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Molecular and biochemical studies of
Arabidopsis thaliana small GTP-binding proteins

Toyoaki Anai

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Summary:

Using a genomic DNA fragment encoding *Arabidopsis thaliana* small GTP-binding proteins, I isolated independent four cDNA clones (*ara-2*, *ara-3*, *ara-4* and *ara-5*) of the same plant species. The deduced amino acid sequences of these genes showed conservation of amino acids in four regions, all of which are present in small GTP-binding proteins, and are important for GTPase/GTP-binding activities. These products were highly homologous to those of the *YPT* genes of *Saccharomyces cerevisiae* and the *ypt* gene of *Schizosaccharomyces pombe* in the regions around amino acid 45, which is thought to be the site interacting with effector molecules. Furthermore, four genes showed common amino acid sequences (Cys-Cys-Xaa-Xaa) at their C termini. Another characteristic feature of this gene family is the presence of Ser in place of Gly in the first conserved region (Gly¹² of mammalian GTP-binding RAS protein). To identify the subcellular localization of ARA proteins, suspension cultured cells of transgenic tobacco cells were analyzed. Consequently, I identified proteins located in the membrane fraction by ARA-4 specific monoclonal antibody. Such ARA proteins was readily released by the detergent such as sodium deoxycholate and NP-40, indicating ARA-4 protein is associated with organelle and / or plasma membrane surface of plant cells. To examine the biochemical properties of ARA proteins, I introduced several deletions and point mutations into *ara* cDNAs. Mutant proteins were analyzed in terms of the GTP-binding or GTP-hydrolysis ability *in vitro*. Results showed that four conserved amino acid sequence regions of ARA proteins were necessary for GTP-binding. Point mutation of Asn at position 126 for ARA-2, 125 for ARA-4, to Ile decreased the

Summary

GTP-binding and the point mutation of Gln at position 72 for ARA-2, 71 for ARA-4 to Leu suppressed the GTP-hydrolysis activity. Thus, plant small GTP-binding proteins possess similar biochemical properties with yeast or mammalian small GTP-binding proteins.

Introduction:

Signal transduction pathways have been extensively studied in eukaryotic cells such as yeast and mammalian cells. Many hormone-receptor proteins and intracellular molecules have been isolated and characterized genetically. But very little is known about the signal transduction pathway in plant cells. One of central problems in plant molecular biology is how external signals are transmitted to the interior of cells.

Many small GTP-binding proteins have been identified in various species of both vertebrates and invertebrates (for reviews, see Bourne et al., 1990; 1991). Some of these molecules are thought to be involved in signal transduction pathways in eukaryotic cells. Small GTP-binding protein-encoding genes make up a multi-gene family in mammalian cells, and they are localized in different intra-cellular membranes, e.g., plasma membrane (Rubins et al., 1990), the Golgi apparatus (Segev et al., 1988; Goud et al., 1990) and the endoplasmic reticulum (Nigam, 1990). The amino acid sequences of their products are conserved in four regions, which are now known to be important for binding and hydrolysis of GTP (Bourne et al., 1990; 1991). Small GTP-binding proteins can be classified into three subfamilies: RAS, RHO and YPT/RAB by differences in a region around amino acid position 35 in RAS which is important both for binding to effector proteins and regulating GTPase activity (GTPase activating protein, GAP; Trahey and McCormik, 1987; Gibbs et al., 1988) and for interaction with effector molecules (Hall, 1990). Mainly from studies in *Saccharomyces cerevisiae*, the YPT/RAB subfamily is known to be localized in the surface of traffic vesicles segregated from the Golgi apparatus

and determining the fate of the vesicles (Segev et al., 1988). YPT1 is also important for cell viability, and in *Saccharomyces cerevisiae* lack of the *YPT* gene causes lethality to cells (Schmitt et al., 1986). The structure of ARA is most similar to that of YPT of *Saccharomyces cerevisiae*. Recently, three *YPT*-like genes were cloned from *Schizosaccharomyces pombe* (Haubruck et al., 1990; Miyake and Yamamoto, 1990). In the case of higher plants, Matsui et al. (1989) were successful to isolate one genomic DNA clone (*ara*) from *Arabidopsis thaliana*, which is supposed to encode a RAS-related small GTP-binding protein. In order to investigate molecular divergency in *ara* gene family, I have attempted to screen *Arabidopsis thaliana* cDNA libraries using genomic *ara* gene.

In the Part 1 of this thesis, I report molecular cloning and characterization of four cDNA clones encoding putative small GTP-binding proteins.

Previously, I isolated cDNA clones encoding small GTP-binding proteins from *Arabidopsis thaliana*, namely the *ara* gene family.

Recently, Kamada et al. (Kamada et al., 1992) reported the production of transgenic tobacco plants possessing a gene encoding the rice small GTP-binding protein. Such plants showed abnormal growth characteristics such as tillering, dwarfism and abnormal flower development. I also obtained similar transgenic tobacco plants containing CaMV 35S promoter and the *ara* cDNA. The suspension cultured cells of such transgenic tobacco were used to identify subcellular localization of ARA proteins.

In the Part 2 of this thesis, I report that RAS-related small GTP-binding proteins locate on the surface of organella and / or plasma membrane of plant cells.

Recently, several small GTP-binding protein genes have been isolated from higher

Introduction

plants (see Table 1). All plant small GTP-binding protein genes so far reported showed highest similarities with YPT/RAB family proteins. Very little is known about biological function of plant small GTP-binding protein in plant cells. And biochemical properties of plant small GTP-binding proteins have not yet been characterized.

In the Part 3 of this thesis, I characterized biochemical properties of genetically engineered ARA-2 and ARA-4 proteins by deletion and site-directed mutagenesis. Probably this is the first report of this kind indicating that preserved amino acid residues are essential for the GTP hydrolysis by plant RAS-related small GTP-binding proteins.

Table 1: Comparison of reported sequences of small GTP-binding protein genes in higher plants.

Table 1: Comparison of reported instances of small GTP-binding protein genes in higher plants.

Organism	Name	Predicted molecular mass (kDa)	Cloning method	Biochemical characterization	Reference
<i>Arabidopsis thaliana</i>	<i>ara</i>	24	Hybridization with v-Ha-ras probe	None	Matsui et al. (1989)
	<i>ara-2</i>	23.9	Hybridization with <i>ara</i> probe	GTP-binding, GTPase, Immuno-precipitation	This paper
	<i>ara-3</i>	23.8	Hybridization with <i>ara</i> probe	None	ibid.
	<i>ara-4</i>	24	Hybridization with <i>ara</i> probe	GTP-binding, GTPase, Immuno-precipitation	ibid.
	<i>ara-5</i>	N. D.	Hybridization with <i>ara</i> probe	None	ibid.
<i>Nicotiana plumbaginifolia</i>	<i>rha 1</i>	21.8	Hybridization with oligonucleotide probe	GTP-binding	Anuntalabhochai et al. (1991)
	<i>rhn 1</i>	21.8	Hybridization with oligonucleotide probe	None	Terryn et al. (1992)
	<i>Np-ypt 3</i>	24.4	PCR	GTP-binding	Dallmann et al. (1992)
<i>Nicotiana tabacum</i>	<i>Nt-rab 5</i>	21.9	PCR	GTP-binding	ibid.
<i>Oryza sativa</i>	<i>rgp 1</i>	24.8	Differential cloning	GTP-binding	Sano et al. (1991)
	<i>rgp 2</i>	23.9	Hybridization with <i>rgp 1</i> probe	None	Youssefian et al. (1993)
	<i>ric 1</i>	22.6	random sequencing	GTP-binding	Kidou et al (1993)
	<i>ric 2</i>	23.9	random sequencing	GTP-binding	ibid.

Organism	Name	Predicted molecular mass (kDa)	Cloning method	Biochemical characterization	Reference
<i>Zea mays</i>	<i>Yptm 1</i>	23.3	Hybridization with oligonucleotide probe	None	Palme et al. (1992)
	<i>Yptm 2</i>	22.5	Hybridization with oligonucleotide probe	None	ibid.
	<i>Pra 1</i>	23.9	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	Nagano et al. (1993)
<i>Pisum sativum</i>	<i>Pra 2</i>	24.9	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Pra 3</i>	N.D.	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Pra 4</i>	24	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Pra 5</i>	24.1	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Pra 6</i>	24	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	GTP-binding	ibid.
	<i>Pra 7</i>	24.2	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Pra 8</i>	22.4	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Pra 9A</i>	22.5	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.

Organism	Name	Predicted molecular mass (kDa)	Cloning method	Biochemical characterization	Reference
<i>Pisum sativum</i>	<i>pra 9B</i>	22.5	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	Nagano et al. (1993)
	<i>pra 9C</i>	22.6	Hybridization with <i>ara-2</i> and <i>ara-4</i> probe	None	ibid.
	<i>Psa-rab</i>	23	Random sequencing	None	Drew et al. (1993)

Molecular cloning and characterization of cDNAs encoding small GTP-binding proteins of *Arabidopsis thaliana*

Materials and methods:

Plant materials and cDNA library

The wild-type strains of *Arabidopsis thaliana* (Landsberg, Columbia, Enkheim, Lapalma and Estland) were a gift from N. Goto (Miyagi College of Education, Miyagi, Japan). A cDNA library of *Arabidopsis thaliana* (λ gt10) was kindly provided by Dr. Werneke (USDA/ARS, Urbana, IL).

Southern-blot hybridization

Arabidopsis thaliana DNA was isolated from whole plants as described in Lichtenstein and Draper (Lichtenstein and Draper, 1985). Plant DNA (10 μ g) was digested with restriction enzymes, subjected to electrophoresis in agarose gel, transferred to nylon membranes and hybridized with *ara*, *ara-2*, *ara-3*, *ara-4* and *ara-5* as probes. The probes were labeled with [α - 32 P]dCTP (3000Ci/mmol) by random priming. Filters were hybridized under low-stringency conditions in a solution consisting of 25% formamide, 6X SSC, 10X Denhardt's solution, 0.5% SDS, 0.2mg per ml of herring sperm DNA and 5×10^8 - 1×10^9 cpm per μ g of DNA probe at 37°C for 12hr and finally washed with 2X SSC and 0.2% SDS at 50°C for 1hr. In high-stringency conditions, filters were hybridized in a solution of 50% formamide, 6X SSC, 10X Denhardt's solution, 0.5% SDS, 0.2mg per ml of herring sperm DNA and 5×10^8 - 1×10^9 cpm per μ g of DNA probe at 37°C for 12hr and finally washed with 0.1X SSC and 0.2% SDS at 60°C for 1hr.

Screening of the cDNA library

About 1×10^5 plaques of a λ gt10 cDNA library of green leaves of *Arabidopsis thaliana* (Columbia) were screened with the labeled *ara* probe. Hybridizations were carried out at 37°C for 12hr in a solution of 25% formamide, 6X SSC, 10X Denhardt's solution, 0.5% SDS, 0.2mg per ml of herring sperm DNA and 5×10^8 - 1×10^9 cpm per μ g of DNA probe. The filters were washed three times with a solution of 1X SSC and 0.1% SDS at room temperature for 15min and finally with 2X SSC and 0.2% SDS at 50°C for 1hr.

Sequencing of DNA

Sequencing was carried out by the method of Sanger et al. (Sanger et al., 1977) with some modification (Mizusawa et al., 1986) (7-deaza dGTP instead of dGTP) on fragments subcloned in M13mp series vectors (Yansich-Perron et al., 1985).

Transformation of Schizosaccharomyces pombe

Schizosaccharomyces pombe (*ras1*, *ypt1* and *ypt3* mutants) were kindly supplied by S. Miyake (Institute of Medical Science, University of Tokyo, Tokyo, Japan). The *ara-4* and *ara-5* cDNAs were cloned in the *Bam* HI site of pREP1 expression vector (Maundrell, 1990). The recombinant plasmids were introduced into *Schizosaccharomyces pombe* by LiAc methods.

Results and discussion:

Southern-blot hybridization of ecotypes of Arabidopsis thaliana

A 1330-bp *Eco* RI-*Eco* RV fragment, which almost covers the *ara*-coding region, was used as a probe for hybridization. Under low-stringency conditions, this probe hybridized weakly to seven DNA fragments besides a 5.5-kb fragment containing the *ara* gene in each *Eco* RI-digested genomic DNA of five wild-type strains, Landsberg, Columbia, Enkheim, Lapalma and Estland (Fig. 1). These fragments were 13, 8.5, 7.0, 4.5, 3.0, 2.3 and 2.0 kb in length. No polymorphism was observed among these wild-type strains. These results suggest that the *Arabidopsis thaliana* genome has some nucleotide sequences homologous to *ara* gene and that these homologs may constitute a gene family. These genes might be conserved in all ecotypes examined and may have been present before their divergence.

Isolation of four ara-related genes from an Arabidopsis thaliana cDNA library

I screened about 1.0×10^5 plaques of a λ gt10 cDNA library of green leaves of *Arabidopsis thaliana* (Columbia) under low-stringency hybridization conditions with a mixture of the 244-bp *Sty* I-*Cla* I, 155-bp *Sty* I-*Sty* I and 222-bp *Bgl* II-*Eco* RV fragments of *ara* genomic clones as probes (Fig. 2 in Matsui et al., 1989). These fragments almost cover the coding region of *ara* genomic sequence. Twenty-six clones hybridized weakly at a frequency of 2.6×10^{-4} and I finally classified four groups of independent cDNA clones by their restriction enzyme digestion patterns and Southern-blot hybridization (Fig. 2 and 3). Each clones contained *ara*-related

genes and the nucleotide sequences differed except for four regions corresponding to conserved amino acid residues of small GTP-binding proteins. The calculated molecular masses of ARA-related proteins were ARA-2, 23923 (216 amino acids); ARA-3, 23833 (216 amino acids); ARA-4, 23979 (214 amino acids). Some of the N-terminal amino acids of ARA-5 have not yet been determined

Genomic Southern-blot hybridization of Arabidopsis thaliana DNA with ara-related genes

As mentioned above the *ara* probe hybridized weakly with seven bands in the *Eco*RI digest of *Arabidopsis thaliana* genomic DNA under low-stringency conditions. Under high-stringency conditions, the *ara-2* fragment hybridized to two fragments of 5.4 and 4.5 kbp, and *ara-3* hybridized to 13-, 4.5- and 0.8-kb-long fragments. Fragments *ara-4* and *ara-5* hybridized to 4.5- and 20-kb-long fragments, respectively (Fig. 4). These hybridization patterns were not changed when filter were washed under more stringent conditions (0.1 X SSC, 65°C). These hybridized bands did not correspond to all the fragments detected in Fig. 1 under the low-stringency hybridization conditions with *ara* probe. It is, therefore, suggested that there are certain number of *ara*-related genes in the genome and *ara* constitutes a multigene family in *Arabidopsis thaliana*, like *ras* in mammalian cells.

Comparison of amino acid sequences of ara-related gene products and other small GTP-binding proteins

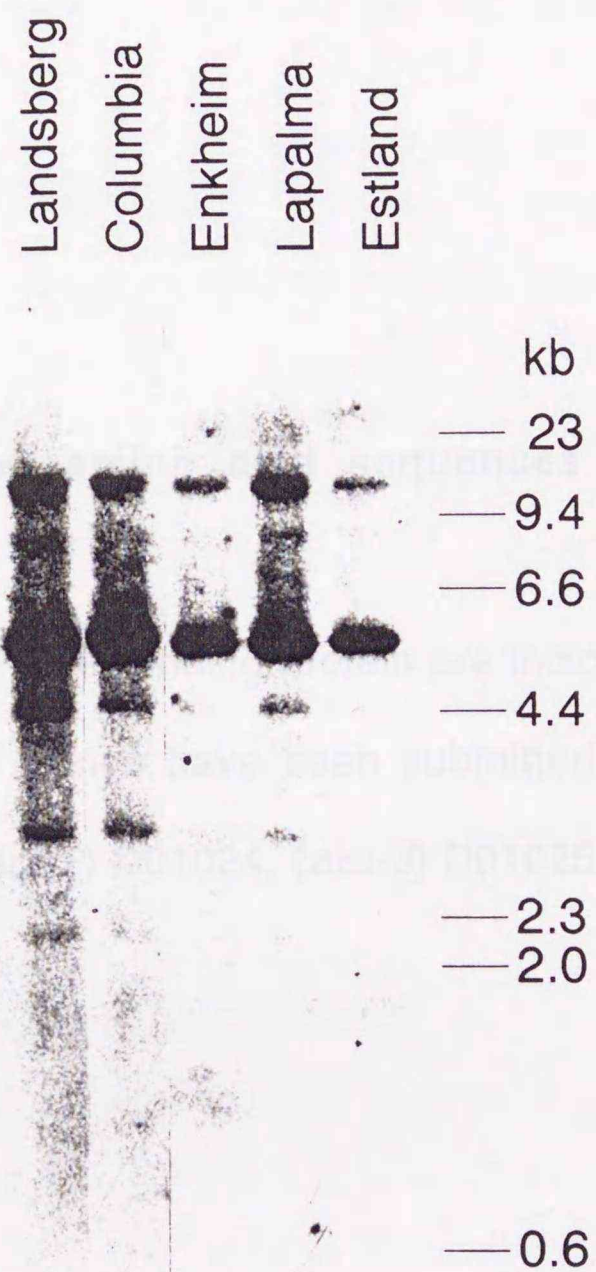
The deduced amino acid sequences of the *ara*-related gene products were

