Biochemical characteristics of a rice (*Oryza sativa* L., IR36) G-protein α -subunit expressed in *Escherichia coli*

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A cDNA encoding the α -subunit of the heterotrimeric G-protein in rice (*RGA1*) was overexpressed in *Escherichia coli* and then isolated by Ni²⁺-nitrilotriacetic acid affinity chromatography. The molecular mass of RGA1 bearing a His tag was approx. 49 kDa. Immunoblot analysis using anti-RGA1 revealed that the RGA1 protein is most abundant in seedling leaves and least abundant in mature roots. It exists at particularly high levels in the immature embryo after pellicle extrusion. In addition, the RGA1 antiserum exhibited a difference in binding affinity for G α proteins from monocots (maize and rice) and dicots (*Arabidopsis*, pea, soya bean and tomato); whereas it cross-reacted with G α proteins of monocots, it did not with those of dicot plants. When bound to guanosine 5'-(γ -thio)triphosphate (GTP[S]), the RGA1 protein was partially protected from tryptic proteolysis. In the

presence of GTP[S], trypsin cleaved the RGA1 protein into four fragments 24, 14, 11 and 5 kDa in size. When RGA1 was bound to GDP, only the 5 kDa polypeptide was seen on SDS/PAGE after trypsin digestion. Photoaffinity labelling with [α -³²P]GTP and a GTP[S]-binding assay revealed that RGA1 incorporated ³²P and showed specific binding to a guanine nucleotide. Guanine binding of RGA1 was affected by the concentration of MgCl₂ (maximum at 2 mM). The rate of guanine nucleotide binding of RGA1 ($k_{on, GTP[S]} = 0.0141 \pm 0.0014 \text{ min}^{-1}$) and, at steady state, the k_{cat} value for GTP hydrolysis ($0.0075 \pm 0.0001 \text{ min}^{-1}$) were very low even at 2 mM MgCl₂. The binding affinity for the nucleotides examined was in the order GTP[S] $\geq$ GTP > GDP > CTP > ATP $\geq$ dTTP.

INTRODUCTION

G-proteins are members of a large family of guanine-nucleotide-binding proteins found widely in eukaryotes. On the basis of subunit composition and size, G-proteins have been broadly classified into heterotrimeric and small (monomeric) G-proteins.

Heterotrimeric G-proteins, consisting of α -, β - and γ -subunits, are known to play roles in signal transduction from cell surface receptors to intracellular effectors [1]. The α -subunits of heterotrimeric G-proteins differ in both guanine-nucleotide-binding affinity and intrinsic GTPase activity. Binding of GTP leads to the dissociation of the α -subunit from the $\beta\gamma$ complex of G-proteins and interaction with downstream effectors, thereby exerting regulatory functions [2–6].

To understand more clearly at a molecular level the physiological roles played by G-proteins in the signalling process, crystal structures have been determined for four related guanine-nucleotide-binding proteins, G α 1 [7,8], transducin [9,10], EF-Tu [11,12] and P²¹ras [13]; thus their nucleotide-binding domains are defined precisely. Many of the amino acid residues that make up the nucleotide-binding pocket in the above four related G-proteins are conserved in the non-contiguous regions of the G-protein α -subunits.

Recently there has been a steady accumulation of biochemical data indicating that G-proteins may be involved in signal transduction in plants. The earlier efforts included measurement of high-affinity binding of GTP, usually using one of the non-hydrolysable GTP analogues, such as guanosine 5'-(γ -thio)triphosphate (GTP[S]) [14]. High-affinity binding of the GTP analogue [γ -³⁵S]GTP[S] to membrane fractions from various species including pea [15], *Arabidopsis* [15,16], maize [15], *Lemna*

paucicostata [17] and zucchini [16] has been reported. By nucleotide competition assays, it has been shown that the guanine-nucleotide-binding proteins from zucchini and *Arabidopsis* membrane are specific for guanine nucleotide [16,18]. In pea and maize [19], the binding affinity of GTP[S] to plasma membranes was increased twofold by treatment with a mastoparan analogue known to activate heterotrimeric G-protein α -subunit in animals. Also, Lubrol PX enhanced GTP[S] binding to plasmalemma in tobacco [20].

Immunological detection to probe GTP-binding proteins has been widely used in plants. By using antisera raised against animal G α or synthetic peptides derived from animal G α sequence, putative G-proteins have been identified from *Arabidopsis* [15,16,21], pea [15,21,22], maize [15,21], zucchini [18], bean [16], *Commelina communis* [16] and soya bean [23]. Also, antisera against synthetic peptides from *Arabidopsis* GP α 1 have been extensively used to probe G α proteins in *Arabidopsis* [21,24,25], carrot [26] and *Sorghum bicolor* [21]. Anti-G α_{ara-1} , anti-G α_{ara-2} and anti-G α_{ara-1} /anti-G α_{ara-2} sera were raised against synthetic peptides corresponding to the conserved GTP-binding domain and the C-terminal region of *Arabidopsis* GP α 1.

It has been reported that proteins of similar size to common G-proteins are ADP-ribosylated by pertussis toxin and cholera toxin in the monocot *L. paucicostata* [17,22,23], pea [22] and soya bean [23]. We previously reported that RGA1 expressed in *E. coli* is ADP-ribosylated by cholera toxin [27].

