 min at 72°C for extension. The resulting products were separated on a 1.4% agarose gel and 1 or 2 μL volume of gel pieces, corresponding to the size of DNA fragment by these pairs of primers, was excised out. The DNA fragment in the gel pieces was eluted into 50 μl of TE for 30 min at room temperature and aliquot of these eluted DNA solution were used as a template for secondary PCR amplification. The secondary PCR was performed using the condition for the primary PCR.

Cloning of PCR product and its characterization

DNA fragments of expected size were excised out from agarose gels and electroeluted (Chung and Na, 1995). The eluted DNA fragments were cloned into *SmaI*-linearlized pTZ19U or pTZ18U through blunt-end ligation. The ligated DNA was transformed into *E. coli* JM107 (Sambrook *et al.*, 1989). The plasmids were isolated by the alkaline method (Sambrook *et al.*, 1989) and its insert sizes were determined by restriction mapping. The sequences of the insert were determined using the dideoxy chain-termination method and were analyzed using the DNasis and PRosis programs.

Preparation of radioactive probe

To obtain a defined size of labelled probe, the plasmids for probe were linearized by cutting the end or close to end of insert (opposite end from T7 promoter) on pTZ plasmid by two restriction enzymes to make sure complete linearization. RNA polymerization reaction was performed using [α-^{32}P] UTP. One μg of template DNA and 50 μCi of [α-^{32}P]UTP was added to a mixture, which contained 0.2 mM of ATP, CTP and GTP, 5 μM UTP, 10 mM DTT, 1x buffer (40 mM Tris · HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl) and 8 units of RNasin. After adding 15~20 units of T7 RNA polymerase, the reaction mixture was incubated for 3~4 h at 37°C. Then 30 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylen cyanol FF) was added, and stored at 4°C.

Southern blot hybridization

Ten μg of genomic DNA were digested with the specified restriction enzymes, extracted with phenol/chloroform and precipitated by 2 volumes of ethanol and resuspended in TE buffer, run on a 0.8% agarose gel, and blotted onto Hybond nylon membrane by the capillary mode of transfer. The membrane was probed with a ^{32}P-labeled riboprobe (about 170 nt) generated by *in vitro* transcription from a mung-bean Rab3 gene or a mung-bean Rho1 gene. The membrane was prehybridized for 30 min at

42°C and hybridized with the riboprobes overnight at 42°C. And it was washed twice at room temperature for 30 min in a solution containing 2x SSC and 0.5% SDS and twice at 65°C for 15 min in a solution containing 0.5x SSC and 0.5% SDS. Filters were exposed for 3 days to X-ray film.

RESULTS AND DISCUSSION

Isolation and sequence analysis of *rab* gene fragments from several plant species

To isolate *rab* gene fragments, two oligonucleotides corresponding to the highly conserved GTP-binding domains, VGDGAVGKT and DTAGQEDY peptide sequences were synthesized and used as primers for PCR amplification. As these primers were degenerate, many different sets of conditions with varying amounts of template genomic DNA, or oligonucleotides and annealing temperatures were tested to obtain clear PCR products in about 170 bp in size. The best condition for the amplification was 100 ng of template DNA and 50 pmole of each oligonucleotide, OLE 1005 and OLE 1006 and annealing for 1 min at 58°C in a 20 μL reaction. The fragments obtained by this PCR reaction were then cloned on pTZ19U and sequences were analyzed.

