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In vitro mutation analysis of *Arabidopsis thaliana* small GTP-binding proteins and detection of GAP-like activities in plant cells

Toyoaki Anai[a,**], Minami Matsui[b], Nobuo Nomura[b], Ryotaro Ishizaki[b], Hirofumi Uchimiya[c,*]

[a]*Department of Botany, Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060, Japan*
[b]*Institute of Gerontology, Nippon Medical School, Nakahara-ku, Kawasaki 211, Japan*
[c]*Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*

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Abstract

Previously, we have reported the molecular cloning of *ara* genes encoding a small GTP-binding protein from *Arabidopsis thaliana*. The criterion based on amino acid sequences suggest that such an *ara* gene family can be classified to be of the *YPT/rab* type. To examine the biochemical properties of ARA proteins, several deletions and point mutations were introduced into *ara* cDNAs. Mutant proteins were expressed in *E. coli* as GST-chimeric molecules and analyzed in terms of their GTP-binding or GTP-hydrolysing ability in vitro. The results indicate that four conserved amino acid sequence regions of ARA proteins are necessary for GTP-binding. A point mutation of Asn at position 72 for ARA-2, or 71 for ARA-4, to Ile decreased GTP-binding and a point mutation of Gln at position 126 for ARA-2, or 125 for ARA-4, to Leu suppressed GTP-hydrolysis activity. Furthermore, certain factors associated with the membrane fraction accelerated GTPase activities of ARA proteins, suggesting the presence of GTPase activating protein(s) (GAP(s)) in the vesicular transport system of higher plant cells.

Key words: Small GTP-binding protein; GTPase; YPT/RAB; Site-drected mutagenesis; GAP; *Arabidopsis thaliana*

1. Introduction

Small GTP-binding proteins have a molecular mass of 20–30 kDa, and possess GTP hydrolysis activity [1,2]. These proteins may act as molecular switches which are turned on by GTP and turned off by hydrolysis of GTP to GDP. This cycle is regulated by at least two other proteins, i.e. GTPase activating protein (GAP) and guanine nucleotide releasing factor (GRF) [1,2]. Three major subfamilies of small GTP-binding proteins have been identified (RAS, ROH and YPT/RAB). The *ras* genes were isolated as an oncogene to transform NIH 3T3 cells, and this gene's products are localized in the plasma membrane. Mammalian *ras* gene products are involved in the control of cell growth and divisions. In *Nicotiana plumbaginifolia* cells, overexpression of the *Saccharomyces cerevisiae RAS2* gene inhibits cell viability and mitotic division [3]. The products of the *rho* gene family are involved in cytoskeletal organization [4,5]. The products of the *YPT/rab* gene family members are located in the Golgi apparatus or endoplasmic reticulum and are believed to be involved in either intercellular vesicular transport or cell growth [6–9].

Recently, several small GTP-binding protein genes have been isolated from higher plants [10–21]. All of the plant small GTP-binding protein genes that have been reported thus far have shown the highest similarity to YPT/RAB family proteins. Very little is known about the biological function of plant small GTP-binding proteins in plant cells. In addition, the biochemical properties of plant small GTP-binding proteins have not yet been characterized.

We previously reported on the molecular characteristics of small GTP-binding protein genes from *Arabidopsis thaliana* (*ara* gene family) [10,11].

In the present study, biochemical properties of genetically engineered ARA-2 and ARA-4 proteins were characterized by deletion and site-directed mutagenesis. Furthermore, we demonstrate for the first time that GTPase activating protein(s) are associated with membranous fractions in plant cells.