Since the α -subunit of heterotrimeric G-proteins also has intrinsic GTPase activity, a GTPase assay has been used to detect this protein [22,28]. Genes encoding G α subunit homologues have been cloned and sequenced in *Arabidopsis* [29], tomato [30], rice [27], *Lotus japonicus* [31] and soya bean [32]. Also, genes

Abbreviations used: Ni²⁺-NTA, nickel-nitrilotriacetic acid; TNT-coupled rabbit reticulocyte lysate, transcription-and-translation-coupled rabbit reticulocyte lysate; TBS, 20 mM Tris/HCl (pH 7.3)/137 mM NaCl; GTP[S], guanosine 5'-(γ -thio)triphosphate; HEDL, 50 mM Hepes/NaOH (pH 8.0)/1 mM EDTA/3 mM dithiothreitol/polyoxyethylene 10-lauryl ether; PEI, polyethyleneimine; DTT, dithiothreitol; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK').

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encoding proteins showing sequence similarities to animal G_{β} subunits have been cloned in *Arabidopsis* [33], maize [33], tobacco [34] and rice [35].

Plant cells respond to a variety of signals from both the environment (light, humidity, temperature, gravity and pathogens) and nascent cells (hormones and nutrients). Evidence that GTP-binding proteins in plants actually participate in signal transduction is largely based on observations that GTP plays an important regulatory role in light-mediated processes. GTP[S] has been reported to mimic the effect of red light on the swelling of mesophyll protoplasts [36] and to block the ATP-induced quenching of chlorophyll fluorescence [37]. Conversely, red light has been shown to inhibit the binding of GTP[S] to *L. paucicostata* membrane extracts by 20–25 % [36]. Results from [γ - 35 S]GTP[S]-binding assays suggest that G-proteins may mediate auxin signal transduction in rice coleoptiles [38]. In etiolated pea, low fluorescences of blue light induced heterotrimeric G-protein activity in the plasma membrane [22]. Activation of stomatal opening by injected GTP[S] and pertussis toxin suggests that G-protein may be involved in guard-cell volume regulation [39]. As effectors downstream of G-protein activation, Ca^{2+} and calmodulin have been proposed to participate in the activation of gene expression and chloroplast development in tomato seedlings [40].

It has been recently reported that heterotrimeric G-protein may be involved in plant defence. Cholera toxin in transgenic plants selectively induces a subset of pathogen-related proteins and cholera toxin microinjected into cells induces the transcription of genes encoding these proteins in tobacco [41]. The elicitor-induced oxidative burst of soya-bean cultured cells was enhanced by internalization of the A subunit of cholera toxin and Fab $G\alpha_{common}$, recognizing a protein of about 45 kDa in soya-bean cell membranes [23].

Here we report the overexpression of a cDNA encoding the rice G-protein α -subunit (RGA1) in *Escherichia coli*. We chose this approach because the quantity of $G\alpha$ protein in plant tissues is very limited. We purified the recombinant protein and examined its functional activity. The biochemical properties, in particular the kinetics of nucleotide binding and GTPase activities, of the purified recombinant protein were characterized, and its nucleotide-dependent structural properties analysed by limited proteolysis.

EXPERIMENTAL

Plant materials and chemicals

Imbibition and growth conditions of rice seeds were described in a previous report [27]. Immature embryos were harvested 36 h after pellicle extrusion. Other plants such as *Arabidopsis*, maize, pea, soya-bean and tobacco were grown for 48 h after germination under the same conditions as the rice seedlings.

Translation-and-transcription (TNT)-coupled rabbit reticulocyte lysate was obtained from Promega. Ni^{2+} -nitrilotriacetate (Ni^{2+} -NTA)-agarose resin was purchased from Qiagen, and polyethyleneimine (PEI)-cellulose TLC reagents were from Aldrich. [α - 32 P]NTPs (ATP, CTP, GTP and UTP; 3000 Ci/mmol) and L-[35 S]methionine were obtained from Amersham. [γ - 35 S]GTP[S] (1000 Ci/mmol) was from Dupont. Most of the other reagents came from Sigma.

Isolation of total membrane proteins

The membranous proteins were prepared by a previously published method with minor modifications [42]. After grinding, filtering and centrifugal fractionation, the resulting pellet was

resuspended in 500 μ l of buffer containing 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 20 mM Hepes/NaOH, pH 8.0, sonicated (twice for 10 s each, on ice) with a probe tip sonicator, and centrifuged at 43000 g for 1 h. The supernatant and pellet were recovered and saved separately. The pellets were resuspended in 200 μ l of 10 mM Tris/Mes, pH 7.2, containing 1 mM DTT and 0.25 M sucrose to give an appropriate protein concentration.

Overexpression of RGA1 in *E. coli* and its purification

In order to overexpress RGA1 in *E. coli* BL21/DE3 (pLysS), we constructed an expression vector, pRGA1A. Mutagenesis was performed on the inserted cDNA of *RGA1* in Bluescript SK(+)II [27]. The oligonucleotide 5'-TTTGATACCATGGGCTC-3' was designed to contain ATG as a translation initiation codon and an *Nco*I site for restriction enzyme recognition. The DNA of a mutagenized clone, named pRGA1P, was digested with both *Nco*I and *Eco*RI. The resulting DNA fragment containing the cDNA insert was introduced into an expression vector, pRSETB, digested with the same restriction enzymes. The construct, called pRGA1A, was transformed into *E. coli* BL21/DE3 (pLysS).