compared with those of other small GTP-binding proteins. The products of all four *ara*-related cDNAs have four conserved regions of amino acid which are found in all small GTP-binding proteins (Fig. 5). Characteristic features of these ARA-related proteins were observed in three regions. One region was around amino acid 45 (corresponding to amino acid 35 of the mammalian RAS). This region (G-2 region in the article of Bourne et al., 1991) is highly conserved within each subfamily, but not between different subfamilies, and is called the effector region (Sigal et al., 1986). GAPs are thought to interact with the *ras* gene product (p21^{ras}) in this region and to stimulate the GTPase activity of p21^{ras} (Trahey and McCormick, 1987; Gibbs et al., 1988). All ARA-related proteins have the sequence Thr-Ile-Gly-Val-Asx-Phe around amino acid 45 (Asx=Asp or Asn) which is also conserved in *YPT1* gene product of *Saccharomyces cerevisiae* (Gallwitz et al., 1983) and those of three *ypt* genes of *Schizosaccharomyces pombe* (Haubruck et al., 1990; Miyake and Yamamoto, 1990; except for Ile instead of Val in *ypt2* and *ara-3*). The second characteristic structure of these proteins is found in their C-terminal amino acid residues. All ARA-related proteins end with Cys-Cys-Xaa-Xaa (the first Xaa is Ser in three of four). YPT/RAB subfamily proteins have two Cys residues at their C-termini. The C-terminal Cys is required for palmitoylation of the YPT1 protein (Molenaar et al., 1988). RAS subfamily proteins end with Cys-Ali-Ali-Xaa (Ali, aliphatic amino acid), in which Cys is polyisoprenylated (Hancock et al., 1989). A small GTP-binding protein from bovine brain (Smg p21B) has Cys-Gln-Leu-Leu and this Cys is geranylgeranylated (Kawata et al., 1990). The specificity of fatty acid modification of the C-terminus of ARA-related proteins remains to be determined. The third

characteristic is a Ser residue in the position, which correspond to highly conserved Gly¹² in RAS subfamily proteins. Substitution of this amino acid residue by Val in RAS results in oncogenicity in mammalian cells (Reddy et al., 1982; Yuasa et al., 1983). The YPT/RAB subfamily proteins also have a Ser residue at this position, suggesting that the *ara* gene family might also belong to the *YPT/rab* gene subfamily. The similarities of the amino acid sequences of small GTP-binding proteins are summarized in Table 1. The ARA-5 product is most similar to the *rab1* gene product of rat brain cDNA (Touchot et al., 1987), showing 74.1% similarity with the latter. The ARA-5 protein also shows high similarity with YPT1 protein of *Saccharomyces cerevisiae* and Ypt1 of the three Ypt proteins of *Schizosaccharomyces pombe*. Gene *ara-5* may be the counterpart of the *ypt1* gene of yeast. The chimeric plasmid of pREP1 (Maundrell, 1990) and *ara-5* did not complement the deletion mutant of *ypt1* gene of *Schizosaccharomyces pombe* (Fig. 6). It is unclear whether lack of the N-terminal few amino acids of this gene product have some influence on protein activity. I also compared the similarity of these ARA-related proteins with 35 independent small GTP-binding proteins known to date (data not shown). Surprisingly, the ARA-3 protein showed 70.6% amino acid similarity to the SAS1 protein of the slime mold *Dictyostelium discoideum* (Table 1). The SAS1 protein is produced maximally during cytodifferentiation (Saxe and Kimmel, 1990). The meaning of this similarity must be elucidated by further studies on the developmental synthesis patterns of these small GTP-binding proteins. Isolation and characterization of small GTP-binding proteins from *Arabidopsis thaliana* may open the way to an understanding of the signal transduction pathway

Fig. 1. Southern-blot hybridization of five *Arabidopsis thaliana* ecotypes with *Eco* RI-*Eco* RV genomic *ara* probe.

Genomic DNAs of five *Arabidopsis thaliana* ecotypes were prepared using CTAB as in the protocols of Lichtenstein and Draper (Lichtenstein and Draper, 1985). DNAs (10µg) were digested with *Eco* RI and electrophoresed in 0.7% agarose gels, denatured and transferred to nylon membrane filters. Filters were hybridized with a ³²P-labeled, random-primed *ara* 1330-bp *Eco* RI-*Eco* RV genomic fragment. Hybridizations were carried out under low-stringency conditions in a hybridization solution containing of 25% formamide/6X SSC/10X Denhardt's solution/ 0.5% SDS/0.2mg per ml of herring sperm DNA using 5×10^8 - 1×10^9 cpm per µg of DNA probe at 37°C for 12hr. The filters were washed three times with a solution of 1X SSC and 0.1% SDS at room temperature for 15 min and finally 2X SSC/ 0.2% SDS at 50°C for 1hr (Southern, 1975) and exposed to Kodak X-ray film with an intensifying screen at -70°C for 24hr.



ara-2

Fig. 2 Nucleotide and deduced amino acid sequences of *ara*-related cDNA clones.

The conserved sequences of small GTP-binding protein are indicated with shaded boxes. The sequences for these clones have been submitted to the GenBank database under accession Nos.: (*ara-2*) D01024, (*ara-3*) D01025, (*ara-4*) D01026, and (*ara-5*) D01027.

ara-2

-33 ATT CCCTAATCCC AAAATTCGAT CTCTCATCTG -1

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1 ATG GCT GGT TAC AGA GCC GAT GAA GAG TAC GAT TAC CTC TTC AAG CTC GTG 51
1 M A G Y R A D E E Y D Y L F K L V 17

52 TTG ATC GGC GAC TCT GGT GTC GGC AAA TCG AAT CTG CTC TCT CGG TTC ACC 102
18 L I G D S G V G K S N L L S R F T 34

103 AAG AAC GAG TTC AAT CTC GAA TCC AAG TCT ACT ATT GGT GTT GAG TTC GCT 153
35 K N E F N L E S K S T I G V E F A 51

154 ACC AAA ACC ACT AAA GTC GAA GGC AAA GTC GTC AAA GCT CAG ATT TGG GAT 204
52 T K T T K V E G K V V K A Q I W D 68

205 ACT GCT GGT CAA GAG AGA TAC CGA GCA ATC ACA AGC GCT TAT TAC AGA GGA 255
69 T A G Q F R Y R A I T S A Y Y R G 85

256 GCT GTT GGT GCT TTG CTT ATT TAC GAC GTG ACA AGG CAC GCA ACA TTT GAG 306
86 A V G A L L I Y D V T R H A T F E 102

307 AAT GCA GCA AGA TGG CTA AGG GAA CTA AGA GGA CAC ACA GAC CCA AAC ATA 357
103 N A A R W L R E L R G H T D P N I 119

358 GTG GTG ATG CTT ATA GGG AAC AAG TGC GAT CTG CGT CAC TTG GTG GCA GTC 408
120 V V M L I G N K C D L R H L V A V 136

409 AAA ACA GAA GAG GCT AAA GCC TTT GCT GAG AGA GAA TCC CTC TAT TTC ATG 459
137 K T E E A K A F A E R E S L Y F M 153

460 GAG ACT TCA GCT CTA GAC GCA ACC AAT GTT GAA AAC GCA TTC ACG GAA GTG 510
154 E T S A L D A T N V E N A F T E V 170

511 CTC ACT CAG ATC CAC AAG ATA GTG AGC AAA AGA AGT GTT GAT GGA GGA GGA 561
171 L T Q I H K I V S K R S V D G G G 187

562 GAG TCG GCT GAT TTA CCG GGA AAA GGA GAG ACG ATA AAC GTT AAA GAA GAC 612
188 E S A D L P G K G E T I N V K E D 204

613 GGA TCG GTT CTT AAG AGA ATG GGT TGC TGC TCT AAC TAG AGTGGGGGGA 661
203 G S V L K R M G C C S N * 216

662 TTGTAGATTT GGCTTGATTT GGTTAGGGTG AGACAACAAA TGATAATCTC TTTGGGTCTT 721

722 GCAATCAAAA AAGAAATAAG AAACGATTAC TTTTAAGATC ATGTGTATGC TTTGGTAACT 781

782 TTGGCCTGTA AGGTCCTTTA AAATTTAAGT AAAAAAAAAA AAAAAA 828

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ara-3

-127 GAAGAAG -121

-120 TAAAATAGAA GAAGCCGGGA AACAAAAGCT AAATCGGGAA CTGGCGCATT TTAGTCGTCC -61

-60 GCCATAACCT TCGCTCTCTG ATTCTTCCGA TTACATCGGT TTTCTTCTCT CTCCGATTCA -1

1 ATG GCT GCT CCA CCT GCT AGA GCC AGA GCT GAT TAC GAT TAT CTC ATT AAG 51
1 M A A P P A R A R A D Y D Y L I K 17

52 CTT CTT TTG ATT GGA GAT AGC GGT GTT GGT AAA AGT TGT CTT CTT CTA CGT 102
18 L L L I G D S G V G K S C L L L R 34

103 TTC TCT GAT GGA TCT TTC ACC ACT AGC TTC ATT ACC ACC ATT GGC ATT GAC 153
35 F S D G S F T T S F I T T I G I D 51

154 TTT AAG ATA AGA ACG ATT GAG CTT GAC GGT AAA CGT ATC AAG CTT CAG ATT 204
52 F K I R T I E L D G K R I K L Q I 68

205 TGG GAT ACC GCT GGT CAA GAG CGG TTT CGG ACT ATC ACC ACT GCT TAT TAC 255
69 W D T A G Q E R F R T I T T A Y Y 85

256 CGT GGG GCA ATG GGC ATT TTG CTG GTG TAT GAT GTC ACA GAC GAG TCA TCC 306
86 R G A M G I L L V Y D V T D E S S 102

307 TTC AAC AAC ATT AGG AAC TGG ATT CGT AAT ATC GAA CAG CAT GCT TCG GAT 357
103 F N N I R N W I R N I E Q H A S D 119

358 AAT GTT AAC AAG ATC TTG GTA GGG AAC AAG GCT GAC ATG GAT GAA AGC AAG 408
120 N V N K I L V G N K A D M D E S K 136

409 AGG GCA GTA CCT ACA GCA AAG GGT CAG GCT CTT GCT GAT GAG TAC GGA ATT 459
137 R A V P T A K G Q A L A D E Y G I 153

460 AAG TTC TTC GAA ACA AGT GCC AAG ACA AAC CTA AAC GTG GAA GAA GTT TTC 510
154 K F F E T S A K T N L N V E E V F 170

511 TTC TCA ATA GGG AGG GAC ATT AAG CAG AGG CTT TCA GAC ACC GAC TCG AGG 561
171 F S I G R D I K Q R L S D T D S R 187

562 GCA GAG CCT GCA ACG ATC AAG ATA AGC CAA ACG GAC CAA GCA GCT GGA GCA 612
188 A E P A T I K I S Q T D Q A A G A 204

613 GGG CAG GCC ACA CAG AAA TCT GCA TGC TGT GGA ACT TAA AAAAGTTTTA 661
205 G Q A T Q K S A C C G T * 216

662 AAAGTGAAGT TTAAGTCGAA AAAAGTTTGG GGGTGAAGTG AAAAATAAG ATATCGTTGG 721

722 GTGCTTATGT AATTTTTTGT TTTATGCCAA CAATTCTGAT TCGTTCTTCT AAATTGTCCT 781

782 TCCTATTTGT ACGAAGGTTT AATCGAAACT TGTATTTTTC TTTTCTAAAA AAAAAAAAAA 841

ara-4

Arabidopsis thaliana

-3 GAA -1

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1 ATG TCA GAC GAC GAC GAG AGA GGC GAA GAG TAC CTC TTC AAG ATT GTT ATC 51
1 M S D D D E R G E E Y L F K I V I 17

52 ATC GGT GAT TCC GCC GTC GGC AAA TCC AAT CTC CTC ACT CGC TAC GCC CGT 102
18 I G D S A V G K S N L L T R Y A R 34

103 AAC GAG TTC AAT CCC AAT TCC AAA GCC ACT ATC GGC GTC GAG TTT CAG ACT 153
35 N E F N P N S K A T I G V E F Q T 51

154 CAG AGC ATG CTC ATC GAC GGC AAA GAG GTC AAA GCT CAG ATT TGG GAT ACC 204
52 Q S M L I D G K E V K A Q I W D T 68

205 GCC GGT CAG GAA CGC TTC CGC GCC GTT ACA TCT GCT TAT TAC CGT GGC GCC 255
69 A G Q E R F R A V T S A Y Y R G A 85

256 GTT GGA GCC CTC GTC GTC TAC GAT ATC ACT CGT AGC TCC ACT TTC GAA AAC 306
86 V G A L V V Y D I T R S S T F E N 102

307 GTC GGT CGC TGG CTC GAT GAG CTC AAC ACT CAT TCT GAT ACA ACA GTA GCA 357
103 V G R W L D E L N T H S D T T V A 119

358 AAA ATG CTT ATT GGG AAC AAA TGT GAT CTC GAG AGC ATA AGA GCG GTG AGT 408
120 K M L I G N K C D L E S I R A V S 136

409 GTT GAG GAA GGC AAG AGT CTT GCT GAG TCT GAA GGT TTG TTT TTC ATG GAG 459
137 V E E G K S L A E S E G L F F M E 153

460 ACC TCT GCG TTG GAC TCG ACC AAT GTA AAG ACG GCT TTC GAG ATG GTT ATT 510
154 T S A L D S T N V K T A F E M V I 170

511 CGG GAG ATC TAT AGC AAC ATT AGT CGA AAG CAA TTG AAC TCT GAT TCT TAC 561
171 R E I Y S N I S R K Q L N S D S Y 187

562 AAA GAG GAG CTG ACT GTG AAT CGT GTT AGC TTG GTT AAG AAC GAG AAC GAA 612
188 K E E L T V N R V S L V K N E N E 204

613 GGG ACA AAG ACA TTC TCT TGC TGT TCG AGG TAA CATCTTTGTA ACGGTTTCTC 665
205 G T K T F S C C S R * 214

666 TTTGTCCTGA GGATTGGTTT CGGGTTGAAT TGTGTTCTCTG GTAAACTTAT AGAAAGGCAG 725

726 AGATATATTC TTATAGTTCT CAGTTAAAGT AAAAGAAGAA GAGAGAAAGA TGTTTTGAGT 785

786 TTCTTTGTTA CGCATACAGA AAAAGTTTGG TTAGTGTATA AGTTATGTTT TCATTTGTAA 845

846 CTTTTGTAAA CTTATGATTG TTAAATCAAT TTTGATATTT TCCAAAAAAA AAAAAAAAAA 905

906 AAAAAAAAAA AAAAAAAAAA AAA 928

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ara-5

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1  TT TTC AAG CTC CTG CTT ATC GGG GAT TCT GGC GTA GGC AAG TCT TGT CTT 50
1      F  K  L  L  L  I  G  D  S  G  V  G  K  S  C  L 16

51 CTT TTG AGA TTC TCT GAT GAT TCT TAT GTA GAA AGT TAC ATT AGC ACT ATT 101
17  L  L  R  F  S  D  D  S  Y  V  E  S  Y  I  S  T  I 33

102 GGA GTC GAT TTT AAA ATT AGG ACT GTG GAA CAA GAT GGC AAA ACA ATT AAG 152
34  G  V  D  F  K  I  R  T  V  E  Q  D  G  K  T  I  K 50

153 CTC CAA ATT TGG GAC ACT GCT GGT CAA GAA CGG TTC AGG ACT ATT ACT AGC 203
51  L  Q  I  W  D  T  A  C  Q  E  R  F  R  T  I  T  S 67

204 AGT TAC TAC CGT GGG GCA CAT GGA ATT ATT ATT GTC TAC GAT GTC ACA GAT 254
68  S  Y  Y  R  G  A  H  G  I  I  I  V  Y  D  V  T  D 84

255 GAA GAA AGC TTC AAT AAT GTC AAG CAA TGG TTG AGT GAA ATT GAT CGT TAT 305
85  E  E  S  F  N  N  V  K  Q  W  L  S  E  I  D  R  Y 101

306 GCT AGT GAC AAT GTC AAC AAA CTC CTT GTT GGA AAC AAG TCT GAT CTT ACT 356
102 A  S  D  N  V  N  K  L  L  V  G  N  K  S  D  L  T 118

357 GAA AAC AGA GCC ATT CCT TAT GAA ACT GCC AAG GCT TTT GCC GAT GAA ATC 407
119 E  N  R  A  I  P  Y  E  T  A  K  A  F  A  D  E  I 135

408 GGG ATT CCT TTT ATG GAG ACT AGT GCA AAA GAT GCT ACA AAC GTA GAA CAG 458
136 G  I  P  F  M  E  T  S  A  K  D  A  T  N  V  E  Q 152

459 GCT TTC ATG GCA ATG TCT GCA TCC ATC AAA GAG AGA ATG GCT AGC CAA CCA 509
153 A  F  M  A  M  S  A  S  I  K  E  R  M  A  S  Q  P 169

510 GCT GGG AAT AAT GCA AGA CCA CCG ACC GTG CAG ATC AGA GGA CAG CCT GTG 560
170 A  G  N  N  A  R  P  P  T  V  Q  I  R  G  Q  P  V 186