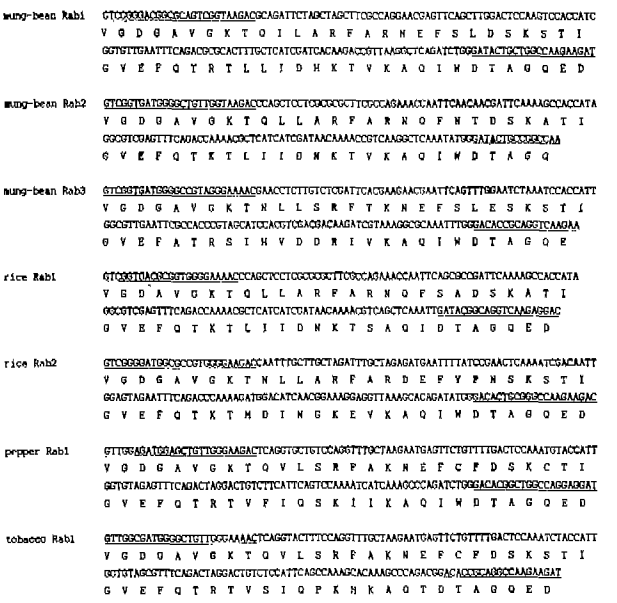

Fig. 1. Sequence analysis of *rab* gene fragments from plants. Nucleotide sequences and the deduced amino acid sequences of the cloned genes from mung-bean, rice, pepper and tobacco are shown. Primer sequences are underlined.

Among 70 candidates, 7 candidates from 4 different species were used for further characterizations.

We obtained seven partial *rab* DNA fragments corresponding to the GTP-binding domains from four different plants, which are three clones from mung-bean, two clones from rice and one clone from pepper and tobacco, respectively. Their nucleotide sequences and the deduced amino acid sequences are shown in Fig. 1. The comparison of amino acid sequence of these candidates with those of all the other known small GTP-binding proteins revealed that they belong to the Ypt/Rab family and are not previously reported. More than two different rab genes in rice, tobacco and mung-bean strongly suggest that *rab* gene in plants might exist as a multigene family.

Sequence comparison of *rab* genes from plants, animals and yeast

An amino acid alignment of all seven cloned genes with other proteins in the Ypt/Rab family is shown in Fig. 2. This comparison clearly demonstrates that regions shown to participate in the binding and

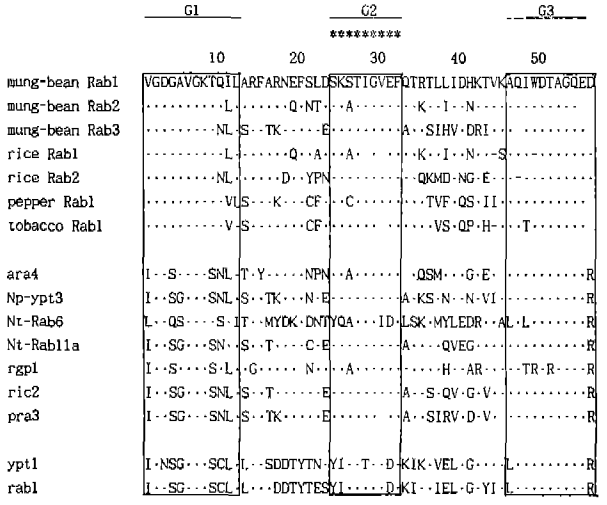

Fig. 2. Sequence comparison of *rab* genes from plants, animals and yeast. Comparison of the amino acid sequences of *rab* genes cloned this study with members of the Ypt/Rab family. Protein sequences are from *ara4* (Anai *et al.*, 1991), *Np-ypt3* (Dallmann *et al.*, 1992), *Nt-Rab6* and *Nt-Rab11a* (Heizel *et al.*, 1995), *rgp1* and *ric2* (Sano and Youssefian, 1991), and *pra3* (Nagano *et al.*, 1993) in addition to yeast *ypt1* (Gallwitz *et al.*, 1983), and human *rab 1* (Haubruck *et al.*, 1989). Amino acid residues identical with those of the uppermost mung-bean Rab1 sequence are indicated by dots. The putative effector-binding domains (G2) are indicated by asterisks.

hydrolysis of GTP and in the coordination of the Mg^{2+} ion for activity are well conserved among these cloned fragments. Characteristic features of these clones were observed in several regions. One region was around residue 30, all of the proteins have the sequence S-K-S/A-T-I-G-V-E-F around the 30 which is also observed in most of the Ypt/Rab family genes from animals and plants. This region (G2 domain) is highly conserved within each subfamily, but not between different subfamilies, and is called the effector region (Sigal *et al.*, 1986), which is thought to interact with other proteins such as the GAP-related protein (Adari *et al.*, 1988). This striking similarity suggested that these proteins interact with identical or very similar GAP proteins. The other characteristic structure of these clones is a Gln residue in place of Cys/Asn at aa 10, which is conserved in other members of Ypt/Rab family proteins. The third characteristic is a conservation of the residues 15, 24 and 25, which are observed in all cloned gene, but show variation in other Ypt/Rab proteins. Amino acid sequence comparison of the cloned gene with other proteins in the Ras superfamily shows that the clones are most highly related to the proteins in the Ypt/Rab family among the three major families of the Ras superfamily proteins. They exhibit 80 to 92 % identity with the plant Ypt/Rab proteins such as ara4, Np-ypt3, Nt-Rab6, Nt-Rab11a, rgp1, ric2 and pra3. The amino acid sequence identity of the clones to the other proteins in the Ypt/Rab family such as the yeast Ypt1 and the human Rab1 protein is 71 to 77%. However, the homology to proteins in the other families such as the human RhoA and the human Ras1 is only 59 to 66% and 55 to 59%, respectively.