E. coli BL21/DE3 (pLysS) transformed with the expression vector pRGA1A, which has a promoter site for T7 RNA polymerase, was inoculated into 50 ml of enriched medium containing 50 μ g/ml ampicillin. The culture was grown at 28 °C overnight and then poured into 10 litres of fresh enriched medium. When an A_{590} of 0.6 was reached, isopropyl β -D-galactopyranoside was added to the culture to a final concentration of 0.4 mM and the incubation continued for another 3 h at 28 °C. The expressed protein was isolated by a published method [43] with some modifications. Cells were centrifuged, resuspended and lysed by a freeze-thaw method. The lysate was then spun and the supernatant recovered and applied directly to a Ni^{2+} -NTA-agarose column. The column was washed with 3 column volumes of buffer A (50 mM Tris/HCl, pH 8.0, 20 mM 2-mercaptoethanol, 0.1 mM PMSF, 20 μ M GDP, 1 mM EDTA, 1 mM $MgCl_2$, 10 % glycerol) containing 500 mM NaCl and 10 mM imidazole, pH 8.0. The proteins were eluted with a 100 ml linear gradient of imidazole (10–250 mM) in buffer A containing 200 mM NaCl and 10 % glycerol; 40 fractions (2.5 ml each) were collected and subjected to SDS/PAGE (10 % gel). The fractions containing a single protein band were combined, dialysed against 3 litres of 50 mM Tris/HCl, pH 8.0, containing 20 mM 2-mercaptoethanol, 0.1 mM PMSF, 10 μ M GDP and 1 mM $MgCl_2$, and concentrated by an Amicon positive-pressure ultrafiltration system before use in the experiments.

Immunoblot analysis

Preimmune serum was collected from an adult New Zealand White rabbit before immunization. Polyclonal antibody was generated by the conventional method and purified as described by Mu et al. [44].

To express only the coding region of *RGA1* of the mutagenized clone, the *Nco*I- and *Bam*HI-digested fragment was ligated into the expression vector pET3d after removal of the corresponding *Nco*I-*Bam*HI fragment from the vector. The clone, named pERGA1, was prepared for *in vitro* transcription and translation using the TNT-coupled rabbit reticulocyte lysate system. pERGA1 (1 μ g) was transcribed and translated in a 50 μ l reaction mixture containing 25 μ l of TNT-coupled rabbit reticulocyte lysate, 20 μ M unlabelled amino acid mixture minus methionine, 30 μ Ci of L-[35 S]methionine and 40 units of ribonuclease inhibitor at 30 °C for 1 h. Membranous proteins from various plant

organs and/or plant species (40 μ g) and the *in vitro* translated protein were separated by SDS/PAGE (10% gel) and transferred to a nitrocellulose membrane. The transfer was performed in transfer buffer containing 193 mM glycine, 25 mM Tris base and 20% methanol for 2 h at 100 V at 4 °C. Afterwards the membrane was submersed in incubation buffer containing 5% non-fat dried milk and 0.1% Tween 20 in 20 mM Tris/HCl (pH 7.3)/137 mM NaCl (TBS) and left overnight at room temperature. The primary antibody, anti-RGA1, diluted 1:2000 in the incubation buffer, was then added to the membrane and the incubation continued for 1 h. The membrane was washed at room temperature (3 $\times$ 20 min) with TBS containing 0.1% Tween 20. Horseradish peroxidase-coupled mouse anti-rabbit serum was used as secondary antibody at a dilution of 1:5000 and incubated with the blot for 1 h. The membrane was then washed as described above. It was developed employing the enhanced chemiluminescence detection method according to the manufacturer's (Amersham) instructions.

Trypsin protection assay

In vitro transcription and translation of the construct pRGA1A were performed under the aforementioned conditions. After the reaction, 100 μ l of sample was loaded on to a 2 ml Sephadex G-50 column equilibrated with 50 mM Hepes/NaOH, pH 7.0, containing 6 mM MgCl₂, 75 mM sucrose, 1 mM EDTA, 1 μ M GDP, and 0.9% cholate. The coloured fractions of the peak region (about 200 μ l) were collected. The translation mixtures (10 μ l) were diluted to 15 μ l with a solution consisting of 50 mM Tris/HCl, pH 7.6, 6 mM MgCl₂, 1 mM EDTA and 1 mM DTT. The reaction mixture was preincubated with or without 25 μ M GTP[S] for 10 min at 30 °C. Then 1 μ l containing 20 pmol of Tos-Phe-CH₂Cl ('TPCK')-treated trypsin was added. The digestion was carried out for 60 min at 30 °C and terminated by the addition of Laemmli sample buffer containing 10 mM benzamidine and 8.8 μ M soya-bean trypsin inhibitor and immediate boiling for 3 min. The products were separated by SDS/PAGE (15% gel), dried and exposed to an X-ray film for 15 h at -70 °C.

Photoaffinity labelling

Column-purified RGA1 (250 ng) was incubated with 0.1 μ M [α -³²P]GTP in 25 μ l of 50 mM Hepes/NaOH, pH 8.0, containing 1 mM EDTA, 100 mM NaCl, 2 mM 2-mercaptoethanol and 0.1% glycerol for 15 min at 4 °C, and then irradiated on ice at a distance of 3 cm for 10 min with mineral light of 254 nm wavelength. After photolysis, reactions were stopped by the addition of 25 μ l of Laemmli sample buffer. The samples were boiled for 3 min, electrophoresed in an SDS/10% polyacrylamide gel. The gel was treated with 50 mM sodium phosphate buffer, pH 5.0, for 30 min at room temperature with low-speed shaking, stained with Coomassie Brilliant Blue R-250, destained, dried and exposed to an X-ray film at -70 °C. After the film had been developed and the protein bands identified, they were excised from the dried gel and their radioactivity quantified in a toluene-based scintillation cocktail.