2. Materials and methods

2.1. Site-directed mutagenesis

Full-length *ara-2* and *ara-4* cDNA fragments were cloned into the pUC118 phagemid vector and in vitro mutagenesis was performed using Kunkel's method [22]. The following oligonucleotides were used: 5′-CTGCTGGTCTAGAGAGATAC-3′ corresponding to 239b to 258b where A was changed to T (codon 72 of the *ara-2* gene); 5′-CTTATAGGGATCAAGTGCGATC-3′ corresponding to 400b to 421b where A was changed to T (codon 126 of the *ara-2* gene); 5′-CCGCCGGTCTGGAACGCTTC-3′ corresponding to 206b to 225b where A was changed to T (codon 71 of the *ara-4* gene) and 5′-CTTATTGGGATCAAATGTGATC-3′ corresponding to 367b to 388b where A was changed to T (codon 125 of the *ara-4* gene).

2.2. Expression and purification of recombinant GST-ARA proteins

Wild-type and mutant *ara* cDNA fragments were cloned into the pGEX expression vector [23]. *E. coli* strain DH5aF′ was transformed with recombinant plasmids and GST-ARA proteins were induced in

* Corresponding author. Fax: (81) (3) 3812 2910.

** *Present address:* Institute of Applied Biochemistry, University of Tsukuba, Tsukuba 305, Japan.

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E. coli with 0.1 mM IPTG for 16 h at 20°C. Pelleted cells were suspended in PBS buffer, and disrupted by sonication (3 min with six 30-s intervals). After centrifugation, Triton X-100 was added to the supernatants in new tubes (0.5% final concentration). Cell extracts obtained in this manner were loaded onto a glutathione-Sepharose column (Pharmacia LKB, Uppsala, Sweden) and washed with PBS buffer containing 0.5% Triton X-100. Recombinant proteins were eluted with a buffer (50 mM Tris-HCl (pH 8.0) and 5 mM glutathione), followed by filtration on a Sephadex G-50 column (Pharmacia LKB).

2.3. Nucleotide binding

GST-ARA-producing *E. coli* cells suspended in Laemmli loading buffer [24] were boiled for 5 min. After centrifugation, supernatants were applied to 15% SDS-PAGE, followed by blotting onto a PVDF membrane (Immobilon-P: Millipore, MA, USA) [25]. Membranes were soaked in binding buffer (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.3% Tween 20 and 0.5 mM EDTA) for 30 min, and incubated in the same buffer which contained 1 nM [α-^{32}P]NTP (3000 Ci/mM; ICN) for 1 h. Membranes were then washed three times with the binding buffer and images were obtained with a Fujix BAS 2000 imaging system (Fuji Film, Tokyo, Japan) [26].

2.4. GTP hydrolysis (GTPase) analysis

GTPase activity was measured by a modified method of Wagner [27]. Recombinant proteins (10 μg) were incubated at 37°C in 100 μl of GTPase reaction mixture (65 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM NaN_3, 5 μM GTP, 33 nM [α-^{32}P]GTP (3000 Ci/mM; ICN) and 1 mM ATP (CTP, UTP or GTP-γ-S)]. Ten μl samples were collected at 1-h intervals and 10 μl 0.5 M EDTA was added to stop the reaction. Two μl were spotted onto a PEI-cellulose TLC plate (MACHERY-NAGEL, Germany), which was then developed in 0.5 M KH_2PO4 (pH 3.4). After drying, the plate was analyzed with a Fujix BAS 2000 imaging system.

2.5. GST-ARA-bound GTP product analysis

Recombinant GST-ARA proteins were incubated at 30°C for 1 h in the GTPase reaction mixture, 10 μl 1 M $MgCl_2$ and anti-ARA antibody-coated protein A-Sepharose or glutathione-Sepharose was then added. After incubating at 4°C for 1 h, the sample was centrifuged (3,000 × *g*, 5 min) and the precipitate was washed three times with washing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 100 mM $MgCl_2$). Binding nucleotides were eluted with 20 μl of 0.5 M EDTA (pH 8.0) and applied to TLC for GTPase assay.