561 GCA CAG AAG AAC GGC TGC TGC TCA ACT TGA TTGACGTGAC CAGCCTAGCA 610
187 A  Q  K  N  G  C  C  S  T  * 195

611 ATATCCTTTC CAATCTTAGA ACACGTGTTT CTTCTTTTGA CTAGGCTCCA ATTCACTACT 670

671 ACTTGGTTTT TACACAACAT CCCCCAAAT CTGTCTCGTC TGTAATTAAG CTCCTTTGGT 730

731 TGGT 734

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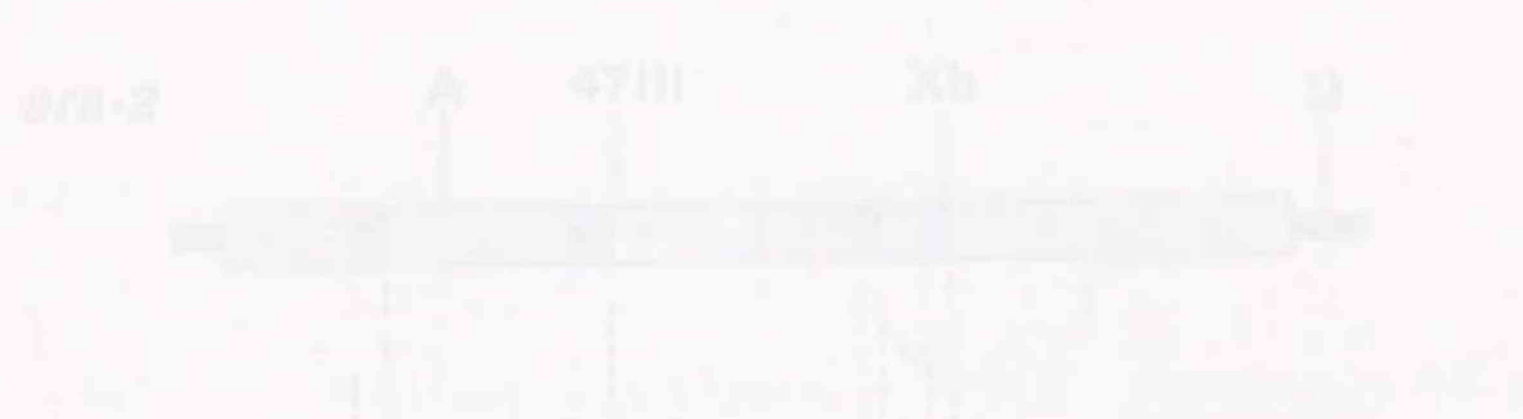
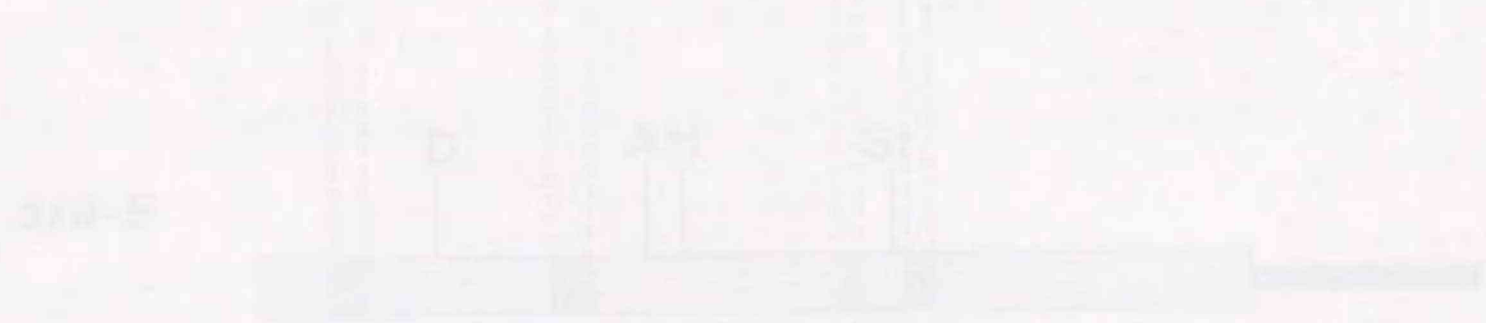
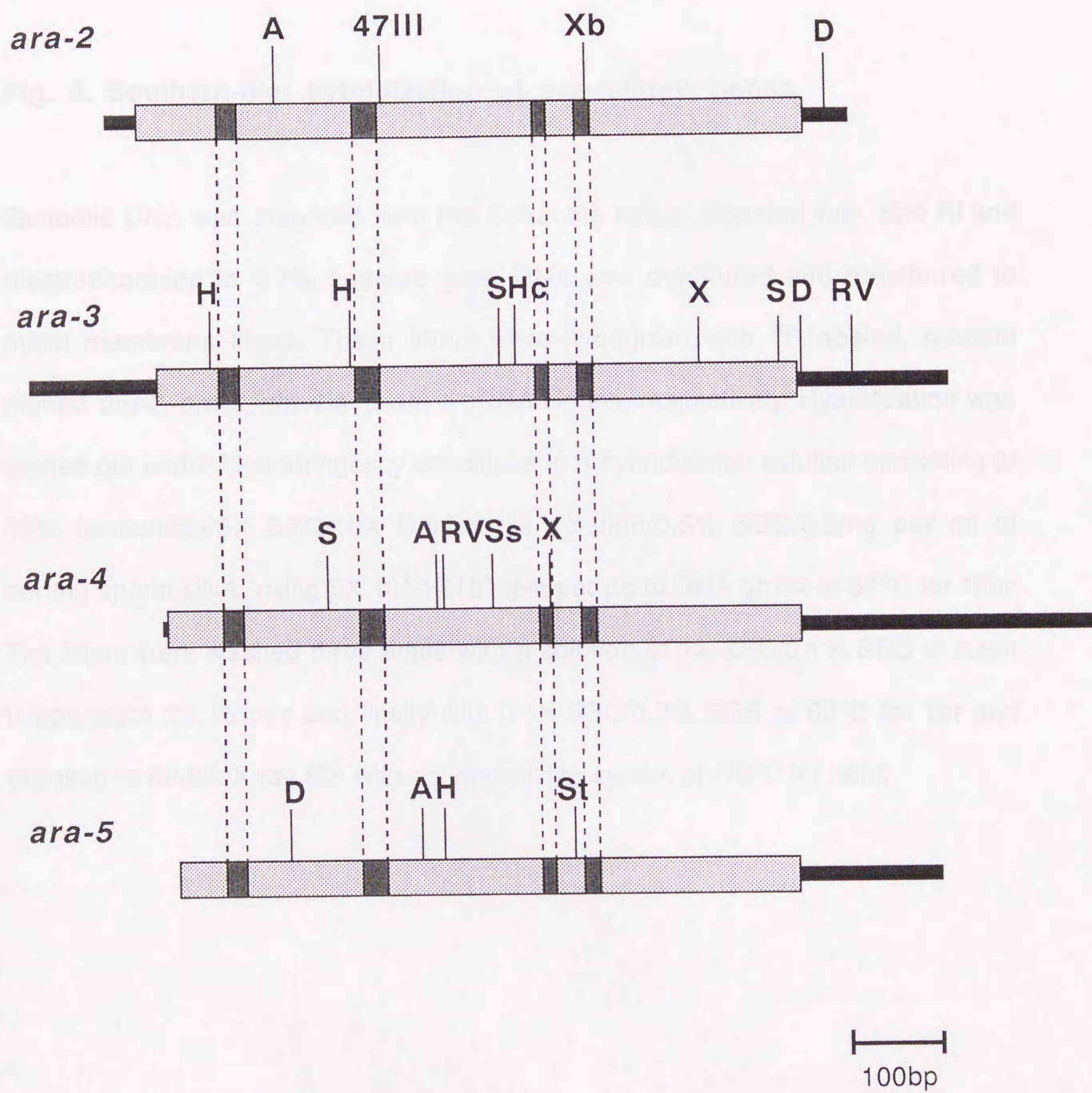



Fig. 3. Restriction maps of four *ara*-related cDNA clones, *ara-2*, *ara-3*, *ara-4* and *ara-5*.

The lightly shaded boxes represent the putative coding regions. Four regions common to all small GTP-binding proteins are represented by black boxes. Abbreviations: 47III, *Eco* 47III; A, *Acc* I; D, *Dra* I; H, *Hin* dIII; Hc, *Hinc* II; RV, *Eco* RV; S, *Sal* I; St, *Sty* I; Ss, *Sst* I; Xb, *Xba* I; X, *Xho* I.





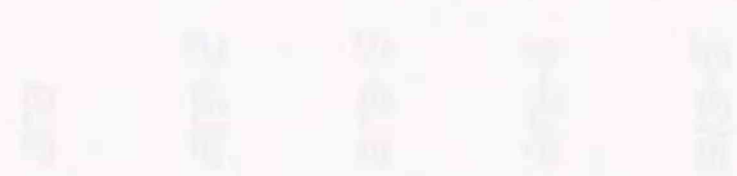


Fig. 4. Southern-blot hybridization of *ara*-related genes.

Genomic DNA was prepared from the Columbia strain, digested with *Eco* RI and electrophoresed in 0.7% agarose gels. DNA was denatured and transferred to nylon membrane filters. These filters were hybridized with 32 P-labeled, random primed *ara-2*, *ara-3*, *ara-4* and *ara-5* cDNA clones, respectively. Hybridization was carried out under high-stringency conditions in a hybridization solution consisting of 50% formamide/6X SSC/10X Denhardt's solution/0.5% SDS/0.2mg per ml of herring sperm DNA, using 5×10^8 - 1×10^9 cpm per μ g of DNA probe at 37°C for 12hr. The filters were washed three times with a solution of 1X SSC/0.1% SDS at room temperature for 15 min and finally with 0.1X SSC/0.2% SDS at 60°C for 1hr and exposed to Kodak X-ray film with an intensifying screen at -70°C for 24hr.

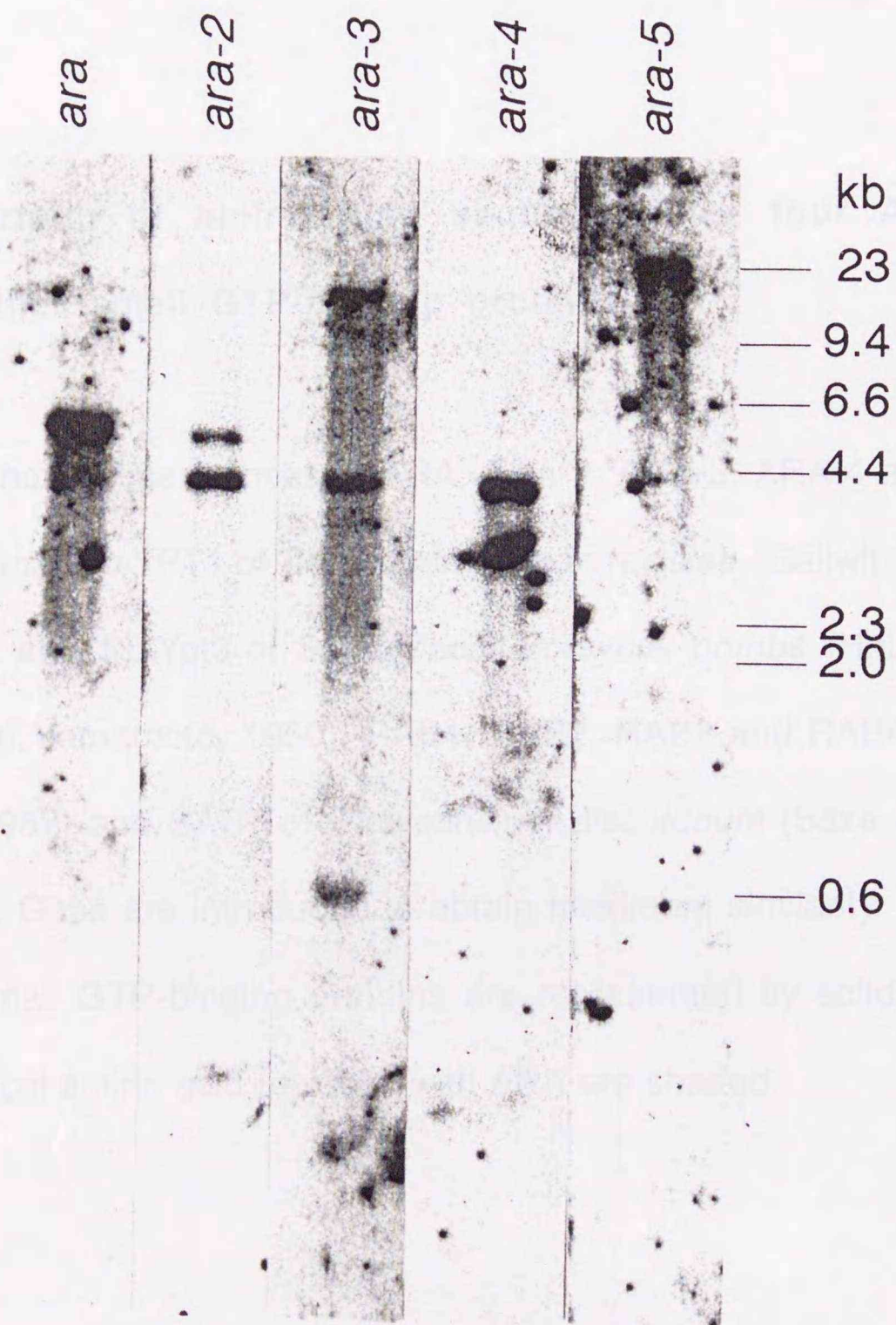


Fig. 5. Comparison of amino acid sequences for four ARA-related proteins and other small GTP-binding proteins.

The deduced amino acid sequences of ARA, ARA-2, ARA-3, ARA-4 and ARA-5 of *Arabidopsis thaliana*, Sc-YPT1 of *Saccharomyces cerevisiae* (Gallwitz et al., 1983) Sp-Ypt1, Sp-Ypt2 and Sp-Ypt3 of *Schizosaccharomyces pombe* (Haubruck et al., 1990; Miyake and Yamamoto, 1990), RAB1, RAB2, RAB3 and RAB4 of rat brain (Touchot et al., 1987) and SAS1 of *Dictyostelium discoideum* (Saxe and Kimmel, 1990) are shown. Gaps are introduced to obtain maximum similarity. Four regions common to all small GTP-binding proteins are represented by solid lines above sequences. Identical amino acid residues with ARA are shaded.

Part 1: Molecular cloning and characterization of cDNAs encoding small GTP-binding proteins of

Arabidopsis thaliana

ARA	MSSDDEGREBYLFRKIVVIGD	SAVGKSNLLSRYARNEFSAN	SKATIGVEFQTQSMIEGKE	60
ARA-2	MAGYRADEEYDYLFKLVLIGD	SGVGKSNLLSRFTKNEFNLE	SKSTIGVEFATKTTKVEGKV	61
ARA-3	MAAPPARARADYDYLIKLLLLIGD	SGVGKSNLLSRFSDGSFTTS	FITTIGIDFKIRTIELDGKR	63
ARA-4	MSDDDERGEREYLFKIVVIGD	SAVGKSNLLTRYARNEFNPN	SKATIGVEFQTQSMILIDGKE	60
ARA-5	VFKLLLLIGD	SGVGKSNLLSRFSDDSYVES	YISTIGVDFKIRTVEQDGKT	49
Sc-YPT1	MNSEYDYLFKLLLLIGD	TGVGKSNLLSRFSDDTYTND	YISTIGVDFKIKTVELDGKT	56
Sp-Ypt1	MNPEYDYLFKLLLLIGD	SGVGKSNLLRFADDTYTES	YISTIGVDFKIRTFELEGKT	56
Sp-Ypt2	MSTKSYDYLIKLLLLIGD	SGVGKSNLLRFSEDSFTPS	FITTIGIDFKIRTIELDGKR	57
Sp-Ypt3	MCQDEYDYLFKIVVIGD	SGVGKSNLLMRFTNEFNIE	SKSTIGVEFATRNIVLDNKK	58
Rab1	MSSMNPYDYLFKLLLLIGD	SGVGKSNLLRFADDTYTES	YISTIGVDFKIRTIELDGKT	59
Rab2	MAYAYLFKYIIIGD	TGVGKSNLLQFTDKRFQPV	HDLTMGVEFGARMITIDGKQ	54
Rab3	MASATDSRYGQKFSSDNQFDYMFKILIIGN	SSVGKTSFLFRYADDSFTPA	FVSTVGIDFKVKTIYRNDKR	70
Rab4			EFQKIINVGGKY	13
SAS1	MTSPATNKSAAYDYLIKLLLLIGD	SGVGKSNLLRFSEDSFTPS	FITTIGIDFKIRTIELEGKR	63
ARA	VKAQIWDTAGQERFRAVISA	YYRGAVGALVVDITRRITF	ESVGRWLDELKIHSDTIVAR	120
ARA-2	VKAQIWDTAGQERYRAITSA	YYRGAVGALLIYDVTRHATF	ENAAARWLRELRGHTDPNIVV	121
ARA-3	IKLQIWDTAGQERFRTITTA	YYRGAMGILLVYDVTDESSF	NNIRNWIRNIEQHASNIVN	123
ARA-4	VKAQIWDTAGQERFRAVISA	YYRGAVGALVVDITRSSTF	ENVGRWLDELNTHSDTIVAK	120
ARA-5	IKLQIWDTAGQERFRTITSS	YYRGAGHIIIVYDVTDEESF	NNVKQWLSEIDRYASDNVVK	109
Sc-YPT1	VKLQIWDTAGQERFRTITSS	YYRGSHGIIIVYDVTQDES	NGVKMWLQSEIDRYATSTVLK	116
Sp-Ypt1	VKLQIWDTAGQERFRTITSS	YYRGAGHIIIVYDVTQDSE	NNVKQWLQSEIDRYAVEGVNR	116
Sp-Ypt2	IKLQIWDTAGQERFRTITTA	YYRGAMGILLVYDVTDKKSF	DNVRTWFSNVEQHASENVYK	117
Sp-Ypt3	IKAQIWDTAGQERYRAITSA	YYRGAVGALIVYDITKQSSF	DNVGRWLKELREHADSNIIV	118
Rab1	IKLQIWDTAGQERFRTITSS	YYRGAGHIIIVYDVTQDES	NNVKQWLQSEIDRYASENVVK	119
Rab2	IKLQIWDTAGQESFRSITRS	YYRGAGALLVYDITRRITF	NHLTTWL EDARQHSNSNMVI	114
Rab3	IKLQIWDTAGQERYRTITTA	YYRGAMGFILMYDITNEESF	NAVQDWSTQIKTYSWDNAQV	130
Rab4	VKLQIWDTAGQERFRSVTTS	YYRGAGALLVYDITSRETY	NALTNWLT DARM LASQNIVI	73
SAS1	IKLQIWDTAGQERFRTITTA	YYRGAMGILLVYDVTDEKSF	GNIRNWIRNIEQHATDSVVK	123
ARA	MLVGNKCDLENIRAVSVVEEG	KALAEFE-GLFFVETSALDS	TNVKTAFEMVILDIYNNVSR	179
ARA-2	MLIGNKCDLRHLVAVSTEEA	KAFARE-GLYFMETSALDA	TNVENAFTEVLTQIHKIVSK	180
ARA-3	ILVGNKADMDESKRAVPTAK	GQALADEYGIKFFETSAKTN	LNVEEVFFSIGRDIKQRLSD	183
ARA-4	MLIGNKCDLESIRAVSVVEEG	KSLAESE-GLFFMETSALDS	TNVKTAFEMVIREIYSNISR	179
ARA-5	LLVGNKSDLTENRAIPYETA	KAFADFI-GIPFMETSADKA	TNVEQAFMAMSASIKERMAS	168
Sc-YPT1	LLVGNKCDLKDKRVVEYDVA	KEFADAN-KMPFLETSALDS	TNVEDAFLTMARQIKESMSQ	175
Sp-Ypt1	LLVGNKSDMVDKVVVEYSVA	KEFADSL-NIPFLETSAKDS	TNVEQAFLTMSRQIKERMGN	175
Sp-Ypt2	ILIGNKCDCEQDQVSFEQG	QALADEL-GVKFLESAKTN	VNVDEAFFTLAREIKKQKID	176
Sp-Ypt3	MLVGNKTDLLHLRAVSTEEA	QAFAAEN-NLSFIETSAMDA	SNVEEAFQTVLTEIFRIVSN	177
Rab1	LLVGNKCDLTTKKVVDYTTA	KEFADSL-GIPFLETSAKNA	TNVEQSFMTMAAEIKKRMGP	178
Rab2	MLIGNKSDLESRRVKKKEEG	EAFAREH-GLIFNETSAKTA	SNVEEAFINTAKEIYEKIQE	173
Rab3	LLVGNKCDMEDERVVSSERG	RQLADHL-GFEFFEASAKDN	INVKQTFERLVDVICEKMSE	189
Rab4	ILCGNKCDLDADREVTFLEA	SRFAQEL-ELMFLETSALTG	ENVEEAFMQCARKILNKIES	132
SAS1	MLIGNKCDMAEKKVVDSSRG	KSLADEY-GIKFLETSAKNS	INVEEAFISLAKDIKKRMID	182
ARA	KQLNSDTYKDELTVNRV----	SLVKDDNSASKQSSGFSCCSST		218
ARA-2	RSVDGGGESADLPKGGE----	TINVKEDGSVLKRMG--CCS-N		216
ARA-3	TDSRAEPATIKISQTDQ----	AAGAGQATQKSA-----CCG-T		216
ARA-4	KQLNSDSYKEELTVNRV----	SLVKNENEGTKT---FSCCS-R		214
ARA-5	QPAGNNARPPTVQIRGQ----	PVAQKNG-----CCS-T		196
Sc-YPT1	QNLNETTQKKEDKGNVN----	LKGQSLTNTGGG-----CC		206
Sp-Ypt1	NTFASSNAKSSVKVGQG----	TNVSQSSSN-----CC		203
Sp-Ypt2	AENEFNSQANNVDLGND----	RTVKKR-----CC		200
Sp-Ypt3	RSLEAGDDGVHPTAGQT--L	NIAPTMDNLNKKKSSSQCC		214
Rab1	GATAGGAEKSNVKIQST----	-----PVKQSGGGCC		205
Rab2	GVFDINNEANGIKIGPQHAA	TNASHGGNQGQGGQAGGGCC		212
Rab3	SLDTADPAVTGAKQGPQ----	----LTDQQAAPHQDCAC		220
Rab4	GELDPERMGSIGYGDAAALR	QLRSPRRTQAPSAQECGC		170
SAS1	TPNEQPQVVQPGTNLGA----	-----NNNKKKACC		208