Isolation and sequence analysis of *rho* gene fragments from several plant species

To isolate *rho* gene fragments, two more oligonucleotides, OLE 1007 and OLE 1009, encoding DGAVGKTCLLI and VGTKLDLRDDK, respectively, in addition to OLE 1005 and OLE 1006, were utilized. OLE 1007 and OLE 1009 are made by considering the codon usage in plants to reduce the degeneracy. Best conditions for the PCR amplification were determined by changing annealing temperature from 36°C to 52°C and by changing the amount of template and oligonucleotides. The optimized conditions were 40 cycles with denaturation for 30 s at 94°C, annealing for 1 min at 36°C and extension for 1 min at 72°C. Five, fifty or five hundreds ng of the cDNA extracted from libraries of mung-bean, rice, *Arabidopsis*, *Gonyaulax* was used as a template. In case of *Allium*, genomic DNA was used instead.

PCR amplification was performed by two steps. The primary PCR was carried out using OLE 1005 and OLE 1009 as primers and resulted in several DNA products. As DNA fragments of about 350 bp in size were expected with this pair of primers, these portion of gel were excised out and eluted. The isolated DNA was used as templates for the secondary PCR. Two internal oligonucleotides, OLE 1006 and OLE 1007, were used as primers in the second reaction and gave rise to the results as shown in Fig. 3 for *Arabidopsis* and rice. Fragments of about 160 bp in size obtained by secondary PCR were then cloned on pTZ18U and their sequences were analyzed.

Six partial *rho* DNA fragments corresponding to the GTP-binding domain, one clone from mung-bean, rice, *Arabidopsis* and *Gonyaulax*, two clones from *Allium*, were obtained. Nucleotide sequences and the deduced amino acid sequences of these cloned *rho* genes were shown in Fig. 4.

Sequence comparison of *rho* genes from plants, animals and yeast

An alignment of the sequences between plant *rho* cDNA fragments, human Rho protein, and yeast Rho proteins is shown in Fig. 5. The proteins en-

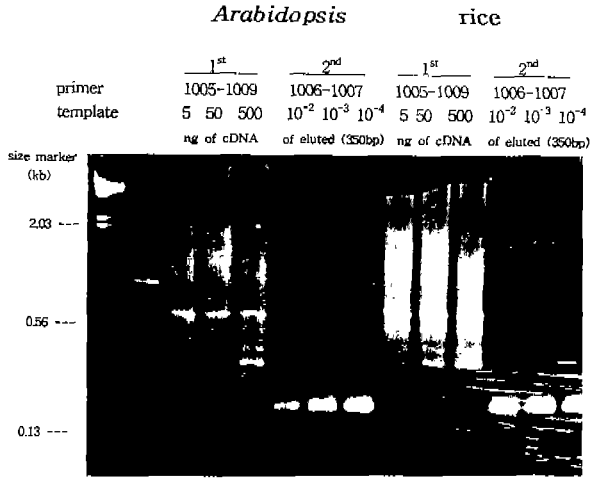

Fig. 3. PCR products amplified by nested PCR. PCR amplification was performed by two steps; OLE 1005 and OLE 1009 were used in the primary PCR, OLE 1006 and OLE 1007 were used in the secondary PCR. Forty cycles of 30 s at 94°C, 1 min at 36°C and 1 min at 72°C was used.