GTP[S] binding and dissociation assay

The degree of [γ -³⁵S]GTP[S] binding was analysed using the published rapid filtration technique [14] with some modifications. Samples were incubated for various periods of time at 30 °C in 200 μ l of 50 mM Hepes/NaOH, pH 8.0, containing 1 mM EDTA, 3 mM dithiothreitol and 0.05% C₁₂E₁₀ (HEDL buffer)

Table 1 Competition by various nucleotides for GTP[S] binding to RGA1

Proteins (500 ng) were incubated at 30 °C in a final volume of 100 μ l with 2 mM MgCl₂, 0.2 μ M [γ -³⁵S]GTP[S] and 250 μ M each nucleotide as competitor. Incubations were terminated after 60 min, and the radioactivity of the bound [γ -³⁵S]GTP[S] was counted. The total concentration of [γ -³⁵S]GTP[S] bound to RGA1 without any competitors was 0.95 pmol, which was taken as 100%

Competing nucleotide	[γ - ³⁵ S]GTP[S] (%)
None	100
ATP	98
CTP	91
GDP	32
GTP	5
GTP[S]	3
dTTP	99

plus 0.2 μ M [γ -³⁵S]GTP[S]. In the competition assay shown in Table 1, the MgCl₂ concentration in all samples was adjusted to 2 mM. The reaction was stopped by the addition of 2 ml of ice-cold buffer B (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂). The samples were then applied to a 0.45 μ m nitrocellulose membrane filter which had been rinsed with 3 ml of buffer B, and the filter-bound samples were washed rapidly four times with 3 ml of buffer B. The filters were dried and counted for radioactivity as above. For a standard sample, [γ -³⁵S]GTP[S] by itself was dotted on to a filter, dried and counted for radioactivity in the same way. To examine the influence of MgCl₂ on [γ -³⁵S]GTP[S] binding to RGA1, as shown in Figure 5(A), each reaction mixture was adjusted to the prescheduled MgCl₂ concentration.

The rate of [γ -³⁵S]GTP[S] dissociation was measured by displacement of [γ -³⁵S]GTP[S] from RGA1 in the presence of a large excess unlabelled GTP[S]. The [γ -³⁵S]GTP[S]-bound RGA1 was first made by incubating RGA1 with 0.2 μ M [γ -³⁵S]GTP[S] for 60 min at 30 °C in 200 μ l of HEDL buffer containing 2 mM MgCl₂. The dissociation reaction was initiated by the addition of unlabelled GTP[S] to a final concentration of 250 μ M. After incubation for various periods of time, the dissociation reaction was terminated using the same method as for the [γ -³⁵S]GTP[S]-binding assay. The residual radioactivity on the nitrocellulose filters was recorded.

GTPase assay

The GTP-hydrolytic assay was carried out by a slightly modified version of the method of Wagner et al. [45]. Briefly, the reaction proceeded in 200 μ l of HEDL buffer containing 2 mM MgCl₂, 1 mM NaN₃, one NTP 4 μ M, 33 nM [α -³²P]GTP (3000 Ci/mmol), 1 mM ATP and 500 ng of RGA1 protein at 30 °C. Aliquots of volume 10 μ l were sampled at appropriate time intervals and added to 10 μ l of 0.5 M EDTA, pH 8.0, to stop the reaction. Then 2 μ l was spotted on to a PEI-cellulose TLC plate which was developed in 0.5 M KH₂PO₄, pH 3.4. After drying, the plate was exposed to an X-ray film.

GTPase activity was also measured using charcoal as previously described in detail [46]. Briefly, 500 ng of RGA1 was incubated at 30 °C in HEDL buffer containing 0.2 μ M [γ -³²P]GTP and appropriate concentrations of MgCl₂. For the measurement of the time-dependent release of ³²P, the reaction was initiated by the addition of the protein prewarmed to the reaction temperature. Aliquots of volume 25 μ l were removed at the scheduled time intervals, added to 775 μ l of 5% (w/v) charcoal in 50 mM

NaH_2PO_4 on ice, and vortex-mixed. The charcoal was removed by centrifugation at 2000 *g* for 15 min, and the total amount of radioactivity in a 400 μl aliquot of the supernatant was measured in a liquid-scintillation counter.

Data reproducibility

Data shown in Figures 5 and 7 are the means of three independent experiments. Those shown in Table 1 are the mean values of duplicate determinations for a single experiment representative of three. All values and drawings were calculated and plotted using the computer program Softwindow Grafit, except for Figure 4 which was drawn using CA-Cricket Graph III program, and represents the mean $\pm$ S.E.M. for each experiment.

RESULTS

Purification of RGA1 protein expressed in *E. coli*

The plasmid constructed for expression of RGA1, pRGA1A, and transformed into *E. coli* BL21/(DE3)/pLysS yielded RGA1 protein up to a concentration of 0.5–1 mg/l of culture, based on an immunoblot analysis. We reported previously that a rice cDNA of the G-protein α -subunit was expected to encode a polypeptide of approximate molecular mass 44.5 kDa [27]. The His-tag-fused RGA1 protein is estimated to be 49 kDa in size. Most of the RGA1 expressed in *E. coli* was found in inclusion bodies. The quantities of RGA1 obtained amounted to about 0.15% of total cellular proteins, the soluble form comprising up to 2% of the yield. The His-tag-bearing RGA1 could be highly purified by using a Ni^{2+} -NTA column, as seen in Figure 1.

Detection of the G-protein α -subunit in total membrane protein of plant tissue

To estimate the relative levels of expression of RGA1 at different developmental stages, we performed immunoblot analyses on extracts of total membranous protein from various rice plant organs at different stages of development. Antibody raised against RGA1 protein was allowed to cross-react with each of the various membrane proteins and yielded a band around the

Figure 1 Purification of RGA1

The extract of *E. coli* BL21 (DE3)/pLysS harbouring the RGA1 gene was loaded on a Ni^{2+} -NTA column, and purified as described in the Experimental section. The fractions collected were assayed on an SDS/10% polyacrylamide gel stained with Coomassie Brilliant Blue. Lane 1, protein molecular mass markers; lane 2, total protein; lane 3, before loading; lane 4, flow through; lane 5, purified RGA1 (500 ng); lane 6, purified RGA1 (2 μg). The arrow on the right indicates the position of RGA1 and the numbers on the left indicate the sizes of the markers in kDa.