2.6. Preparation of proteins from plant cells

Three-day-old suspension-cultured cells of *Arabidopsis thaliana* were homogenized with a grinding buffer (50 mM HEPES-KOH (pH 8.0), 330 mM sorbitol, 2 mM EDTA, 100 μM PMSF, 10 μg/ml leupeptin and pepstatin A). The filtrates were separated by differential centrifugation (1,000 × *g*, 5 min; 3,000 × *g*, 10 min; 10,000 × *g*, 20 min and 100,000 × *g*, 60 min). Pellet fractions at each step were collected and kept at −80°C. The supernatant fraction was also saved under the same conditions.

2.7. Measurement of GTPase activating (GAP) activity of plant extracts

To analyze GAP activity, protein samples prepared from *Arabidopsis thaliana* cells were mixed with GST-ARA-2 or GST-ARA-4 in the GTPase reaction mixture. After 60 min incubation at 30°C, to the reaction mixture was added 1 M $MgCl_2$ (final concentration 100 mM), and glutathione-Sepharose, followed by 1 h incubation at 4°C.

The complexes of GST-ARA and guanine nucleotides were collected by centrifugation (5,000 × *g*, 5 min). The pellets were washed with TBS buffer containing 100 mM $MgCl_2$ three times, and 0.5 M EDTA (pH 8.0) was added to release nucleotides, and spotted to TLC as described earlier.

3. Results and discussion

3.1. GTP-binding ability

Using deleted mutants (confirmed deleted proteins by Coomassie brilliant blue staining) and those with a point mutation in *ara*-2 and *ara*-4 cDNAs, a GTP-binding experiment was carried out as shown in Fig. 1. The wild-type and some mutated proteins showed binding to [α-^{32}P]GTP. Deletion up to the point of the *Rsa*I site (11 amino acids for ARA-2, 10 amino acids for ARA-4 from the initiation codon) had no affect, while further deletions apparently diminished GTP-binding ability. The results of the GTP-binding experiments clearly indicate that conserved regions such as GTP-binding common sequences (Boxes I–IV in Fig. 1A), seem to be essential. A point mutation at Gln (72 for ARA-2, 71 for ARA-4, from the initiation codon) did not affect GTP-binding ability, but mutation at Asn (126 for ARA-2, 125 for ARA-4, from the initiation codon) completely inhibited nucleotide binding. This region is known to act as a guanine ring-associated loop (Box III: Asn-Lys-Cys-Asp) [28]. In the case of yeast YPT1 protein, a similar mutation showed a dominant lethal phenotype in yeast cells [29,30] and no GTP-binding activities in vitro [27,31].

3.2. GTP hydrolysis

A GTP hydrolysis experiment was carried out in wild-type and point mutated GST-ARA proteins. As shown in Fig. 2A and B, both ARA-2 and ARA-4 showed hydrolysis of GTP to GDP, which was significantly inhibited by a mutation at the Asn site (Fig. 2C and D). In mammalian RAS protein, a similar mutation increased GTP-binding ability due to decreased GTPase activities in vivo [32]. The rate of GTPase activities in GST-ARA-2 and GST-ARA-4 were 0.006 min^{-1} and 0.0015 min^{-1}, respectively. GTPase activities in other studies are as follows: RAS, 0.02 min^{-1} [33]; ROH, 0.1 min^{-1} [34]; YPT1, 0.006 min^{-1} [27]; Smg 25A, 0.04 min^{-1} [35]; RAB1A, 0.009 min^{-1} [31]; and RAB1B, 0.006 min^{-1} [31]. The Gln→Leu substitution decreased GTP hydrolytic activity by about 40%. The rate of GTP hydrolysis in the Asn→Ile substitution was about 30% of that in the wild-type. GST alone could not hydrolyze GTP (data not shown). These results show that these point mutations affect GTP hydrolysis activity in the same way as RAS and YPT proteins [27,32].

3.3. Effects of nucleotides on GTP hydrolysis by ARA

The effects of nucleotides, such as ATP, CTP, UTP and GTP-γ-S, on the GTP hydrolysis activities of GST-ARA proteins were measured. As shown in Fig. 3, only GTP-γ-S (non-hydrolyzable analog of GTP) significantly inhibited GTP hydrolysis.