Table 1. Calculation of similarity among various small GTP-binding proteins

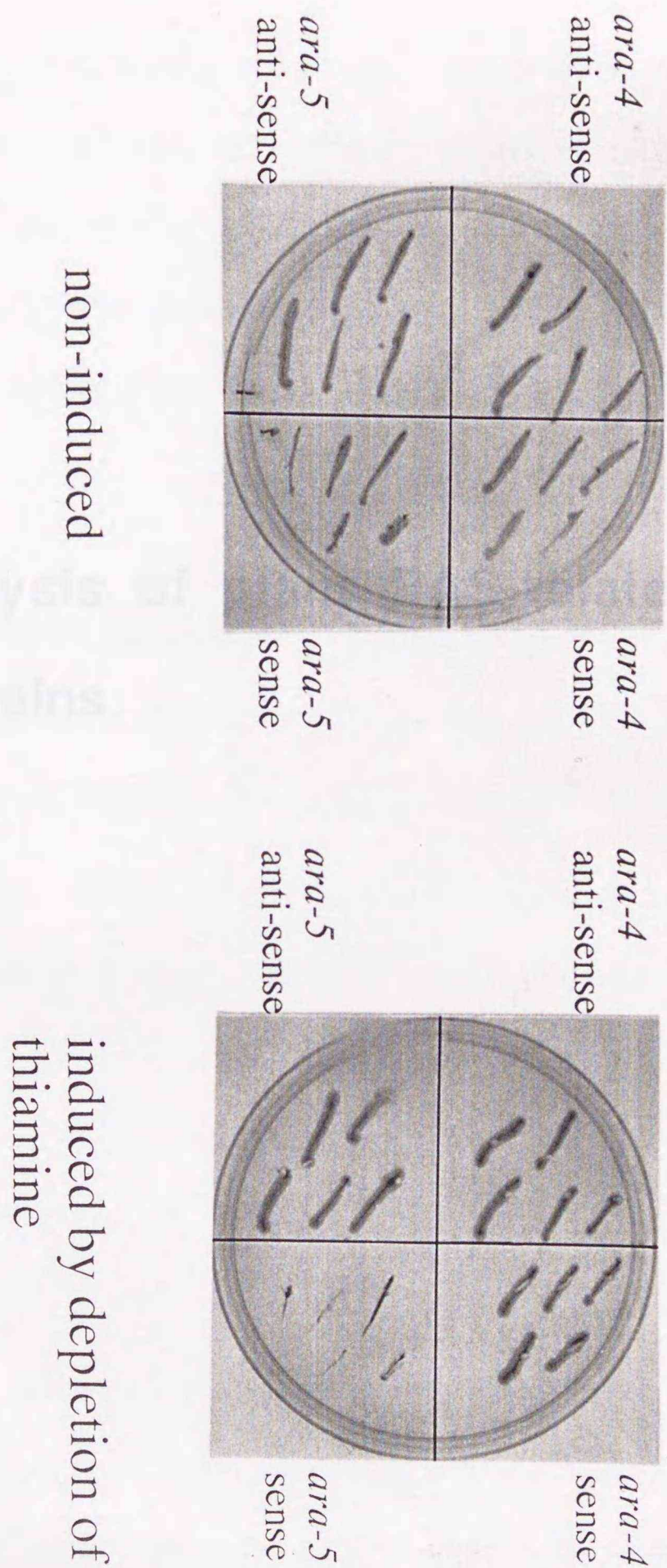
^a Proteins are ARA-2, ARA-3, ARA-4, ARA-5 and ARA of *Arabidopsis thaliana*, Sc-YPT1 (Gallwitz et al., 1983), Sc-RAS1, Sc-RAS2 (Powers et al., 1984) and Sc-SEC4 (Salminen and Novick, 1987) of *Saccharomyces cerevisiae*, Sp-Ypt1, Sp-Ypt2, Sp-Ypt3 (Haubruck et al., 1990; Miyake and Yamamoto, 1990) and Sp-RAS (Fukui and Kaziro, 1985) of *Schizosaccharomyces pombe*, RAB1, RAB2, RAB3 and RAB4 of rat brain (Touchot et al., 1987) and SAS1 of *Dictyostelium discoideum* (Saxe and Kimmel, 1990).

^b The amino acid sequence similarity between ARA-related proteins and other small GTP-binding proteins was analyzed using SEQFP or SEQHP of UWGCG (University of Wisconsin Genetics Computer Group) or IDEAS programs (Kanehisa, 1984) on a VAX minicomputer in the Institute of Medical Science, University of Tokyo.

Proteins	Percent similarity				
	ARA-2	ARA-3	ARA-4	ARA-5	ARA
ARA-2					
ARA-3	42.9				
ARA-4	56.1	40.2			
ARA-5	45.3	59.4	41.7		
ARA	56.5	39.7	83.8	44.6	
<i>Sc</i> -YPT1	45.7	51.0	44.8	64.8	45.1
<i>Sp</i> -Ypt 1	45.5	54.1	42.8	68.8	43.6
<i>Sp</i> -Ypt 2	43.7	69.7	45.2	54.6	42.6
<i>Sp</i> -Ypt 3	62.9	46.2	59.1	52.2	52.8
Rab 1	45.7	59.3	43.4	74.1	41.3
Rab 2	45.7	47.3	46.7	46.4	43.3
Rab 3	35.6	48.3	36.9	45.6	43.8
Rab 4	46.3	43.0	40.4	39.9	38.2
<i>Sc</i> -SEC4	39.9	52.3	38.9	51.8	41.5
<i>Sc</i> -RAS1	33.7	32.8	31.5	33.3	30.7
<i>Sc</i> -RAS2	32.1	31.2	33.3	34.8	34.2
<i>Sp</i> -Ras	34.5	34.0	32.8	32.4	32.4
SAS1	45.9	70.6	48.8	56.9	48.2

Fig. 6. Expression of *ara*-related genes in *Schizosaccharomyces pombe*.

ara-4 and *ara-5* genes were introduced to *Schizosaccharomyces pombe*. These genes were expressed by a promoter regulated by thiamine condition. *ara-4* sense; *ara-4* gene in a sense orientation to the promoter, *ara-4* anti-sense; *ara-4* gene in an anti-sense orientation to the promoter; *ara-5* sense; *ara-5* gene in a sense orientation, *ara-5* anti-sense; *ara-5* gene in an anti-sense orientation. *Schizosaccharomyces pombe* cells were grown in SSA medium plus adenine with (non-induced) or without (induced) 2 μ M of thiamine.



Materials and Methods

Cell cultures

Transgenic tobacco plants (*Nicotiana glauca* L. cv. Petite Havana 581) expressing the chimera gene of CaMV 35S promoter (Gardner et al., 1981) and *gusA* were used for the induction of callus (Fig. 1A). Suspension cultured cells were maintained in MS liquid medium (Murashige and Skoog, 1962) containing 3 mg/L 2,4-D and 0.5 mg/L 6-BA at 25°C.

Part 2:

Subcellular analysis of plant RAS-related small GTP-binding proteins

Cells (Fig. 1B) were harvested into R. cell strain D-100. Transformed cells were kept at 20°C until the OD value reached 0.5-0.6, followed by the addition of IPTG (0.1 mM final concentration). After 16 hr, cells were harvested and GST-RAS fusion proteins were purified with glutathione Sepharose column chromatography (Pharmacia LKB, Uppsala, Sweden).

Subcellular fractionation

Total 200 µg cell-suspension cultured cells were homogenized with the grinding buffer (50 mM HEPES-KOH pH 8.0, 250 mM sucrose, 5 mM EDTA, 100 mM PMSF, 10 µM leupeptin and 10 µM pepstatin A) and passed through 4 layers of cheesecloth. The filtrate was separated with differential centrifugation (1,000 ×g, 5 min; 3,000 ×g, 10 min; 10,000 ×g, 20 min and 100,000 ×g, 1 hr).

Materials and Methods:

Cell cultures

Transgenic tobacco plants (*Nicotiana tabacum* L. cv. Petite Havana SR1) possessing the chimeric gene of CaMV 35S promoter (Gardner et al., 1981) and *ara* cDNA were used for the induction of callus (Fig. 1A). Suspension cultured cells were maintained in MS liquid medium (Murashige and Skoog, 1962) containing 2mg/l 2, 4,-D and 0.2mg/l kinetin at 25°C.

ARA proteins preparation

Full-length *ara-2* and *ara-4* cDNA fragments (this paper) fused to the 3' end of GST gene in pGEX expression vector (Smith and Johnson, 1988). Recombinant plasmids (Fig. 1B) were transformed into *E. coli* strain DH5 α F'. Transformed cells were kept at 20°C until the OD₆₀₀ value reached 0.4 to 0.5, followed by the addition of IPTG (0.1mM final concentration). After 16 hr, cells were harvested and GST-ARA fusion proteins were purified with Glutathione-Sepharose column chromatography (Pharmacia LKB, Uppsala, Sweden).

Subcellular fractionation

Three day-old-suspension cultured cells were homogenized with the grinding buffer [50 mM HEPES-KOH (pH 8.0), 330 mM sorbitol, 2 mM EDTA, 100 μ M PMSF, 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A] and filtered through 4 layers of cheese cloths. The filtrates were separate with differential centrifugations (1,000 Xg, 5min; 3,000 Xg, 10min; 10,000 Xg, 20min and 100,000 Xg, 1hr).

The 100,000 Xg pellet was suspended in the grinding buffer containing different concentrations of salts or detergents. After 1 hr treatment on ice bath, proteins were separated by the high speed centrifugation (100,000 Xg, 1 hr) to the pellet and the supernatant fraction.

Antibody preparation and Immuno-blot analysis

Monoclonal antibodies were raised against purified GST-ARA proteins (Fig. 2). Antibodies were purified with protein A affinity column chromatography (Ampure PA Kit; Amersham Japan, Tokyo, Japan). Proteins from each fraction suspended in the Laemmli loading buffer (Laemmli, 1970) were boiled for 5 min. After a centrifugation (10,000 Xg, 5 min), supernatants were applied to the 15 % SDS-PAGE, followed by blotting to PVDF membrane (Immobilon-P: Millipore, MA, USA) (Towbin et al., 1979). Membranes were incubated in the blocking buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 5 % skimmilk] for 30 min, followed by 30 min incubation in the TBS-T buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.5 % Triton X-100] containing purified antibodies. After three washes with the TBS-T buffer, the membranes were incubated in the TBS-T buffer containing alkaline phosphatase conjugated anti-mouse antibody (Jackson, PA, USA) for 30 min. After washing the membrane, ARA proteins were visualized with NBT and BCIP.

Results and Discussion:

Subcellular fractionation

Using 3 day-old-suspension cultured cells of transgenic tobacco plants expressing *ara-4* gene, cell homogenates were fractionated by the differential centrifugation. As seen in Fig.4, immunological detection of ARA-4 protein was seen in 3,000 Xg, 10,000 Xg and 100,000 Xg pellets. This result clearly suggests that ARA-4 protein locates mainly in the membranous fraction of organella and / or plasma membrane.

Solubilization of proteins from membranes

To see whether or not membrane associated ARA-4 protein is integrated into the membrane or anchored to the membrane surface, 100,000 Xg pellet fraction was treated with NaCl, sodium deoxycholate, NP-40 and Urea. After treatments with such reagents, proteins were separated by a centrifugation (100,000 Xg, 1hr). As seen in Fig. 5 (A) and (B), the detergent such as sodium deoxycholate and NP-40 released ARA-4 protein from membranous fraction to the supernatant fraction. Neither NaCl nor Urea at high concentrations did affect to release ARA-4 protein to the supernatant. In as much as ARA proteins have a putative fatty acid modification sequence in the C-terminal (this paper), ARA proteins may locate on the surface of membrane, possibly by lipid linkage.

Presence of ARA proteins in A.thaliana

In order to confirm whether the observed instances in transgenic tobacco cells which over-produce ARA proteins were also true in *A.thaliana*, the similar

experiments were carried out. The result indicated that ARA-4 protein was clearly seen in the membrane fraction of the same plants (Fig.6). Similar results were also obtained using monoclonal antibodies against ARA-2 protein. The ARA proteins showed the highest similarities with YPT / RAB sub-family proteins among the small GTP-binding proteins. The products of *YPT* and *rab* gene family members showing conserved GTP-binding domains may play a role in vesicular transport of proteins (Chavrier et al., 1990; Goud et al., 1990; Nigam et al., 1990; Gorvel et al., 1991). Hence, it is likely that ARA proteins may associate with organella membrane such as Golgi apparatus. Further investigation using the immuno-electron-microscopy to identify precise location of ARA proteins is in progress.

Fig. 1. Construction map of expression plasmids pBIN19-35S-*ara*-NOS and pGEX-*ara*-N.

a) The chimeric gene consisting of Cauliflower mosaic virus 35S promoter-*ara* gene was cloned into pBIN19. These plasmids were transferred into *Nicotiana tabacum* cv. SR1 via *Agrobacterium* mediated transformation.

b) The *ara*-cDNAs were cloned into the *Eco* RI site of pGEX (Smith and Johnson, 1988). Products of these cDNA were fused with GST protein.

Amp^r, ampicillin resistant gene. Km^r and NPTII, kanamycin resistant gene. tac-pro, fusion promoter of tryptophan-lac. 35S-pro, 35S promoter of Cauliflower mosaic virus (Gardner et al. 1981). NOS-ter, terminator of nopaline synthase gene (Bevan 1984).