Figure 2 Immunoblot analysis of membrane proteins resolved by SDS/PAGE

Membrane fractions (40 μg of protein) from various species (A) and rice organs (B) were separated by SDS/PAGE and the resolved proteins transferred to nitrocellulose. Immunoblot analysis was performed as described in the Experimental section using the affinity-purified anti-RGA1 at a 1:2000 dilution as the probe. As a control, preimmune serum was used. Immunoblots performed with preimmune serum are shown below the corresponding protein blots. The ERGA1 lane contains the *in vitro* translated protein from the pERGA1 clone used as standard. Size markers are indicated on the left.

44.5 kDa position (Figure 2) that corresponds to the expected size of a product of the *RGA1* gene [27], as previously reported in *Arabidopsis* [25]. RGA1 is present at a lower level in leaves and roots of 4-week-old plants than in the same organs of 2-day-old seedlings. After pellicle extrusion, immature embryonic RGA1 was also revealed at a higher level than in leaves and roots of 4-week-old plants.

When we analysed the expression of the homologous protein in different plant species, we found that the level of proteins cross-reacting with the RGA1 antibody was high in monocots such as maize and rice, but low in dicots, for example *Arabidopsis*, pea, soya bean and tomato, as seen in Figure 2(A). Since equal amounts of membranous protein had been loaded, these data indicate that the G-protein α -subunit of monocots recognizes anti-RGA1 with higher affinity than does the homologous protein of dicots.

In summary, using antiserum against RGA1 we have shown by immunoblot analysis of rice organs detectable levels of a 44.5 kDa immunoreactive membranous protein in both seedlings and mature plants. Protein levels were highest in seedling leaves and lowest in mature roots. The gene is also highly expressed in immature embryos. Considering the level of detection, we see a significant difference in the affinity of the RGA1 antiserum for the respective proteins in monocot and dicot plants.

Analysis of trypsin proteolysis protection

Most G α proteins are substantially protected from tryptic proteolysis when activated with a non-hydrolysable analogue of GTP, such as GTP[S]. The tryptic-cleavage pattern of G α depends on whether or not a GDP or GTP is bound at the active site. The *in vitro* translated RGA1 was subjected to digestion by Tos-Phe-CH₂Cl-treated trypsin at a protease to substrate ratio of 1:10 (w/w). The reaction was terminated by adding excess amounts of soya-bean trypsin inhibitor and the results were

Figure 3 Protection of RGA1 from tryptic proteolysis

The *in vitro* translated proteins were preincubated for 10 min with or without 25 μ M GTP[S] followed by digestion with 20 pmol of trypsin for 60 min at 30 °C. Proteolysis was terminated by the addition of 10 mM benzamidine/8.8 μ M soya-bean trypsin inhibitor. The samples were analysed by SDS/PAGE (15% gel) followed by autoradiography. + and - indicate reactions with or without the corresponding additives. Standard protein size markers expressed in kDa are indicated on the left.

analysed (Figure 3). When the *in vitro* translated RGA1 had been allowed to react with GTP[S] as ligand, trypsin produced four protein fragments of apparent molecular masses of 24, 14, 11 and 5 kDa. In the presence of GDP as ligand (results not shown), however, only one fragment of 5 kDa was produced, as in the absence of ligand. However, since the protein band at the 26 kDa position in the control did not react with the anti-RGA1 serum, this may be an artifact produced by the translation mixture.

Analysis of GTP binding by photoaffinity labelling

A photolysable analogue, 8-N₃-GTP, has been utilized in the past to determine which of the G-protein subunits contain the

nucleotide-binding site [47]. Instead of 8-N₃-GTP, we used an α -³²P-labelled GTP in photoaffinity labelling. Figure 4 shows the degree of photoincorporation of [α -³²P]GTP into RGA1. It shows the protein-staining profile of RGA1 in (a), and in (b) the autoradiogram after photolabelling with [α -³²P]GTP. Changes in MgCl₂ concentration changed the degree of photoincorporation (Figure 4A), with maximal photoincorporation occurring at 2 mM MgCl₂. In addition, we also examined the binding specificity of RGA1 to guanine nucleotide by using GTP, GTP[S], ATP, CTP and GDP as competitors. At a concentration of 100 μ M, GTP[S], a GTP analogue, but not ATP, effectively blocked photoincorporation. Figure 4(B) shows the relative percentage of ³²P incorporation measured with a liquid-scintillation counter. The values for the various competitors are 16.5% for GTP[S], 48.8% for ATP, 33.8% for CTP, 24.5% for GDP and 20.5% for GTP. When the concentrations of GTP[S] as competitor of [α -³²P]GTP was increased to 200 μ M in the presence of 2 mM MgCl₂, the photoincorporated amounts of ³²P gradually decreased (Figure 4C).

Time course of GTP[S] binding and RGA1 protein binding specificity

More evidence that RGA1 is a GTP-binding protein can be derived from the analysis of [γ -³⁵S]GTP[S]-binding activity. As shown in Figure 5(A), the rate of binding of [γ -³⁵S]GTP[S] to RGA1 is low. It does not reach the maximal value even after 60 min of incubation. The data, however, can be fitted to a single exponential curve for estimating maximal binding values. When the GTP-binding activity of RGA1 was measured after 60 min at 30 °C in the presence of various concentrations of MgCl₂, it was found to be highest at 2 mM (Figure 5A). At 2 mM, $k_{on, GTP[S]}$, the binding rate constant of [γ -³⁵S]GTP[S] to RGA1, is first-order (0.0141 ± 0.0014 min⁻¹). Binding rates of GTP[S] to RGA1 also reached maximum at 2 mM MgCl₂. When the concentration of MgCl₂ was increased to 100 mM, the binding rate constant decreased to 0.0008 ± 0.0001 min⁻¹. RGA1 also bound [γ -³⁵S]-GTP[S] in a dose-dependent manner as shown in Figure 5(B). Scatchard-plot analysis reveals that this protein binds maximally at 1.06 mol of nucleotide/mol of protein with a K_s value of 240 nM (inset of Figure 5B). The straight line indicates