3.4. GST-ARA protein-guanine nucleotide complex analysis

In this experiment, after induction of GST-ARA proteins with [α-^{32}P]GTP, the same reaction mixture was treated with anti-ARA protein monoclonal antibody-coated protein A-Sepharose complexes or glutathione-

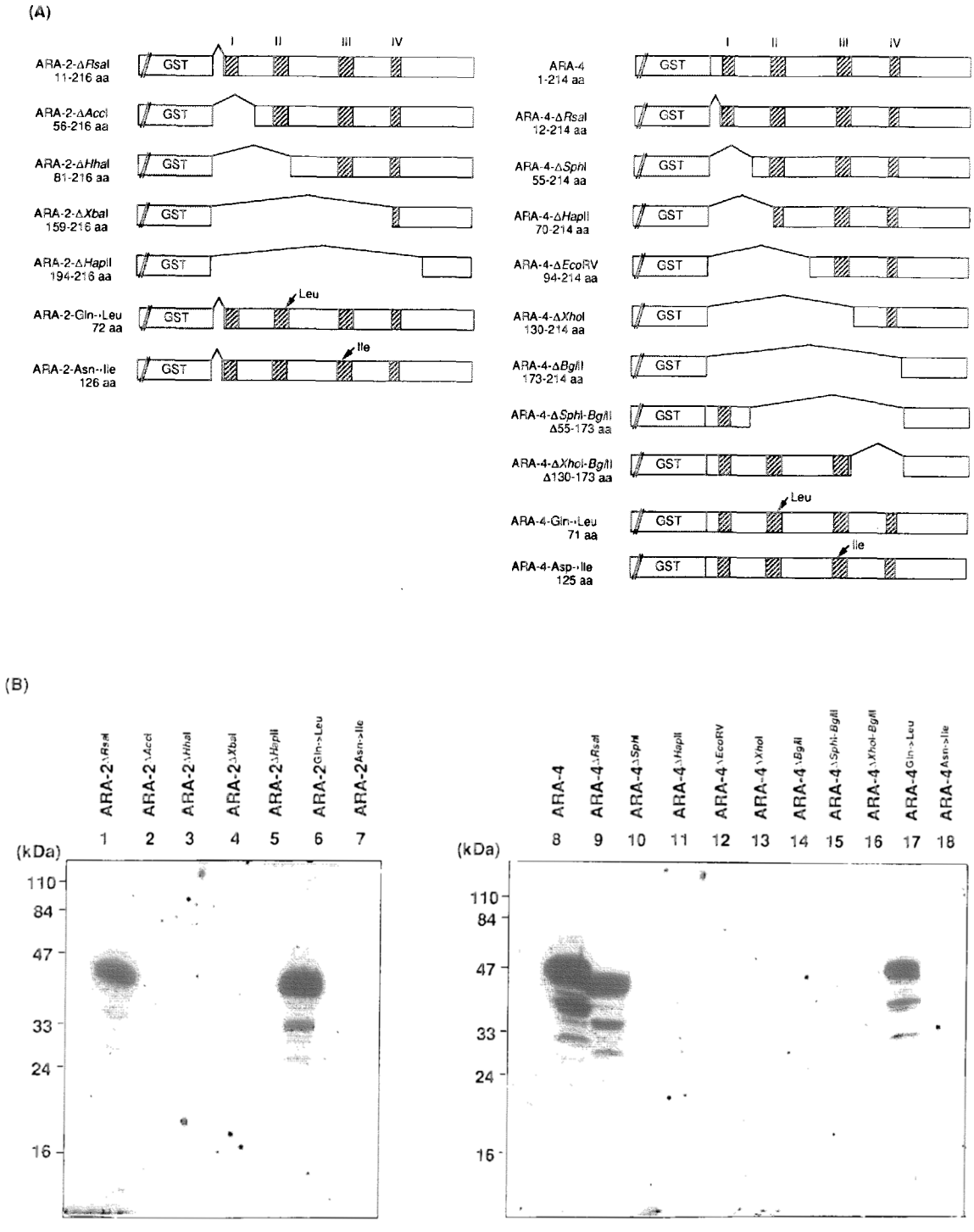

Fig. 1. GTP-binding analysis of recombinant GST-ARA proteins. (A) Deleted *ara* cDNAs were generated using restriction endonucleases, and point mutations with oligonucleotides were introduced by the method of Kunkel. Wild-type and mutated *ara* cDNA fragments were cloned into pGEX expression vectors. Recombinant ARA proteins were expressed as GST-ARA fusion proteins. Boxes (I–IV) were conserved in all of the small GTP-binding proteins. (Box I, GDSAVGKS; Box II, DTAGQE; Box III, LVGNKCDL; Box IV, ETSA; Bold typeface indicates conserved in all GTPase [40]. Underlined amino acid residues were changed by site-directed mutagenesis.) Numbers indicate the positions of respective amino acids of ARA protein. (B) Total *E. coli* lysates, producing recombinant GST-ARA proteins, were solubilized in buffer containing 1% SDS by boiling for 5 min. Proteins separated by SDS-PAGE (15%) were transferred onto a PVDF membrane, and then used for the GTP-binding assay. The presence of proteins separated by SDS-PAGE was confirmed by CBB staining.