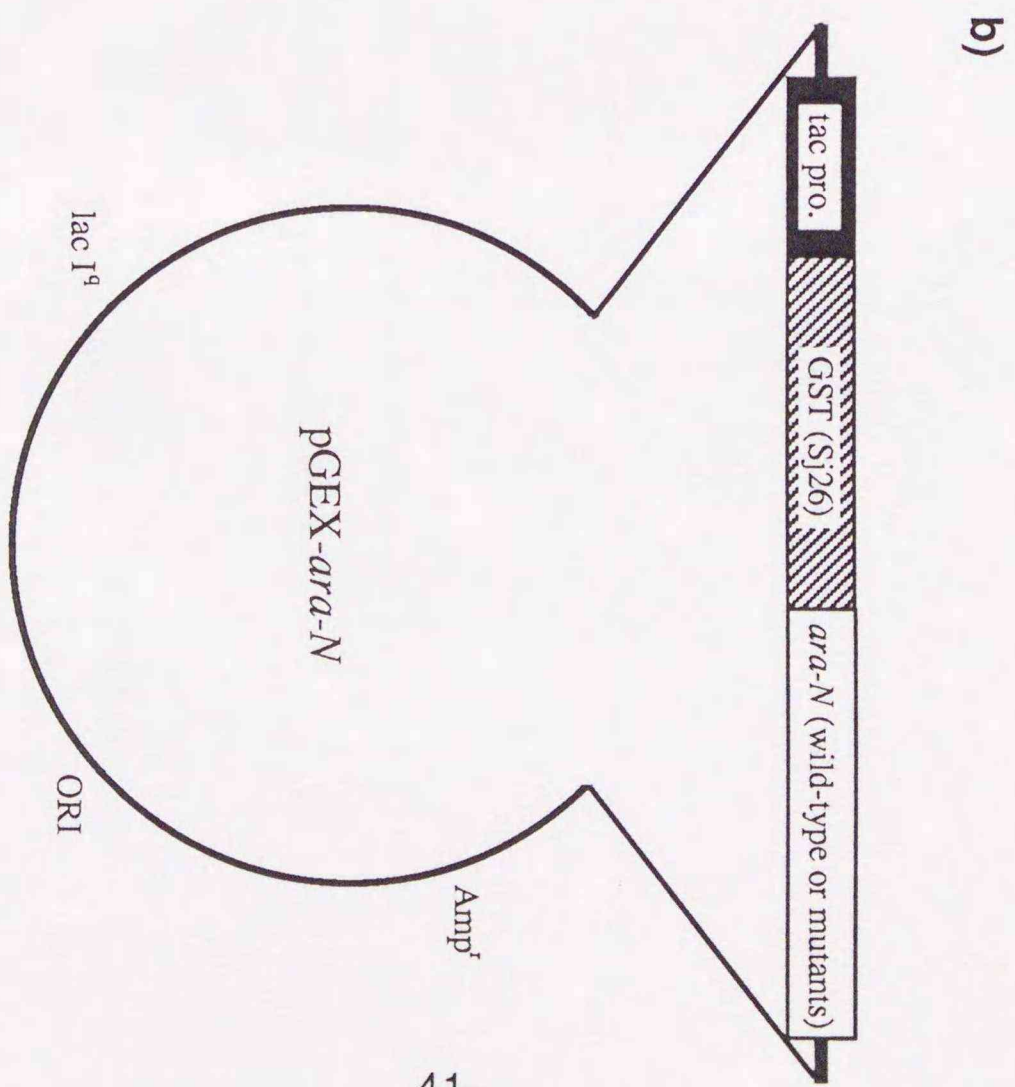
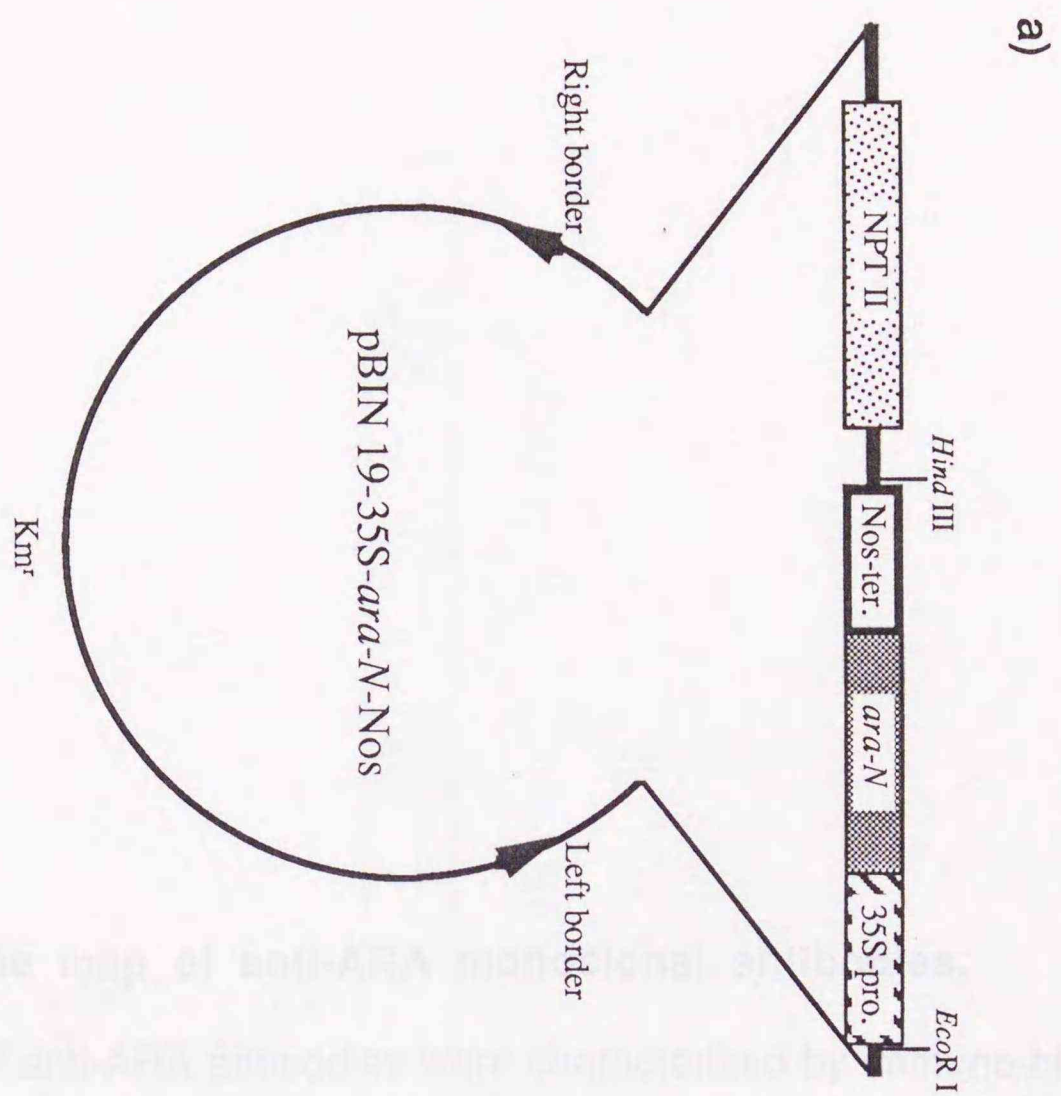
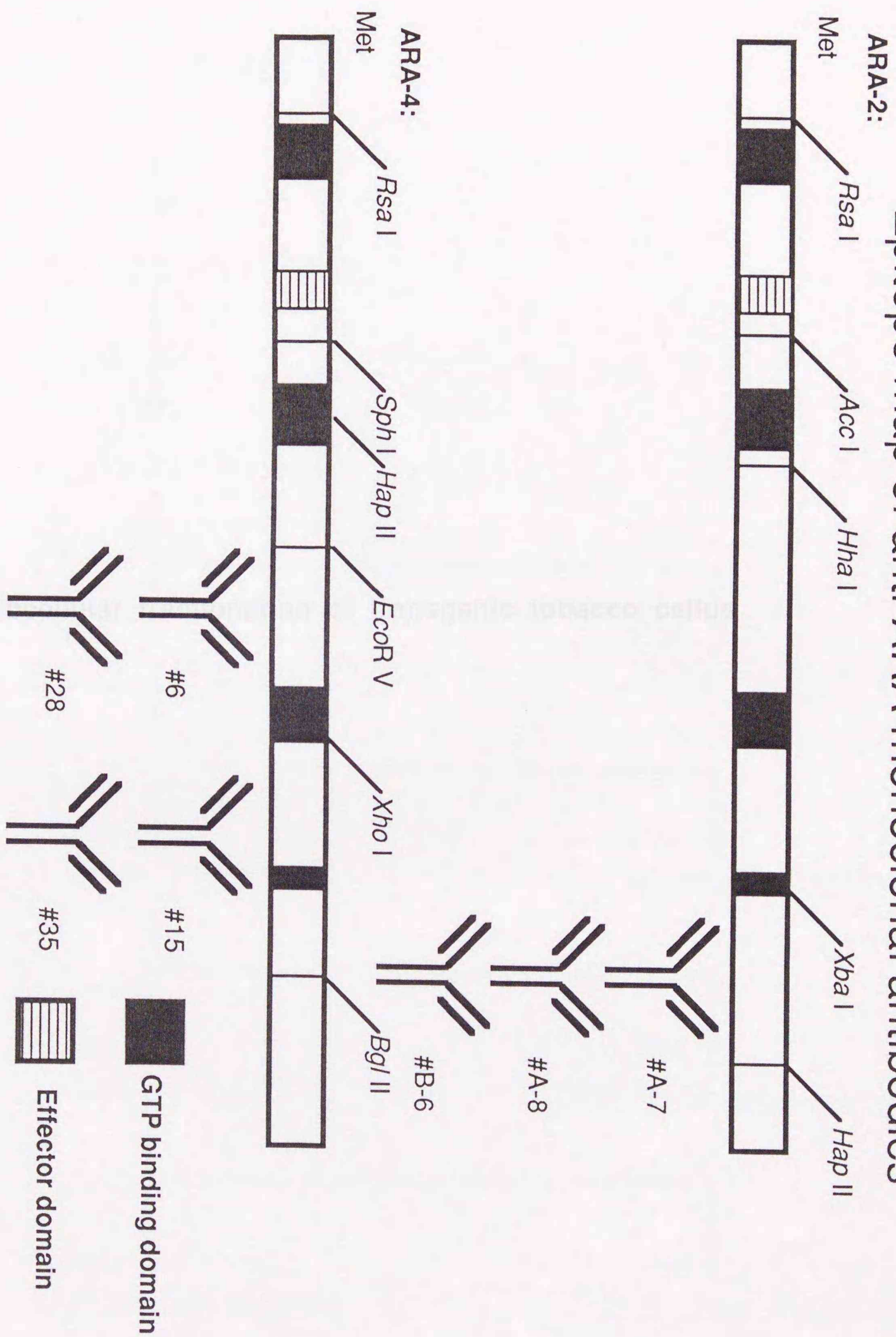


Fig. 2. Epitope map of anti-ARA monoclonal antibodies.

The epitopes of anti-ARA antibodies were characterized by immuno-blotting of ARA deleted clones.

Epitope map of anti-ARA monoclonal antibodies



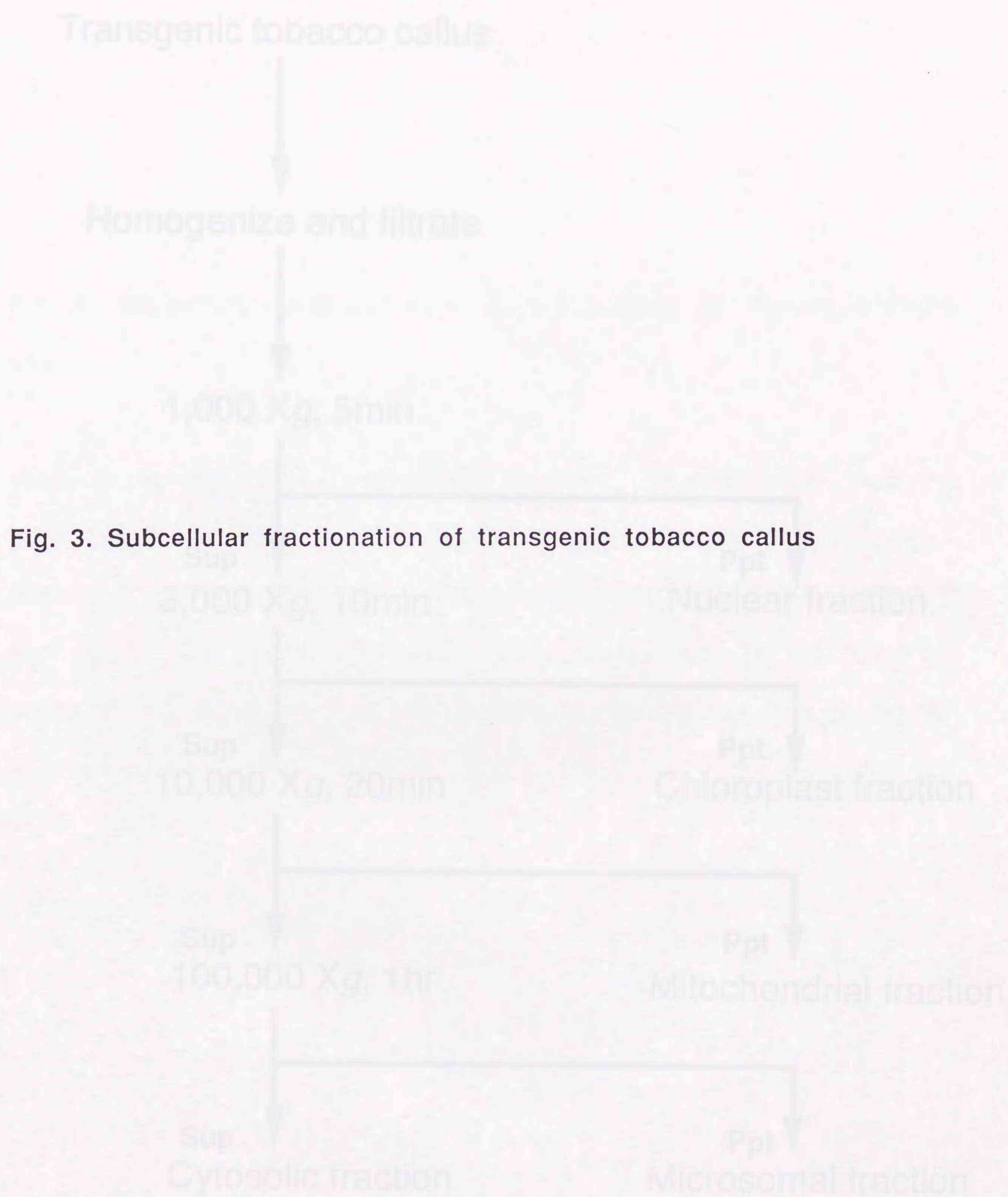
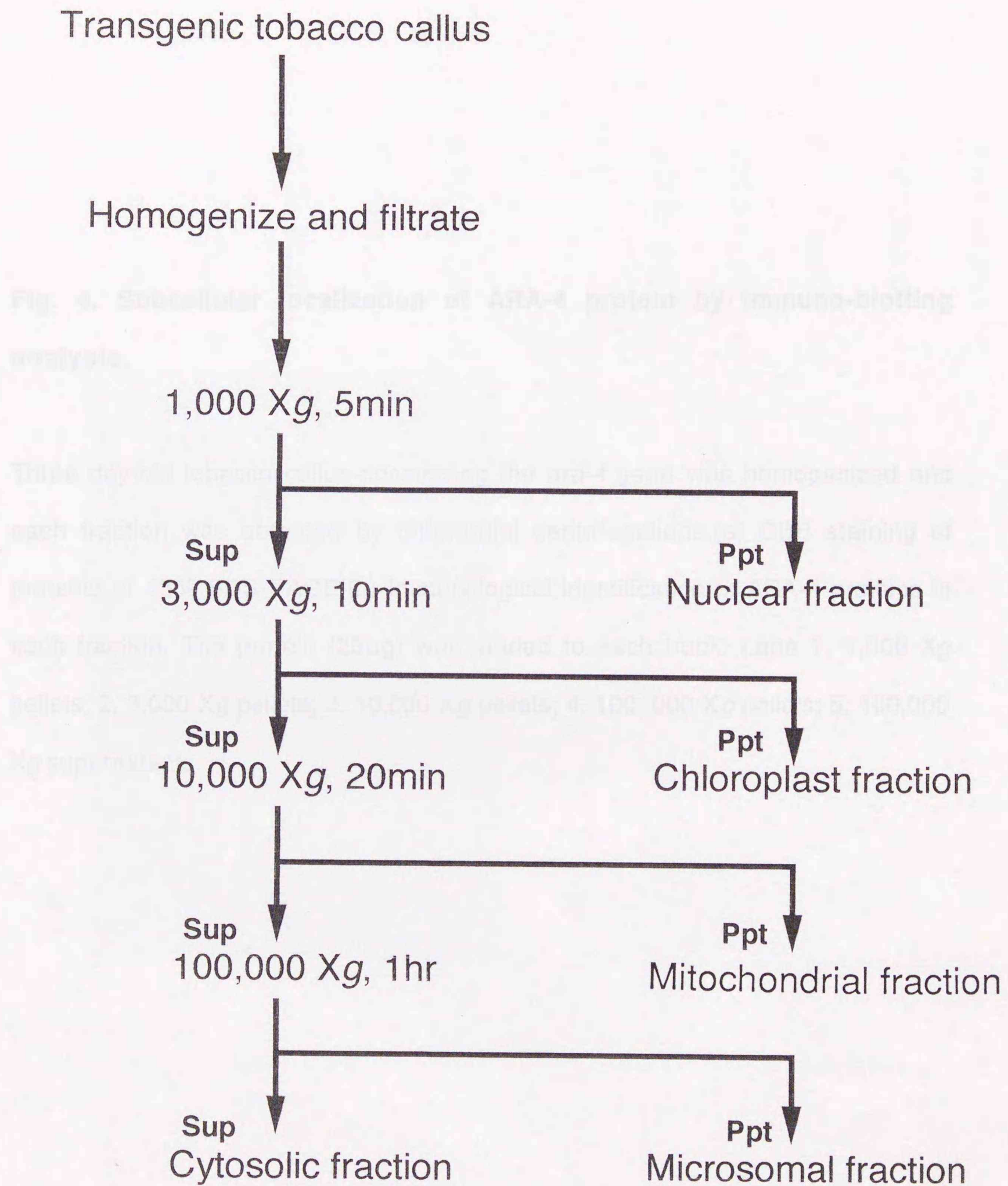