Figure 4 Photoaffinity labelling of RGA1 with [α -³²P]GTP

RGA1 (250 ng) was incubated with 0.1 μ M [α -³²P]GTP in 25 μ l of 50 mM Hepes/NaOH, pH 8.0 containing 1 mM EDTA, 100 mM NaCl, 2 mM 2-mercaptoethanol and 0.1% glycerol plus the indicated additions. The samples were photolysed and the protein fragments detected as described in the Experimental section. Photoaffinity labelling was performed in the presence of various MgCl₂ concentrations (A) and in the presence of nucleotide (B) and GTP[S] (C) competition. (a) and (b) show the protein gel profiles and their autoradiograms respectively. Abbreviations: C, control; GT, GTP; AT, ATP; CT, CTP; GD, GDP; GS, GTP[S]. The relative percentages of ³²P incorporation are indicated by the height of the columns.

Figure 5 $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ -binding activity of RGA1

(A) Time course of $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ binding to RGA1 under specific MgCl_2 concentrations. RGA1 (500 ng) was incubated at 30 °C with 0.2 μM $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ and 0.5 mM (●), 2 mM (○), 10 mM (□) or 100 mM (■) MgCl_2 ; △, Control (0.1% BSA). The reaction conditions are described in the Experimental section. Aliquots (20 μl) were withdrawn at the indicated time points, filtered and counted for radioactivity. (B) Dose-dependent binding of $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ to RGA1. RGA1 (500 ng) was incubated in the presence of 2 mM MgCl_2 with various doses of $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ for 60 min as described in the Experimental section. The inset shows the Scatchard plots. (C) Dissociation kinetics of $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ from RGA1. Protein (500 ng) was preincubated with 0.2 μM $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ for 60 min, which was then chased with 250 μM unlabelled GTP[S] for 120 min at 30 °C. Aliquots were withdrawn at the indicated times. The reaction was terminated and the residual radioactivity of $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ bound to the protein was determined as described in the Experimental section.

Figure 6 GTPase activity of RGA1

(A) Protein (500 ng) was incubated in the presence of 2 mM MgCl_2 at 30 °C with 33 nM $[\alpha\text{-}^{32}\text{P}]\text{GTP}$. Aliquots from the reaction mixture were withdrawn, separated into GTP and GDP on a PEI-cellulose TLC plate, and visualized by autoradiography. (B) The reactions were performed as described in (A) except that $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ were used as substrates. The positions of NTPs (ATP, CTP, GTP and UTP), NDPs (ADP, CDP, GDP and UDP) and the origins are indicated by arrows. The numbers under the autoradiogram indicate the incubation times (h).

a single binding site for $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ in RGA1. The kinetics of dissociation of GTP[S] from RGA1 were calculated as described in the Experimental section. Once bound, GTP[S] dissociation from RGA1 became very slow. More than 50% of the labelled nucleotide remained bound 272 min after the addition of a 1250-fold excess of unlabelled GTP[S] at 2 mM MgCl_2 (Figure 5C). The half-time and rate constant of $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ dissociation from RGA1 were calculated to be approx. 272 min and

Figure 7 GTPase activity of RGA1 at steady-state

(A) GTPase activity in the presence of various MgCl_2 concentrations. Protein (500 ng) was incubated at 30 °C with 0.2 μM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and 0.5 mM (□), 2 mM (●), 10 mM (○), 50 mM (■) or 200 mM (△) MgCl_2 . Aliquots (25 μl) were withdrawn at the indicated times, and the release of ^{32}P was determined as described in the Experimental section. (B) Effects of nucleotide competitors on the GTPase activity in the presence of 2 mM MgCl_2 . Reactions were performed as in (A) except that MgCl_2 was fixed at 2 mM and nucleotide competitors were added to a final concentration of 50 μM. ●, ATP; □, CTP; ■, GDP; △, GTP[S]; ○, control (no competitor).

$0.002547 \pm 0.00027 \text{ min}^{-1}$ respectively in the presence of 2 mM MgCl_2 (Figure 5C).

The competition of various purine and pyrimidine nucleotides, such as ATP, CTP, dTTP, GDP, GTP and GTP[S], with $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$ for binding to RGA1 was examined. Of the nucleotides tested at concentrations as high as 1000-fold over $[\gamma\text{-}^{35}\text{S}]\text{GTP[S]}$, only the guanine nucleotides were effective (Table 1).

GTPase activity of the RGA1 protein

Since most heterotrimeric G-protein α -subunits are known to have intrinsic GTPase activity, we examined the hydrolysing activity of RGA1 in changing NTPs to NDPs including GTP to GDP. Analysis of the GTPase activity of this protein on a PEI-cellulose/TLC plate revealed that the free forms of GDP gradually increased as the incubation time progressed, whereas addition of BSA in the place of RGA1 to the reaction mixture or no protein at all, did not produce this phenomenon (Figure 6A). Also, the hydrolytic activities of RGA1 for ATP, CTP and UTP

Table 2 Rates (min^{-1}) of GTP hydrolysis according to MgCl_2 concentration and coexistence of competing nucleotide

The GTP-hydrolytic rates were determined by using the computer program Softwindow Grafitt from Figure 7 and are expressed as mean $\pm$ S.E.M.