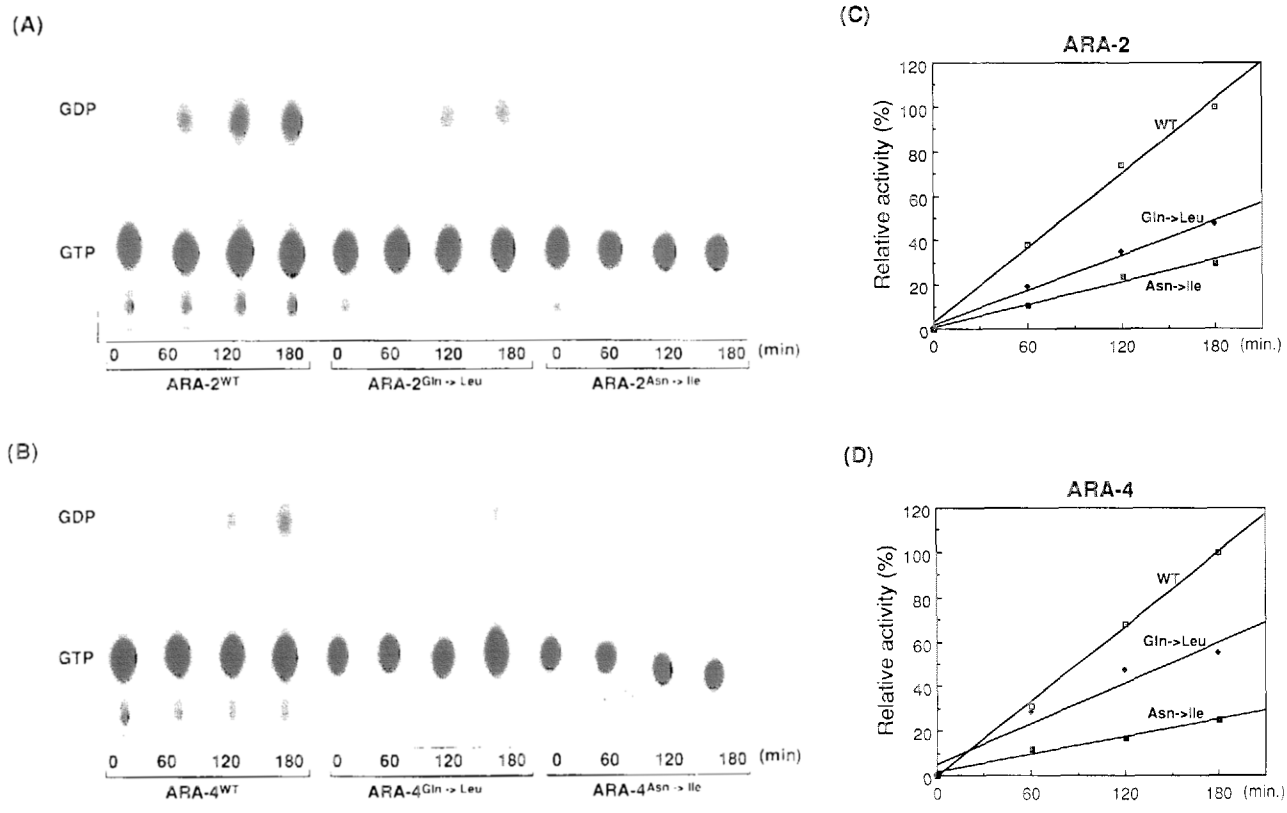

Fig. 2. GTP hydrolysis analysis for GST-ARA mutant proteins. Wild-type (WT) and point mutated GST-ARA proteins (Gln→Leu or Asn→Ile) were incubated in the reaction buffer containing [α-^{32}P]GTP. Samples of the reaction mixtures were collected at 60 min intervals and EDTA was added to stop the reaction. Reaction mixtures were spotted onto a PEI-cellulose TLC plate which was then analyzed using a Fujix BAS 2000 imaging system. Autoradiograms of GTP hydrolysis by (A) GST-ARA-2 protein and derivatives, (B) GST-ARA-4 protein and derivatives. Quantitative GTP hydrolysis by (C) GST-ARA-2 protein and derivatives and (D) GST-ARA-4 protein and derivatives.