Fig. 3. Subcellular fractionation of transgenic tobacco callus



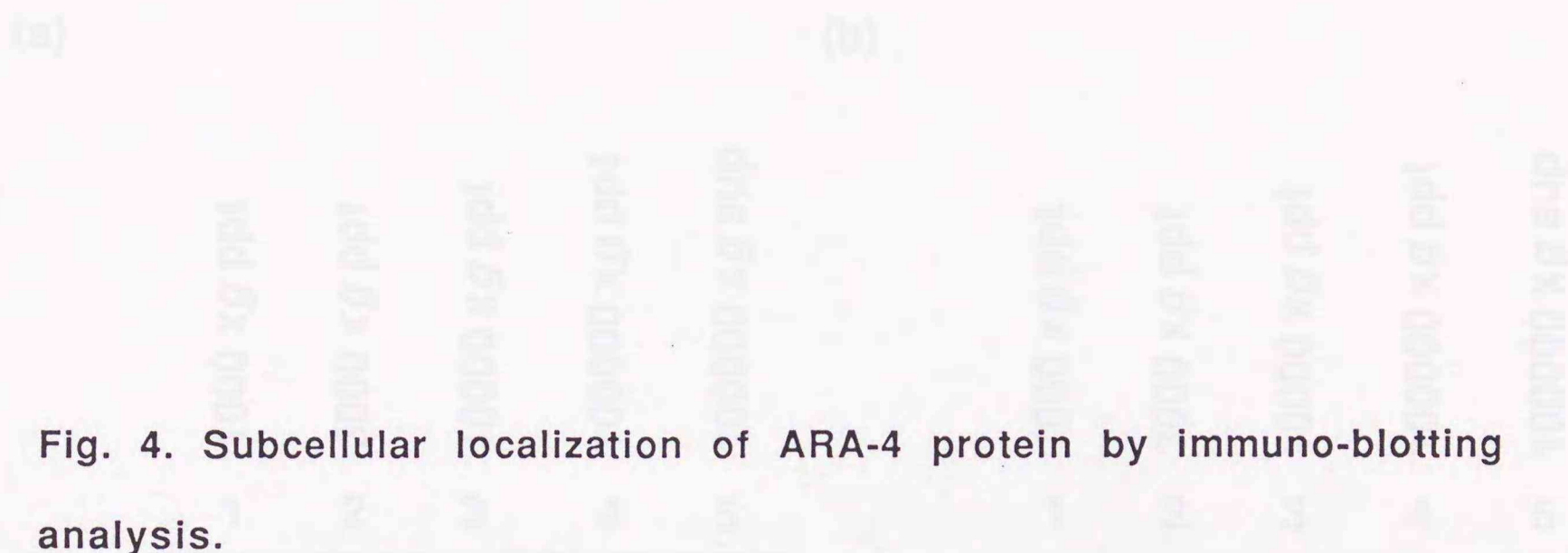
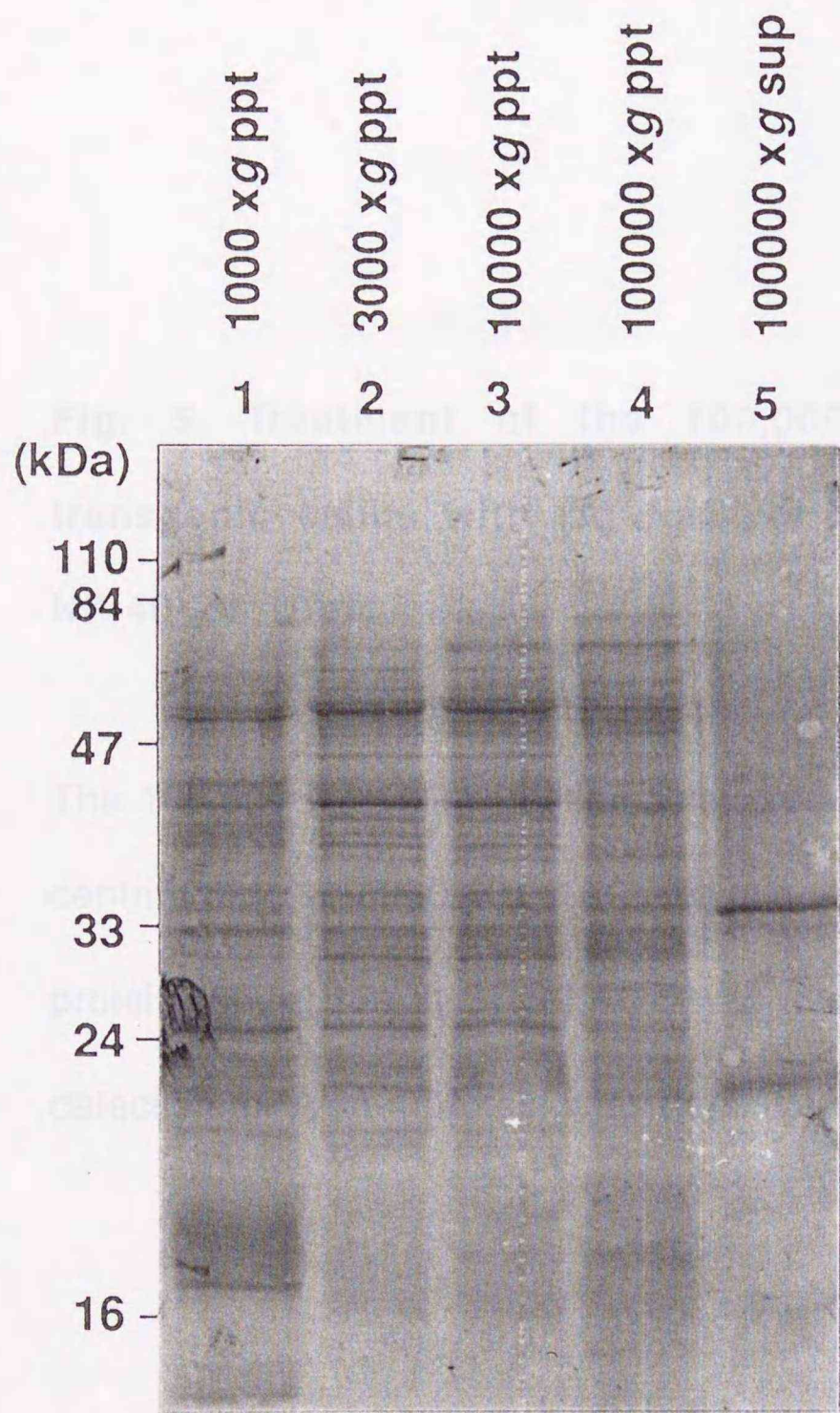


Fig. 4. Subcellular localization of ARA-4 protein by immuno-blotting analysis.

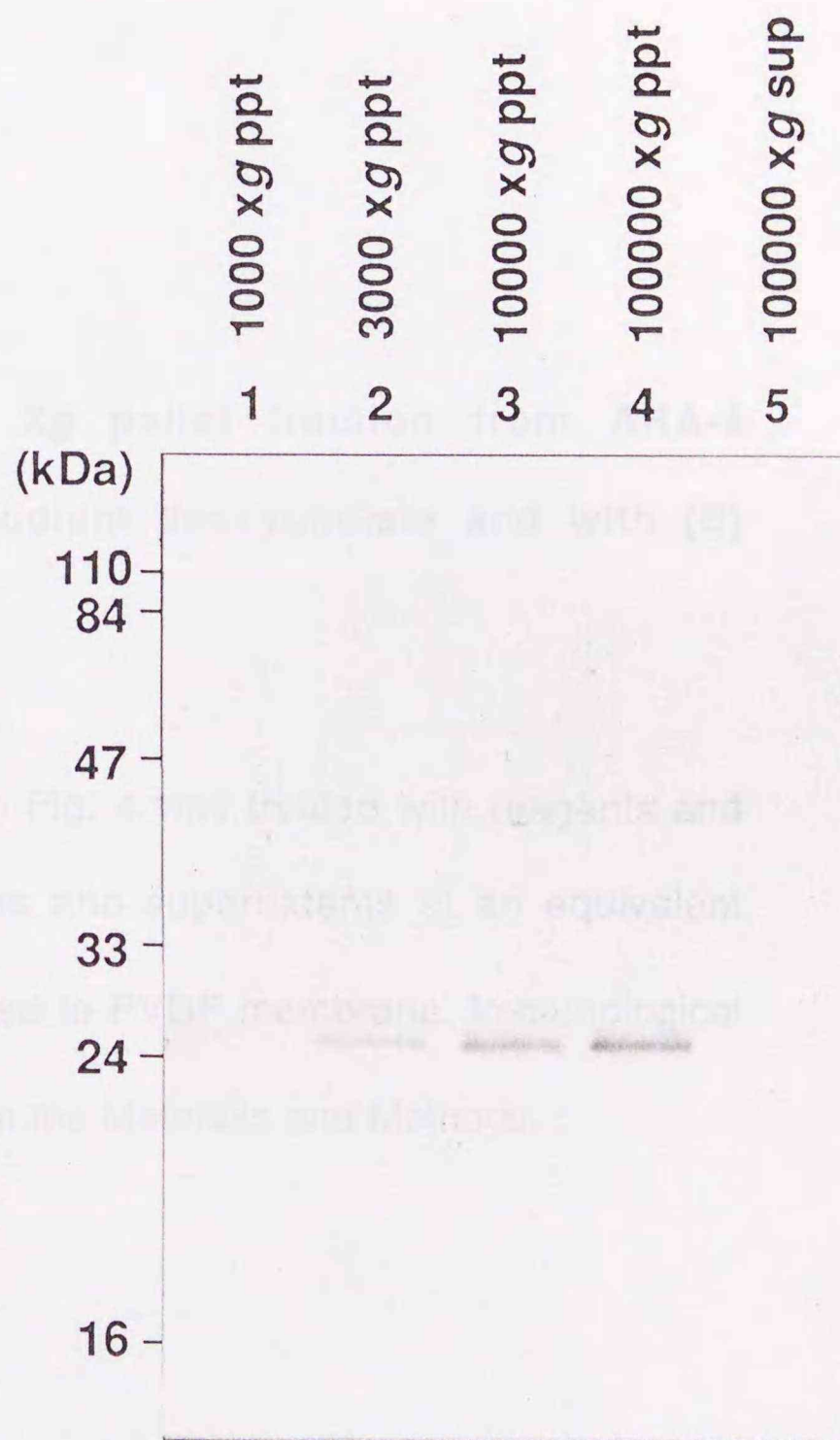
Three day-old tobacco callus possessing the *ara-4* gene was homogenized and each fraction was obtained by differential centrifugations.(a) CBB staining of proteins in 15% SDS-PAGE.(b) Immunological identification of ARA-4 proteins in each fraction. The protein (25µg) was loaded to each truck. Lane 1, 1,000 Xg pellets; 2, 3,000 Xg pellets; 3, 10,000 Xg pellets; 4, 100, 000 Xg pellets; 5, 100,000 Xg supernatants.

(a)



CBB stain

(b)

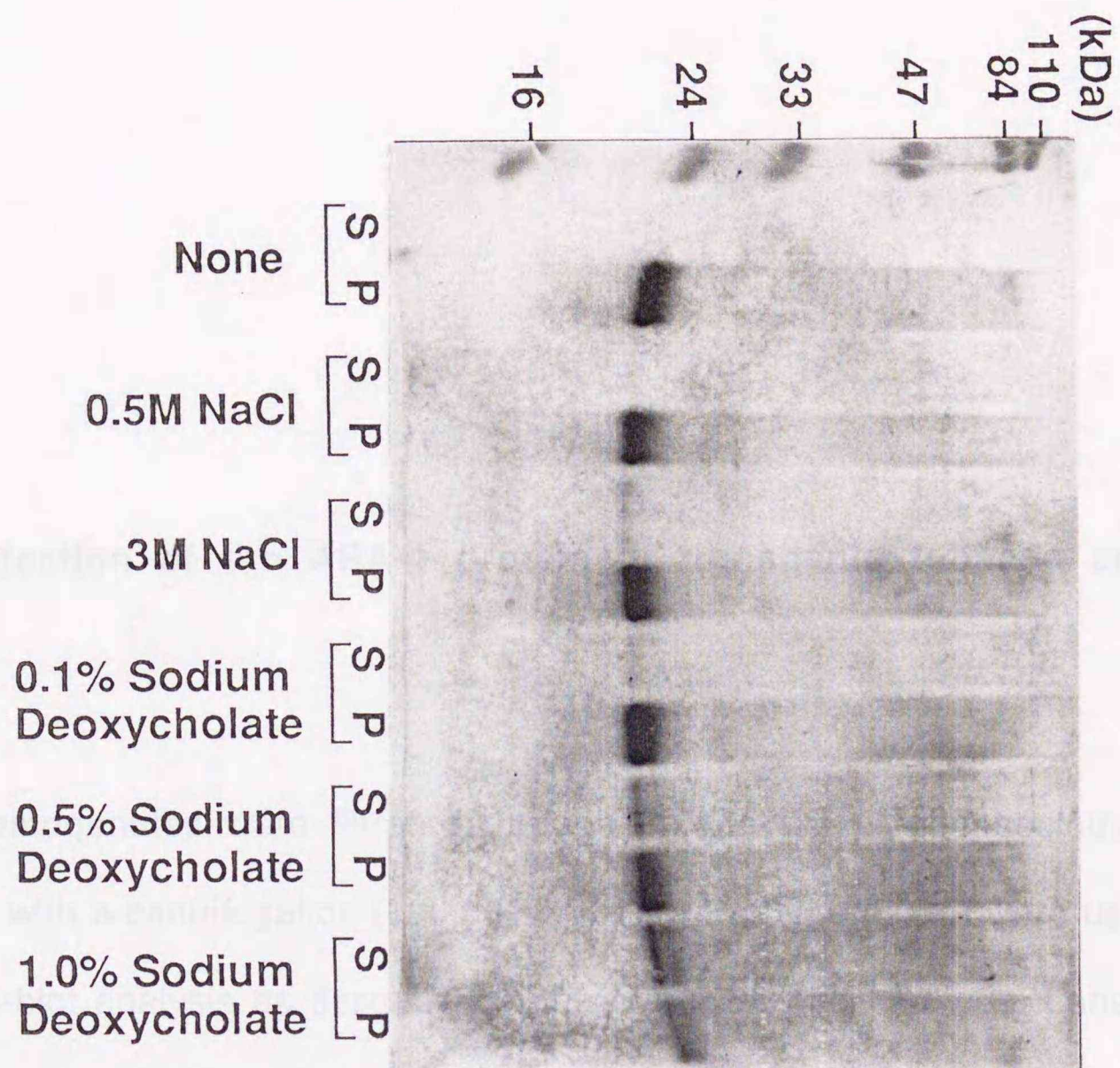


anti-ARA-4

Fig. 5. Treatment of the 100,000 Xg pellet fraction from ARA-4 transgenic callus with (A) NaCl or Sodium deoxycholate and with (B) NP-40 or Urea.

The 100,000 Xg pellet fraction illustrated in Fig. 4 was treated with reagents and centrifuged (100,000 Xg, 1hr). Both pellets and supernatants at an equivalent protein (50 μ g) run in SDS-PAGE was blotted to PVDF membrane. Immunological detection for ARA-4 protein is as described in the Materials and Methods.

(A)



(B)

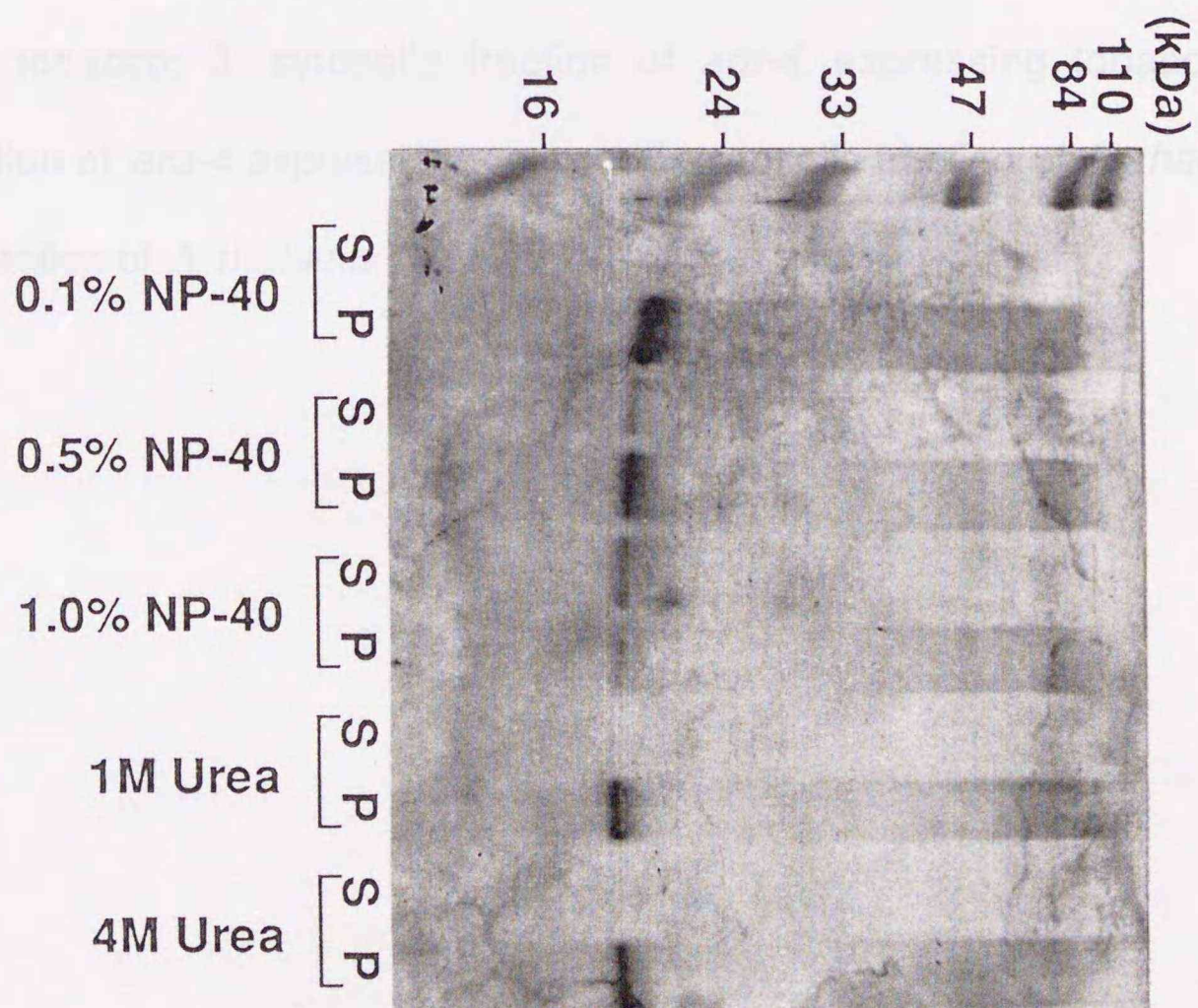


Fig. 6. Detection of the ARA-4 protein in transgenic tobacco and *A. thaliana*.

The leaf homogenates were filtrated through four layers of cheesecloths and fractionated with a centrifugation (100,000 Xg, 1hr). Proteins (50µg) were used for the Immuno-blot analysis as described in the Materials and Methods. Lanes: 1 , cytosolic fraction (S) of untransformed tobacco; 2, membrane fraction (P) of untransformed tobacco; 3, cytosolic fraction of *ara-4* expressing tobacco; 4, membrane fraction of *ara-4* expressing tobacco; 5, cytosolic fraction of *A. thaliana*; 6, membrane fraction of *A. thaliana*.