$[\text{MgCl}_2](\text{mM})$	Steady-state rate (k_{cat}) (charcoal assay)
0.5	0.0061 ± 0.0003
2	0.0075 ± 0.0001
10	0.0070 ± 0.0002
50	0.0042 ± 0.0001
200	0.0016 ± 0.0001

Nucleotide (50 μM)	Steady-state rate (k_{cat}) (charcoal assay)
None	0.0075 ± 0.0001
ATP	0.0063 ± 0.0003
CTP	0.0055 ± 0.0003
GDP	0.0020 ± 0.0002
GTP[S]	0.0013 ± 0.0001

were examined. As shown in Figure 6(B), no hydrolysed products (ADP, CDP and UDP) were detected.

We also employed another method using charcoal to measure the hydrolysing capability of the G α protein. The protein was incubated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ in the presence of MgCl_2 , and GTP hydrolysis was determined by measuring production of free phosphate as described in the Experimental section. We have already reported above that photolabelling of RGA1 with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ depends on MgCl_2 concentration (Figure 4A). In the photolabelling experiment, we also found that the hydrolytic activity of RGA1 is dependent on the reaction time. Figure 7(A) shows the changes in GTPase activity of RGA1 in the presence of various MgCl_2 concentrations at 30 °C. Similarly to the above photolabelling experiment on GTP binding, near-maximal hydrolysis occurred at a 2 mM MgCl_2 . The steady-state rate constants (k_{cat}) for GTP hydrolysis of RGA1 in the presence of 0.5, 2, 10, 50, and 200 mM MgCl_2 were 0.0061 ± 0.0003 , 0.0075 ± 0.0001 , 0.0070 ± 0.0002 , 0.0042 ± 0.0001 and $0.0016 \pm 0.0001 \text{ min}^{-1}$ respectively (Table 2).

In addition, assays of the GTPase activity of RGA1 in the presence of various nucleotide competitors and 2 mM MgCl_2 were carried out. As in the photoaffinity labelling experiment, at a concentration of 50 μM , the GTP analogue GTP[S], but not ATP, effectively competed with GTP in the hydrolytic activity of RGA1. Of the competitors examined, guanine nucleotides were the most specific (Figure 7B, Table 2.) On the basis of these results we conclude that the rice G-protein α -subunit expressed in *E. coli* (RGA1) specifically binds GTP, and we calculated that, at steady-state, the specific activity for GTP hydrolysis has a k_{cat} value of $0.0075 \pm 0.0001 \text{ mol of P}_i/\text{mol of protein per min}$ in the presence of 2 mM MgCl_2 .

DISCUSSION

We expressed the cDNA encoding the rice G-protein α -subunit in *E. coli* using pRSETB, a plasmid expression vector that contained the T7 RNA polymerase promoter and was designed to produce the native α -subunit. After two affinity-purification steps in the presence of 0.1 % Triton-X 100, we obtained about 100 μg of soluble RGA1 protein from a 1-litre culture. The

purified protein was used in antibody production and in its biochemical characterization.

Results from tissue-specific immunoblot analysis indicates that RGA1 is present in all the rice tissues examined so far and that it appears immediately after germination. Furthermore the level of RGA1 is highest in young developing organs. As the organs age, the level of RGA1 decreases, suggesting a role for it in development. It has also been shown that GP α 1 is differentially expressed during development in *Arabidopsis* [25]. Using the anti-G α ara-2 IgG, heterotrimeric G-protein α -subunit analogues were found associated with membrane-free cytoskeletal and nuclear fractions isolated from carrot cells [26].

A cell-free transcription and translation system can provide some advantages over various cellular systems. Because a specific mRNA could be added to the *in vitro* translation mixture, the desired protein appeared as only one radioactive band. During α -subunit activation, the replacement of GDP by GTP is associated with a conformational change. This becomes apparent in the difference in sensitivity to trypsin degradation [48–50]. When a non-hydrolysable guanine nucleoside triphosphate analogue, such as GTP[S], is bound to G α or other members of the G α family, a 2 kDa N-terminal peptide is cleaved off, leaving a stable 37–39 kDa fragment, depending on the particular α -subunit in question. Similarly, tryptic cleavage of G α connected to the ligand GTP[S] yields a stable fragment that is 1 kDa smaller than the native molecule [51]. When the G α subunit is bound to GDP as the ligand, the 37–39 kDa fragment is reduced further to relatively stable 25–26 kDa and 17–18 kDa polypeptides [49]. For the *in vitro* translated G α subunit, similar tryptic fragments ranging from 35 to 37 kDa were generated in the absence or presence of GTP[S]; however, at high concentrations of trypsin, the GDP form was fully degraded, whereas the complete digestion of the GTP[S]-liganded form required a ratio of trypsin to substrate of over 1000:1. When the *in vitro* translated RGA1 was digested by trypsin for 60 min with an approximate 10:1 ratio of trypsin to substrate, 24, 14, 11 and 5 kDa fragments were generated in the presence of GTP[S].

In several instances it has been demonstrated that the binding activity of a GTP-binding protein can be examined by incubating protein-containing membranes with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ [52]. However, binding of GTP to the 40 kDa G α protein was not observed with this technique [53]. Also, a number of methods have been used to probe G-protein structure and function, and each method has provided some information about the functional domains involved in GTP/GDP binding, GTPase activity, effector activation, receptor association and $\beta\gamma$ subunit interaction. Even though the G-proteins have been crystallized [7–13], much of the structural information could be indirectly derived from mutational studies [51,54], epitope mapping [55–57] and photocross-linking [58,59]. Photoactivatable GTP analogues have been used to identify and map the guanine-nucleotide-binding site of G-protein α -subunits [60,61]. Subsequently, 8-N $_3$ -GTP has been used to identify and characterize various G-proteins and photo-reactive nucleotides. After a brief exposure to UV light of a particular wavelength, 8-N $_3$ -GTP covalently cross-links to the protein. Hence, by utilizing 8-N $_3$ -GTP labelled with ^{32}P at the α , β or γ position, it should be possible to incorporate radioactivity into the G α protein. Photoaffinity labelling of RGA1 using $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ instead of 8-N $_3$ -GTP proceeded well at 4 °C. By examining the photoincorporation of ^{32}P into RGA1 at various concentrations of MgCl_2 , we confirmed that the GTP-binding property of RGA1 is dependent on MgCl_2 concentration. In animal systems photoincorporation of 8-N $_3$ -GTP does not require MgCl_2 [14], and bivalent cations bind non-specifically to G α proteins. Of the bivalent cations tested in the previous report