Sepharose. Immuno-precipitated samples were analyzed by TLC. As shown in Fig. 4, mutation at Gln clearly suppressed hydrolysis of GTP to GDP. Mutation at Asn also substantially reduced the hydrolysis of GTP. The

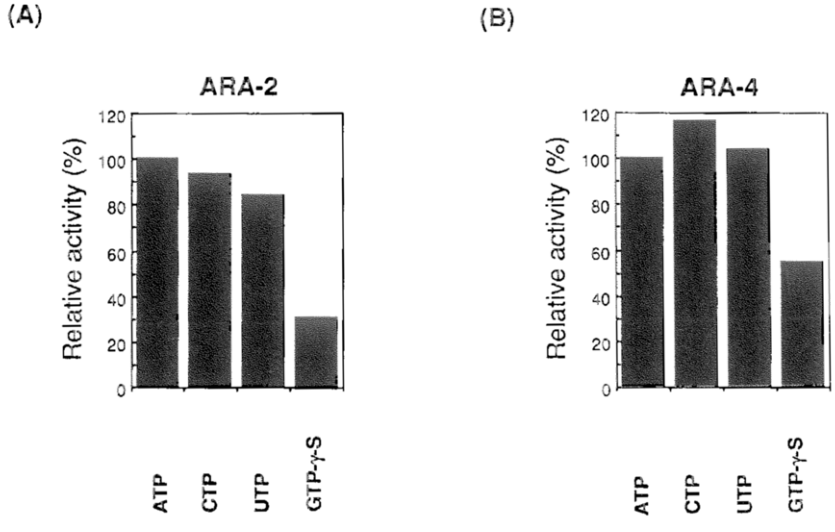

Fig. 3. Effects of different nucleotides on GTP hydrolysis by GST-ARA proteins. GST-ARA-2 (A) and GST-ARA-4 (B) proteins were incubated in reaction buffer containing different nucleotides (1 mM ATP, 1 mM CTP, 1 mM UTP and 1 mM GTP-γ-S) for 1 h. GTP hydrolysis activity is shown relative to that of the buffer that contained only labeled GTP.

results obtained with different monoclonal antibodies and glutathione-Sepharose were similar.