Part 2: Subcellular analysis of plant RAS-related small GTP-binding proteins



Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

Materials and Methods

Site-directed mutagenesis

Full-length *Arabidopsis thaliana* *rac-G* cDNA fragments (this paper) were cloned into pUC119 plasmid vector and *in vitro* mutagenesis was carried out by using Kunkel method (Kunkel *et al.*, 1985). The following oligonucleotide primers were used: 5'-CTGCTGCTTACAGGATAC-3' corresponding to 225 to 257b where A was changed to T (position 225 of *rac-G* gene); 5'-CTGATAGGATCAGTGGATC-3' corresponding to 400b to 431b where A was changed to T (position 125 of *rac-G* gene); 5'-CCCGCCGCTGTGTGAAAGTTG-3' corresponding to 205b to 225b where A was changed to T (position 125 of *rac-G* gene).

Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

Expression and purification of recombinant GTP-*rac* proteins

Wild-type and mutant *rac* cDNA fragments were cloned into pGEX-3 expression vector (Smith and Johnson, 1998). E. coli strain BL21 (DE3) was transformed with recombinant plasmids and GEX-3A₆ protein was added to E. coli with 0.1 mM IPTG for 18 hr at 20°C. Harvested cells were resuspended in PBS buffer and disrupted by sonication (Bioruptor, 300 W, 30 s intervals). After centrifugation, the supernatants in new tubes were mixed with Triton X-100 (1.5% final concentration). Cell extracts were loaded onto 150 µm Superose 6 column (Pharmacia LKB, Uppsala, Sweden) and washed with PBS buffer containing 0.5% Triton X-100.

Materials and Methods:

Site directed mutagenesis

Full-length *ara-2* and *ara-4* cDNA fragments (this paper) were cloned into pUC118 phagemid vector and *in vitro* mutagenesis was carried out by using Kunkel method (Kunkel T. A., 1985). The following oligo nucleotides were used: 5'-CTGCTGGTCTAGAGAGATAC-3' corresponding to 239b to 257b where A was changed to T (codon 72 of *ara-2* gene); 5'-CTTATAGGGATCAAGTGCGATC-3' corresponding to 400b to 421b where A was changed to T (codon 126 of *ara-2* gene); 5'-CCGCCGGTCTGGAACGTTC-3' corresponding to 206b to 226b where A was changed to T (codon 71 of *ara-4* gene) and 5'-CTTATTGGGATCAAATGTGATC-3' corresponding to 367b to 388b where A was changed to T (codon 125 of *ara-4* gene).

Expression and purification of recombinant GST-ARA proteins

Wild-type and mutant *ara* cDNA fragments were cloned into pGEX expression vector (Smith and Johnson, 1988). *E. coli* strain DH5 α F' was transformed with recombinant plasmids and GST-ARA proteins were induced in *E. coli* with 0.1mM IPTG for 16hr at 20°C. Pelleted cells were suspended in PBS buffer, and disrupted by sonication (3min with six 30sec intervals). After centrifugation, the supernatants in new tubes were added with Triton X-100 (0.5% final concentration). Cell extracts thus obtained were loaded onto Glutathione-Sepharose column (Pharmacia LKB, Uppsala, Sweden) and washed with PBS buffer containing 0.5% Triton X-100.

Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

Recombinant proteins were eluted with a buffer [50mM Tris-HCl(pH 8.0) and 5mM glutathione], followed by filtration with Sephadex G-50 column (Pharmacia LKB).

Nucleotide binding

GST-ARA-producing *E. coli* cells suspended in Laemmli loading buffer (Laemmli 1970) were boiled for 5min. After a centrifugation, supernatants were applied to 15% SDS-PAGE, followed by blotting to PVDF membrane (Towbin et al. 1979). Membranes were soaked in binding buffer [50mM Tris-HCl (pH7.5), 5mM MgCl₂, 0.3% Tween 20 and 0.5mM EDTA] for 30min, and incubated in the same buffer but containing 1nM [α -³²P] NTP (3000Ci/mM; ICN) for 1hr. Membranes were then washed three times with the binding buffer and visualized pictures were obtained by Fujix BAS 2000 imaging system (Fuji film, Tokyo, Japan) (Lapetina and Reep 1987) (Fig. 1).

GTP hydrolysis (GTPase) analysis

GTPase activity was measured by modified methods of Wagner et al. (Wagner, P., 1987). Recombinant proteins (10 μ g) were incubated at 37°C in 100 μ l of the GTPase reaction mixture [65mM Tris-HCl (pH7.5), 10mM MgCl₂, 0.5mM DTT, 1mM NaN₃, 5 μ M GTP, 33nM [α -³²P] GTP (3000Ci/mM; ICN) and 1mM ATP (CTP, UTP or GTP γ S)]. Ten μ l samples were collected at 1hr intervals followed by the addition of 10 μ l 0.5M EDTA to stop the reaction. Two μ l were spotted onto PEI-cellulose TLC plate (MACHERY-NAGEL), which was then developed in 0.5M KH₂PO₄ (pH 3.4). After drying, the plate was analyzed by Fujix BAS 2000 imaging system (Fig. 3).

Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

GST-ARA-bound GTP product analysis

Recombinant GST-ARA proteins were incubated at 30°C for 1hr in the GTPase reaction mixture, which was then added with 10 μ l 1M MgCl₂ and anti-ARA antibodies coated protein A-Sepharose or Glutathione-Sepharose. After incubated at 4°C for 1hr, the precipitate of a centrifugation (3,000 xg, 5min) was washed three times with washing buffer [50mM Tris-HCl (pH 7.5), 150mM NaCl and 100mM MgCl₂]. Binding nucleotides were eluted with 20 μ l of 0.5M EDTA (pH8.0) were applied to TLC as the same conditions for GTPase assay (Fig. 6).

Results:

GTP-binding ability

Using deleted mutants and those with point mutation in ARA-2 and ARA-4 cDNAs, GTP-binding experiment was carried out as shown in Fig. 2. The wild-type and some mutated proteins showed binding to [α - 32 P]GTP. Deletion up to the point of *Rsa*I site (10 amino acid for ARA-2, 11 amino acid for ARA-4 from the initiation codon) did not affect the GTP-binding whereas further deletions apparently diminished GTP-binding. Point mutation at a point of Gln (72 amino acid for ARA-2, 71 amino acid for ARA-4 from the initiation codon) didn't affect the GTP-binding ability, but mutation at Asn (126 for ARA-2, 125 for ARA-4 from the initiation codon) completely inhibited nucleotide binding.

GTP hydrolysis

GTP hydrolysis experiment was carried out for wild-type and point mutated GST-ARA proteins. As shown in Fig. 4 (A and B) both ARA-2 and ARA-4 showed hydrolysis of GTP to GDP but mutation at Asn site significantly inhibited which is presented in Fig. 4 (C and D). The estimated rate of GTP hydrolysis was about 0.006min^{-1} (GST-ARA-2^{WT}) or 0.002min^{-1} (GST-ARA-4^{WT}). Yeast YPT1 protein and mammalian *rab1B* showed the same rate as GST-ARA-2 protein (0.006min^{-1}) (Wagner et al., 1987; Touchot et al., 1989). The Gln->Leu substitution decreased the GTP hydrolytic activity about 60% level. The GTP hydrolytic rate of Asn->Ile substitution about 30% of wild-type hydrolysis rate. GST alone could not hydrolyze

Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

GTP. These results show that these point mutations affect the GTP hydrolysis activity as same as RAS and YPT proteins (Wagener et al., 1987; Bos, 1989).

Effect of nucleotides to GTP hydrolysis by ARA

Effect of nucleotide such as ATP, CTP, UTP and GTP- γ -S on GTP hydrolysis activities of GST-ARA proteins was studied. Results shown in Fig. 5 indicated that only GTP- γ -S (non-hydrolyzable analog of GTP) substantially inhibited GTP hydrolyzing reaction.

GST-ARA protein-guanine nucleotide complex analysis

In this experiment, after induction GST-ARA proteins with [α - 32 P]GTP, the same reaction mixture was treated with anti-ARA proteins monoclonal antibody-protein A-Sepharose complexes or glutathione-Sepharose. Immuno-precipitated sample was analyzed by TLC. The result in Fig. 7 shows that mutation at the point of Gln clearly induced the suppression of hydrolysis of GTP to GDP. In the case of mutation at the position of Asn, the amount of GTP held by the protein was substantially reduced. The result was consistent in different monoclonal antibodies and glutathione-Sepharose.

Discussion:

Present investigation was carried out to investigate biochemical properties of RAS-related proteins of plant origin. For this purpose I constructed GST-ARA fusion proteins, partially deleted mutant proteins and point mutated proteins. The results of GTP-binding experiments clearly indicate that conserved regions like GTP-binding common sequences (Box I to IV in Fig.2A) seem to be essential. Point mutation at 126 for ARA-2 and 125 for ARA-4 amino acid residues resulted in the inhibition of GTP-binding. Concerning GTPase activity of GST-ARA protein rate, of hydrolysis of GTP was greatly reduced in the point mutation Asn->Ile (Asn 126 for ARA-2, Asn125 for ARA-4). This region was known to act as guanine ring associated loop (Box III: Asn-Lys-Cys -Asp) (deVos et al., 1988). In the case of yeast YPT1 protein, the similar mutant showed dominant-lethally phenotype in yeast cells (Schmitt et al., 1986 and 1988) and no GTP-binding activities *in vitro* (Wagner et al., 1987; Touchot et al., 1988). Our experiment where ARA protein-nucleotide complexes were quantitated clearly showed that Gln position is important for hydrolysis of GTP to GDP. In mammalian RAS protein, similar mutation showed increase of GTP binding form due to decreased GTPase activities *in vivo* (Bos, 1989). Rate of GTPase activities in GST-ARA-2 and GST-ARA-4 was 0.006 min^{-1} and 0.0015 min^{-1} , respectively. Reported instances of rate of GTPase activities were followings: RAS; 0.02 min^{-1} (Gibbs et al., 1984), RHO; 0.1 min^{-1} (Anderson et al., 1987), YPT1; 0.006 min^{-1} (Wagner et al., 1987), SMG 25A; 0.04 min^{-1} (Kikuchi et al., 1988), RAB1A; 0.009 min^{-1} (Touchot et al., 1989) and RAB1B ; 0.006 min^{-1} (Touchot et al., 1989).

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Furthermore, specific inhibition of GTPase activities by GTP- γ -S in ARA protein was confirmed. These results clearly indicated that plant RAS-related proteins possess similar biochemical properties to YPT/RAB type proteins with respects to GTP-binding and hydrolysis activities. Proposed model for the biochemical properties of plant ARA proteins is illustrated in Fig. 8. It will be important to assess biological properties of ARA proteins *in vivo* conditions.

Fig. 8. The model illustrating GTP-binding analysis.

Fig. 1. The method illustrating GTP-binding analysis.

GTP-binding assay

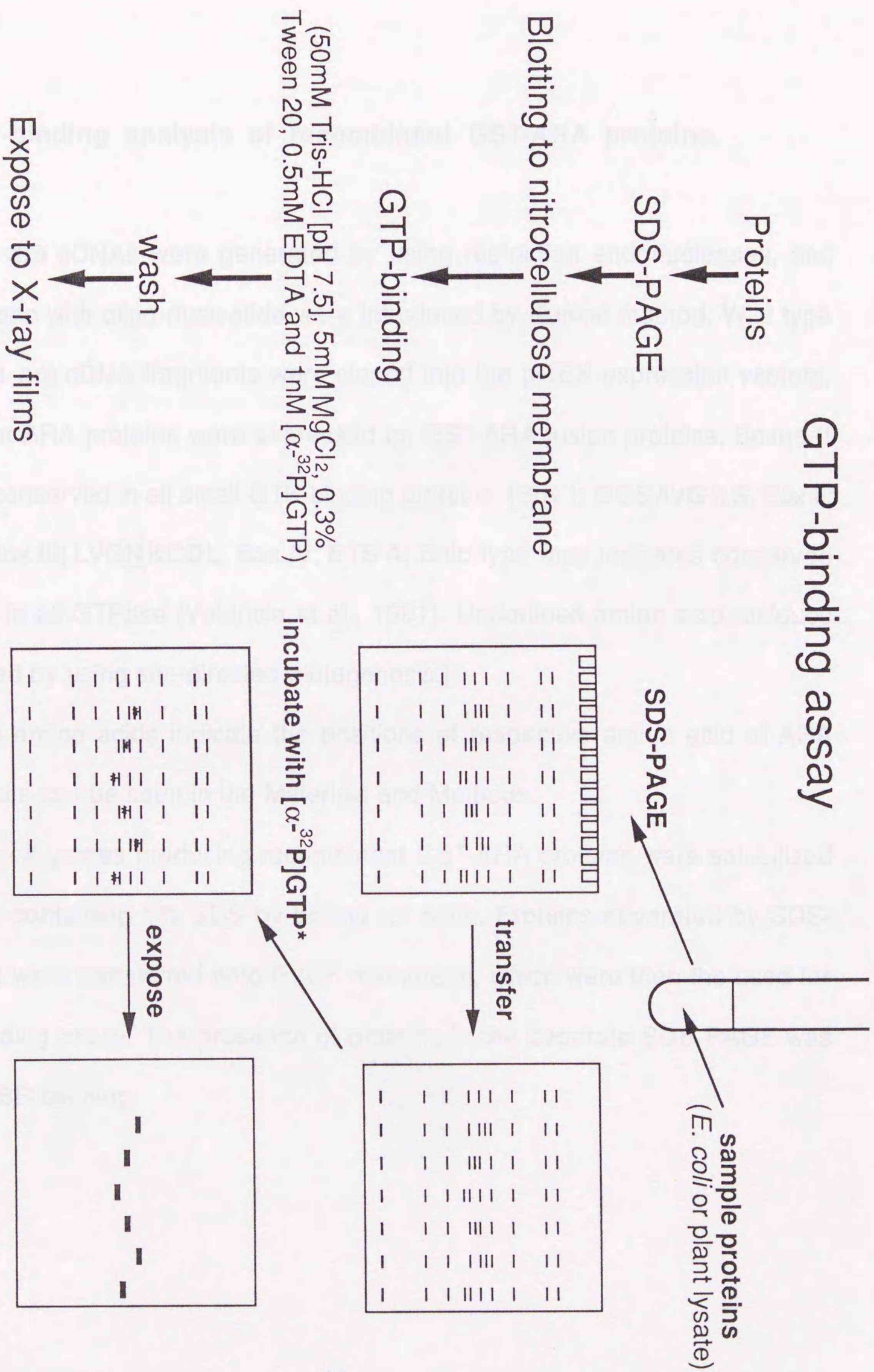


Fig. 2. GTP-binding analysis of recombinant GST-ARA proteins.

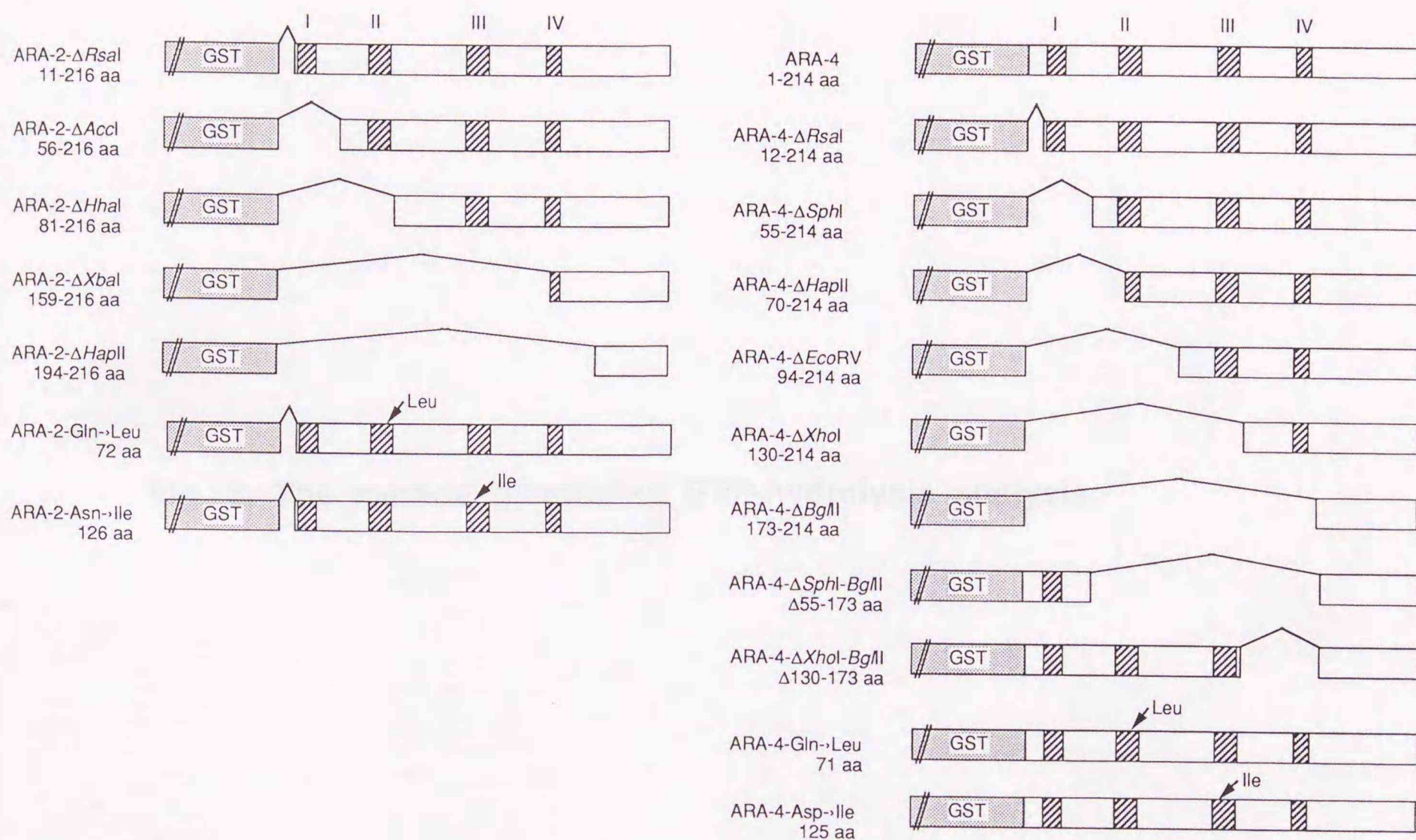
(A) Deleted *ara* cDNAs were generated by using restriction endonucleases, and point mutations with oligo nucleotide were introduced by Kunkel method. Wild type and mutated *ara* cDNA fragments were cloned into the pGEX expression vectors. Recombinant ARA proteins were expressed as GST-ARA fusion proteins. Boxes (I to IV) were conserved in all small GTP-binding proteins. [Box I; **GDSAVGKS**, Box II; **DTAGQE**, Box III; **LVGNKCDL**, Box IV; **ETSA**: Bold type face indicates conserved amino acids in all GTPase (Valencia et al., 1991). Underlined amino acid residues were changed by using site-directed mutagenesis.]