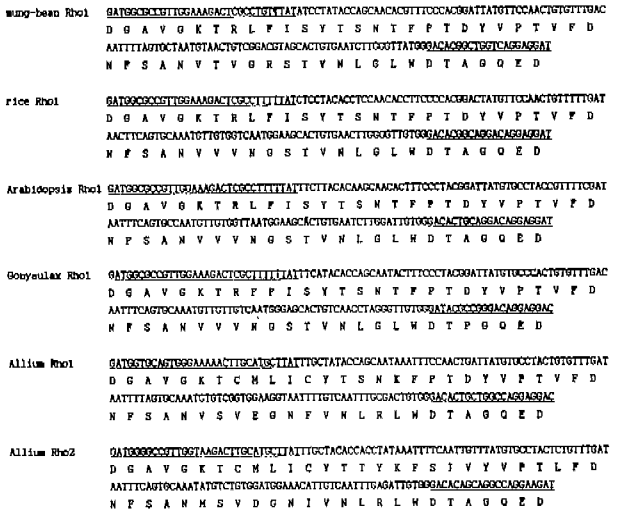

Fig. 4. Sequence analysis of *rho* gene fragments from plants. Nucleotide sequences and the deduced amino acid sequences of the cloned genes from mung-bean, rice, *Arabidopsis*, *Gonyaulax* and *Allium* were shown. Primer sequences are underlined.

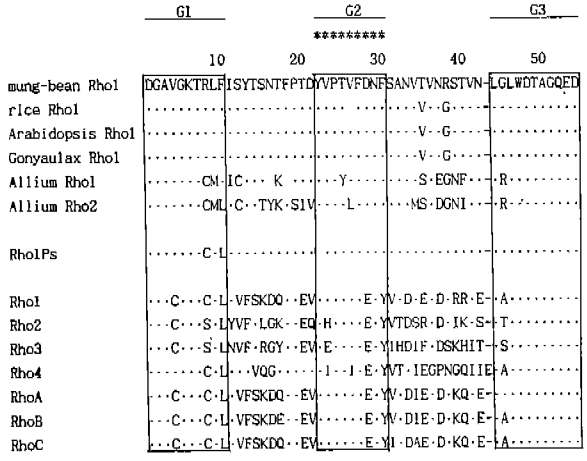

Fig. 5. Sequence comparison of *rho* genes from plants, animals and yeast. Comparison of the amino acid sequences of *rho* genes cloned in this study with members of the Rho family. Protein sequences are from RholPs (Yang and Watson, 1993), yeast Rho1 and Rho2 (Madaule *et al.*, 1987), Rho3 and Rho4 (Matsui *et al.*, 1989), human RhoA (Yeramian *et al.*, 1987), RhoB and RhoC (Chardin *et al.*, 1988). Amino acid residues identical with those of the uppermost mung-bean Rho1 sequence are indicated by dots. The putative effector-binding domains (G2) are indicated by asterisks.

coded by these *rho* genes display 86 to 98% similarity to the *Pisum sativum* RholPs, the plant *rho* gene isolated previously, and 67 to 82% similarity to the yeast Rho1, Rho2, Rho3 and Rho4 protein, respectively. The cloned cDNAs also exhibit amino

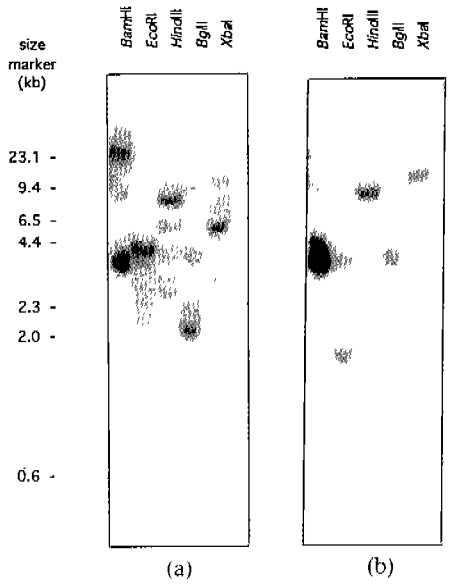

Fig. 6. Southern blot analysis of the mung-bean *rab* gene (a) and *rho* gene (b). Mung-bean genomic DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), *Bgl*II (lane 4) and *Xba*I (lane 5), fractionated in a 0.8% agarose gel, transferred to a nylon membrane. Hybridization was carried out using a riboprobe from mung-bean Rab3 clone or mung-bean Rho1 clone.