3.5. GAP (GTPase activation protein) activity

Fig. 5 shows the result of GAP activities in fractionated cell extracts. As seen in Fig. 5a, the GAP-like factor for ARA-2 protein was located in the 100,00 × *g* pellet of *Arabidopsis thaliana* cells. The protenous nature of this factor was proven by the fact that boiling the sample diminished GAP activities (data not shown). A similar factor also activated GTPase activities of the ARA-4 protein (Fig. 5b). Enhancement of GAP-like activities were parallel to the increased amounts of protein extracts (Fig. 5b). In yeast cells YPT1 and SEC4 protein may participate in intra-cellular vesicle transport [36]. The presence of such a small GTP-binding protein activating factor (GAP) was also reported [37–39].

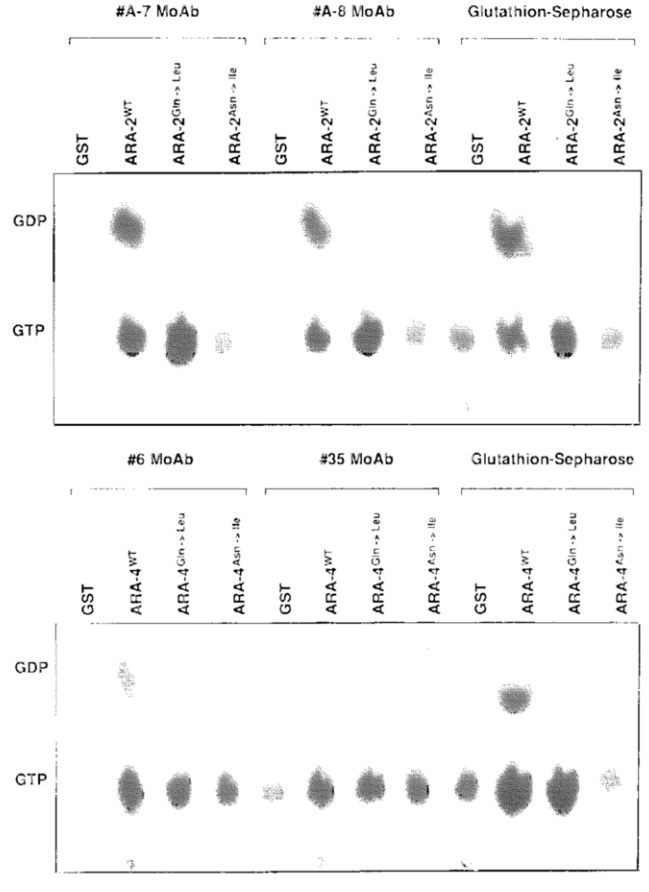

Fig. 4. Immuno precipitation of GST-ARA protein-guanine nucleotide complexes. Proteins were incubated in the buffer containing [α-^{32}P]GTP at 30°C for 60 min. Complexes of GST-ARA protein and nucleotide were precipitated with anti-ARA antibody-coated protein A-Sepharose (#A-7MoAb and #A-8MoAb: anti-ARA-2 monoclonal antibodies, #6MoAb and #35MoAb: anti-ARA-4 monoclonal antibodies) or glutathione-Sepharose. Binding nucleotide was recovered using EDTA. Nucleotide was spotted onto a PEI-cellulose TLC plate and developed. After drying, the plate was analyzed using a Fujix BAS 2000 imaging system. (#A-7 and #A-8 monoclonal antibodies specifically react with wild-type and mutant ARA-2 proteins. #6 and #35 monoclonal antibodies specifically react with wild-type and mutant ARA-4 proteins.)