[14], Mg^{2+} , Mn^{2+} , Ba^{2+} and Sr^{2+} were effective in supporting binding and activation, but all of these cations were required in greater concentrations. Fe^{2+} , Cu^{2+} , Co^{2+} , Cd^{2+} and Ni^{2+} caused irreversible loss of activity. Univalent cations, such as Na^+ , K^+ , NH_4^+ and tris(hydroxymethyl)methylamine, have no effect.

However, our results show that photoincorporation of ^{32}P strongly depends on $MgCl_2$ concentration, with a maximum at 2 mM $MgCl_2$. Furthermore photoincorporation into RGA1 using various nucleotides as competitors revealed that RGA1 binds the guanine nucleotide specifically at 100 μM concentrations of the competitors (Figure 4B). A similar competition pattern has been reported by Casey et al. [53]. The photoaffinity labelling experiment in Figure 4(B) and $[\gamma\text{-}^{35}S]GTP[S]$ -binding assay in Table 1 showed that the nucleotide-binding specificity of RGA1 protein is decreased under the conditions of the photoaffinity labelling reaction in comparison with those of the $[\gamma\text{-}^{35}S]GTP[S]$ -binding assay. Such differences in relative nucleotide competition (%) for RGA1 protein may be caused by reaction conditions such as temperature and buffer compositions. In addition, the photoincorporation into RGA1 at a saturating concentration of $[\gamma\text{-}^{35}S]GTP[S]$ as binding competitor for $[\alpha\text{-}^{32}P]GTP$ resulted in a very faint band (Figure 4C).

The rate of binding of $GTP[S]$ to RGA1 reached a maximum at 2 mM $MgCl_2$ (Figure 5A). The rate of dissociation of $GTP[S]$ from RGA1 is very low at $0.002547 \pm 0.00027 \text{ min}^{-1}$ (Figure 5C), about 60–140-fold lower than those observed for $G_s\alpha$ [62] and $G_o\alpha$ [63] in animals.

The GTP-hydrolytic activity of RGA1 was completely dependent on $MgCl_2$ concentration. Varying the $MgCl_2$ concentration over the range 0.5–200 mM revealed that the rate of GTPase activity was highest at 2 mM $MgCl_2$. GTPase activity of RGA1 was also specific for guanine nucleotides as revealed by both photoaffinity labelling and the $GTP[S]$ -binding assays. Figures 6 and 7 confirm that RGA1 has GTPase activity and that it has a low k_{cat} value compared with the GTP-hydrolytic rates of many heterotrimeric G-protein α -subunits in animals [62]. We estimated the apparent first-order rate constant of GTP hydrolysis (k_{cat}) to be $0.0075 \pm 0.0001 \text{ min}^{-1}$ in the presence of 2 mM $MgCl_2$ (Table 2).

In summary, our studies identify the GTP-binding and -hydrolytic activity of RGA1, a rice $G\alpha$ protein expressed in *E. coli*. Immunoblot analysis using anti-RGA1 serum shows that the RGA1 protein is developmentally expressed in rice. In addition, the RGA1 serum exhibited a difference in binding affinity for the $G\alpha$ proteins of monocot and dicot plants.

G-proteins have been shown to be important molecular switches in animals and simple eukaryotes. Receptor and effector molecules have been found in animals. However, even though the existence of G-proteins in the plant kingdom is fairly well documented, little progress has been made in identifying the receptors and effectors that activate or respond to them. Recently, possible candidates for cytosolic effectors involved in signal transduction in plants have been proposed, but they have not been fully characterized. Evidence for the presence of cAMP in plants has been controversial, but Lusini et al. [64] showed that cAMP formation by adenylate cyclase from castor bean is stimulated by GTP. The phosphatidylinositol turnover pathway has been documented in plants [65], thus phospholipases are possible effectors. Dillenschneider et al. [66] found that guanine nucleotides stimulated the release of phosphoinositol derivatives from membranes isolated from *Acer pseudoplatanus*.

It has recently been reported that phospholipase D can be stimulated by the G-protein activators mastoparan, ethanol and cholera toxin in *Chlamydomonas eugametos* and carnation petals [67]. The plant genes that encode phospholipase D homologues

from castor bean [68], rice [69], maize [69] and *Arabidopsis* [70] have been cloned.

Ion channels are important effectors in animals, and they may also play a similar role in plants, because their interaction with toxin-sensitive G-proteins has been reported in bean [71–75]. Also, at least some of the receptors belonging to the seven-transmembrane-span receptors superfamily have been reported [76,77].

We therefore expect that our findings will provide valuable information on the cross-talk between heterotrimeric G-proteins and receptor or effector molecules involved in plant signal-transduction systems.

We are grateful to Giesela Hoscheck in the U.S.A. and Dr. Chang Deok Han in Gyeongsang National University (GNU) for critical reading of the manuscript. This work was supported by grants from Plant Molecular Biology and Biotechnology Research Center (PMBBRC) in GNU (Chinju, Korea) sponsored by the Korea Science & Engineering Foundation (KOSEF) in Korea.

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