acid sequence similarity to other Rho-related proteins such as *S. cerevisiae* CDC42 (80 to 83%) and human Rho proteins (75 to 79%). The observed homology suggests that the cloned cDNAs from various plants belong to the Rho family. An amino acid alignment of the cloned cDNA fragment with its homologs from other species demonstrates that regions participating in GTP binding and hydrolysis are well conserved among these proteins. Indeed, the G1 (GDGASVGK), G2 (PYVEFDNF), and G3 (WDTAGQE) domains are almost identical. The homology included not only these sequence motifs, but also along most of 160 bp segments. But, in several amino acid residues, characteristic features specific to plants were observed. These are Asp at residue 28, Phe at residue 30, and Gly at residue 44, whose corresponding amino acids in yeast and animals are Glu, Tyr, and Ala, respectively. Generally, amino acid sequences of yeast and animal Rho proteins are less conserved than those of plant Rho proteins.

Genomic Southern analysis of rab and rho gene in mung-bean

To examine the genomic complexity of the *rab*

and *rho* gene, Southern blot analysis was performed. Ten μg of restricted genomic DNA as specified in Figure 6 hybridized with ^{32}P-labeled riboprobe of mung-bean Rab3 clone. Figure 6(a) shows that strongly hybridized fragments in the genomic DNA of mung-bean upon digestion by *Bam*HI, *Eco*RI, *Hin*dIII, *Bgl*II and *Xba*I existed, respectively. In addition to these strongly hybridizing fragments, three or four weakly hybridizing fragments were detected in each lanes. The results suggest that *rab* constitutes a multigene family in the mung-bean genome. These conclusions agree with the facts that there are three cloned *rab* genes as shown in Figure 2.

The same blot was hybridized with ^{32}P-labeled riboprobe of mung-bean Rho1 clone. Figure 6(b) shows that there exist one or two strongly hybridizing fragments and several weakly hybridizing fragments in each lane. Some of these strongly hybridizing bands are also detected by mung-bean Rab3 probe as shown in Figure 6(a), but the number and intensity of hybridizing fragments are different from each other. It is, therefore, suggested that there is a certain number of mung-bean Rho1-related genes in the genome and that *rho* constitutes a multigene family in mung-bean, like *rab* in mung-bean.

The high levels of sequence similarity between plant Rab and Rho proteins with their homologues in animals and yeast suggest that these group of proteins also perform highly conserved basic cellular functions in plants. Recently, many approaches to understand small GTP-binding proteins in plants have been attempted. In addition to expression analysis and biochemical study, functional complementation of yeast mutants and functional analysis using transgenic plants were used as a mean of studying the function of these proteins. For example, *Arabidopsis thaliana A.t.Rab6* gene whose protein product is homologous to the mammalian Rab6 and yeast *Ryh1/Ypt6* protein was able to complement the temperature-sensitive phenotype of the *YPT6* null mutant in yeast (Bednarek *et al.*, 1994) and *bra*, a cDNA clone isolated from *Brassica napus* can complement the cold-sensitive yeast mutation *ypt1-1* with defects at the early stage of the vesicle transport (Park *et al.*, 1994). Sequence homology and functional complementation in yeast strongly suggest that Rab proteins are likely to be involved in the vesicle transport in plants.

The function of the Rho proteins in plants is not known at all. However, existence of these *rho* genes in various plant species from lower to higher plant species and its striking similarity in amino acid sequence and conservation of functional domains suggest that these Rho proteins could be the functional counterpart of animal and yeast Rho proteins. In plants, the evidence that the actin cytoskeleton plays a key role in the spatial control of cell growth, division and cell morphogenesis has now been increased (Seagull *et al.*, 1987; Wick, 1991). So the possibility that Rho proteins regulate various cellular processes linked to the actin cytoskeleton is expected.

The DNA fragments isolated in this study are identified to be parts of a member of the Ypt/Rab or Rho family. It is intriguing that the Ras proteins, one of the small GTP-binding protein, have not been identified in plants so far. The reason of the failure to identify plant Ras proteins is thought to be that proteins similar to Ras in function are also present in plants, but they are not similar to those in animals and simple eukaryotes at the sequence level (Ma, 1994).

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