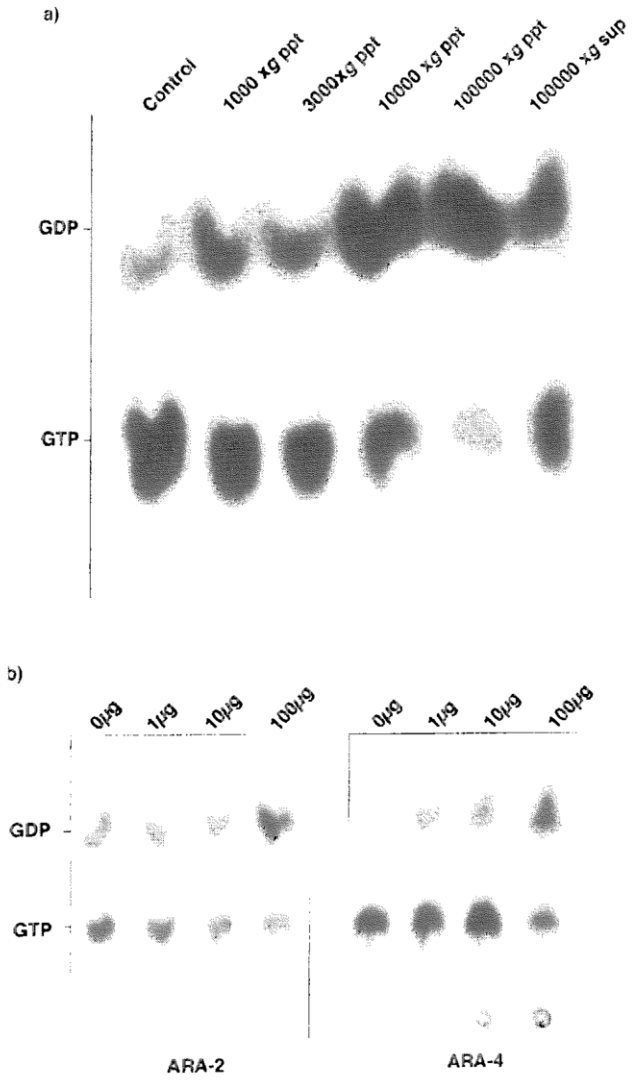

Fig. 5. GAP like activities of plant cell extracts. (a) GAP like activities of *Arabidopsis thaliana* cell fractions to GTPase activity of ARA-2 protein. GST-ARA-2 protein were incubated in the reaction mixture containing 100μg of cell fractions from *Arabidopsis thaliana* (1,000 × *g* ppt, 3,000 × *g* ppt, 10,000 × *g* ppt, 100,000 × *g* ppt, 100,000 × *g* sup) at 30°C for 60 min. Complexes of GST-ARA-2 protein and nucleotide were precipitated with glutathione-Sepharose. Binding nucleotide was recovered using EDTA. Nucleotide was spotted on a PEI-cellulose TLC plate and developed. After drying, the plate was analyzed using a Fujix BAS 2000 imaging system. (b) Effects of protein contents in terms of GAP like activities. GST-ARA-2 or GST-ARA-4 protein were incubated in the reaction mixture containing different amount of (1 μg, 10 μg or 100 μg) membranous fraction of *Arabidopsis thaliana*.

3.6. Conclusion

In this work, we demonstrate that the biochemical nature of ARA-2 and ARA-4 is very similar to the YPT/RAB type small GTP-binding protein, possessing distinctive GTPase activities. Furthermore, we demonstrate for the first time that GTPase activating activity was

located mainly in the membranous fraction of the plant cells. In order to understand the precise molecular interactions, cloning and characterization of GAP-like protein gene(s) in plants need to be carried out.

More recently, the plant RAB1 and RAB7 protein homologs, isolated from soybean (*sRab1* and *sRab7*) and *Vigna aconitifolia* (vRab1) have been found to be involved in the biogenesis of peribacteroid membrane (PBM) in legume [21]. Induction of the srab1 and vrab7 genes directly correlates with membrane proliferation in nodules, however, reducing the expression of these genes (anti-sense constructs) resulted in reduction in the size of root nodules, compartmentalization of bacteria and the efficiency of root nodules in fixing nitrogen. Thus, it is most likely that the ARA protein may also function in a similar fashion.

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