Numerals of amino acids indicate the positions of respective amino acid of ARA protein. Details can be seen in the Materials and Methods.

(B) Total *E. coli* lysates producing recombinant GST-ARA proteins were solubilized in the buffer containing 1% SDS by boiling for 5min. Proteins separated by SDS-PAGE (15%) were transferred onto PVDF membrane, which were then the used for the GTP-binding assay. The presence of proteins in the separate SDS-PAGE was confirmed CBB staining.

Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

(A)



(B)

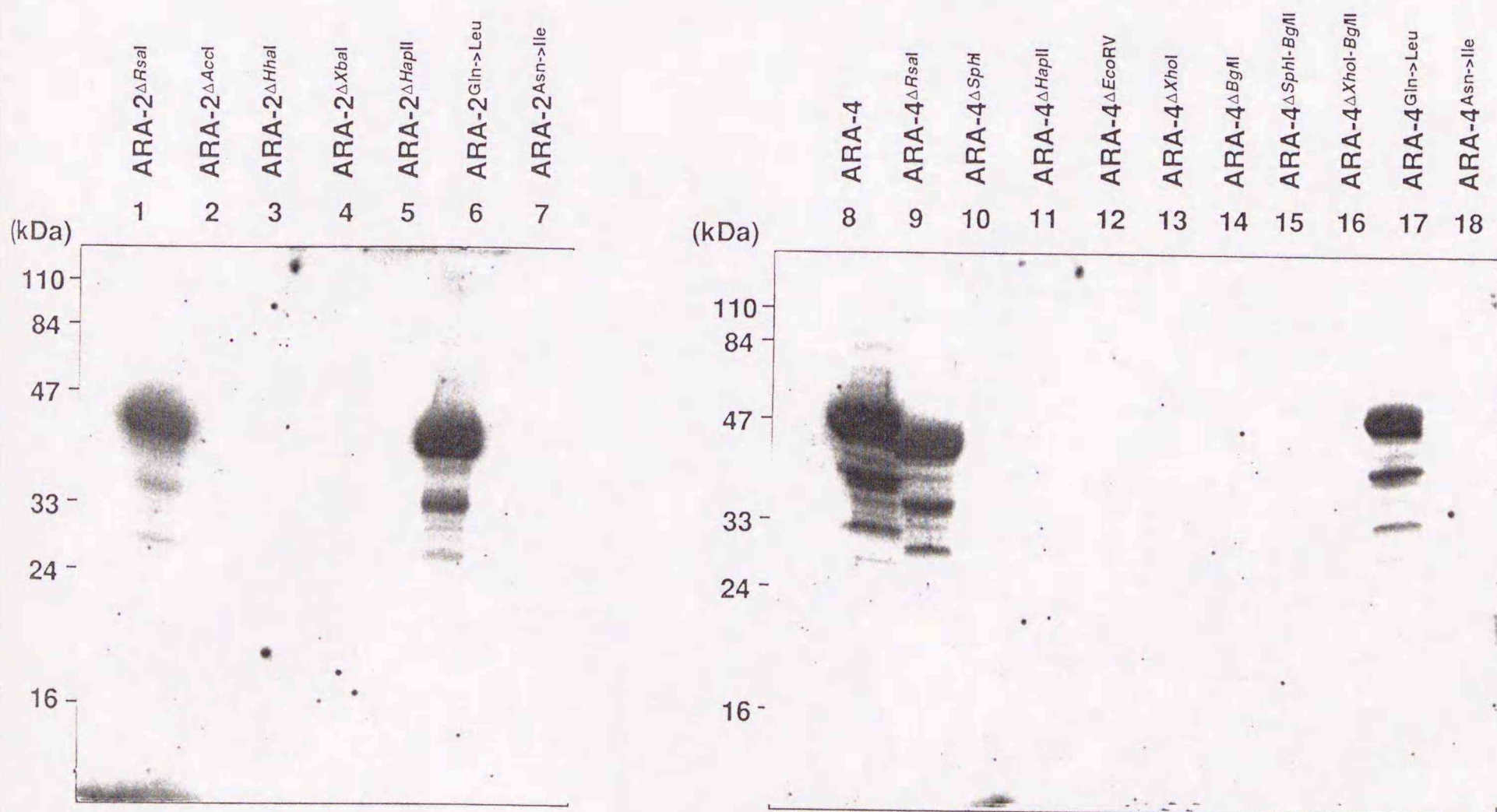
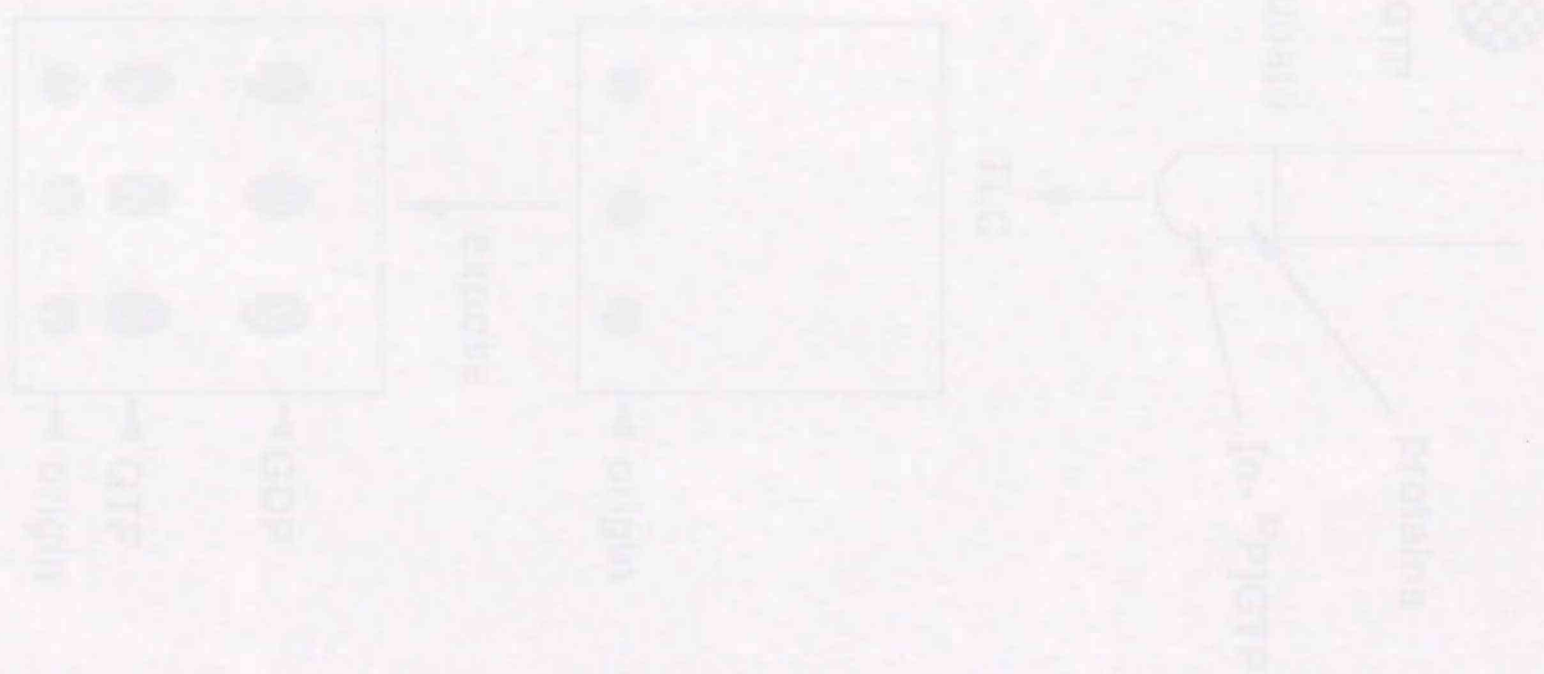


Fig. 3. The method illustrating GTP-hydrolysis analysis.



GTP hydrolysis assay

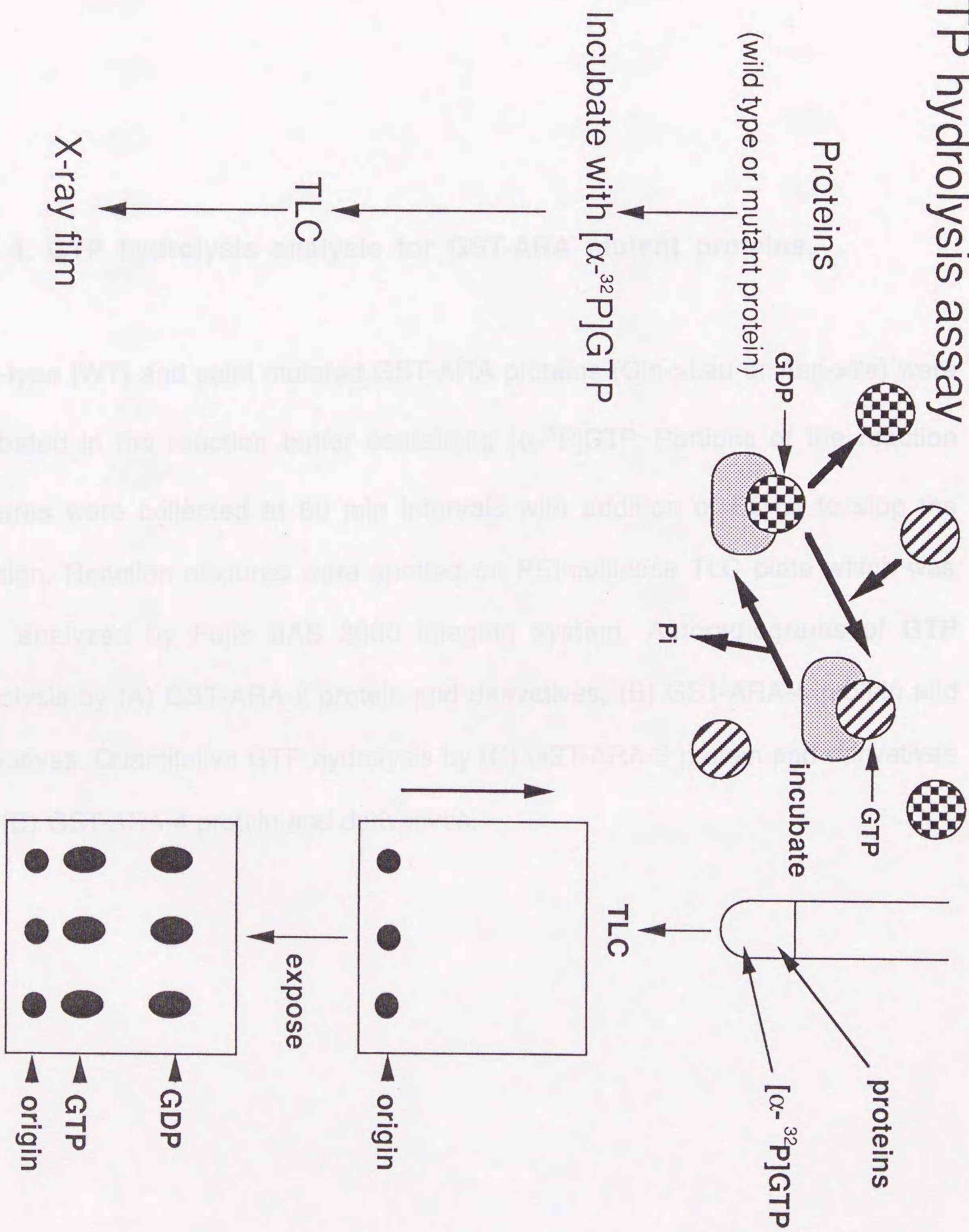
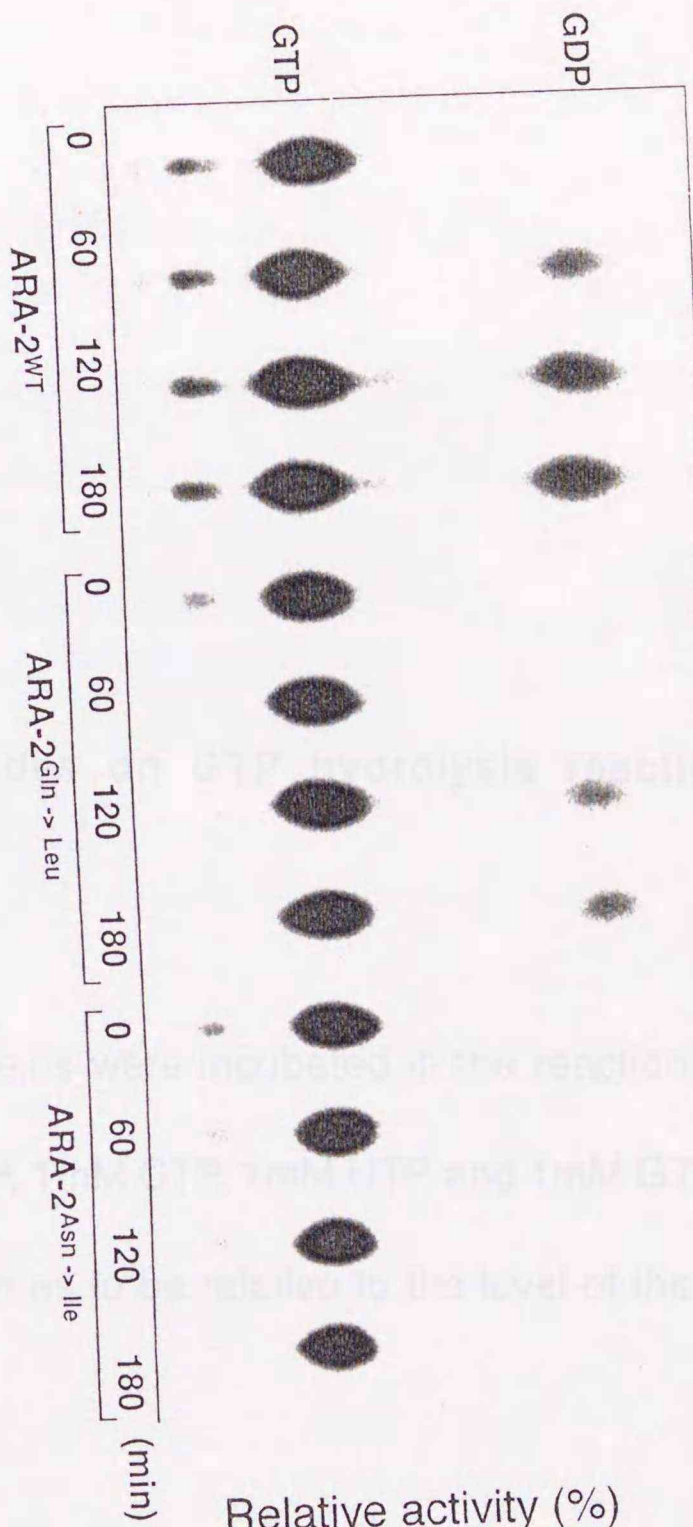


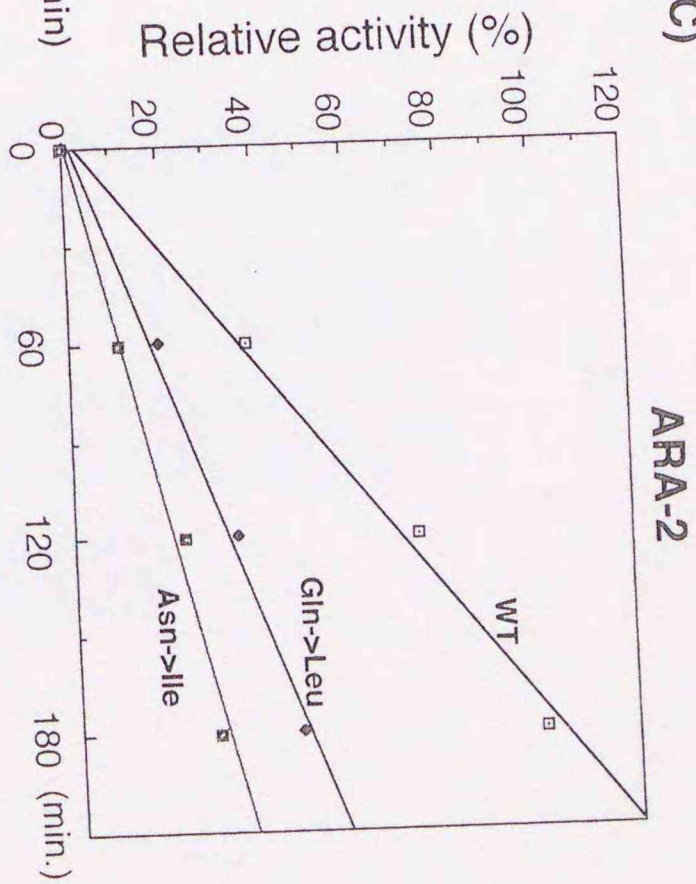
Fig. 4. GTP hydrolysis analysis for GST-ARA mutant proteins.

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. Portions of the reaction mixtures were collected at 60 min intervals with addition of EDTA to stop the reaction. Reaction mixtures were spotted on PEI-cellulose TLC plate which was then analyzed by 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.

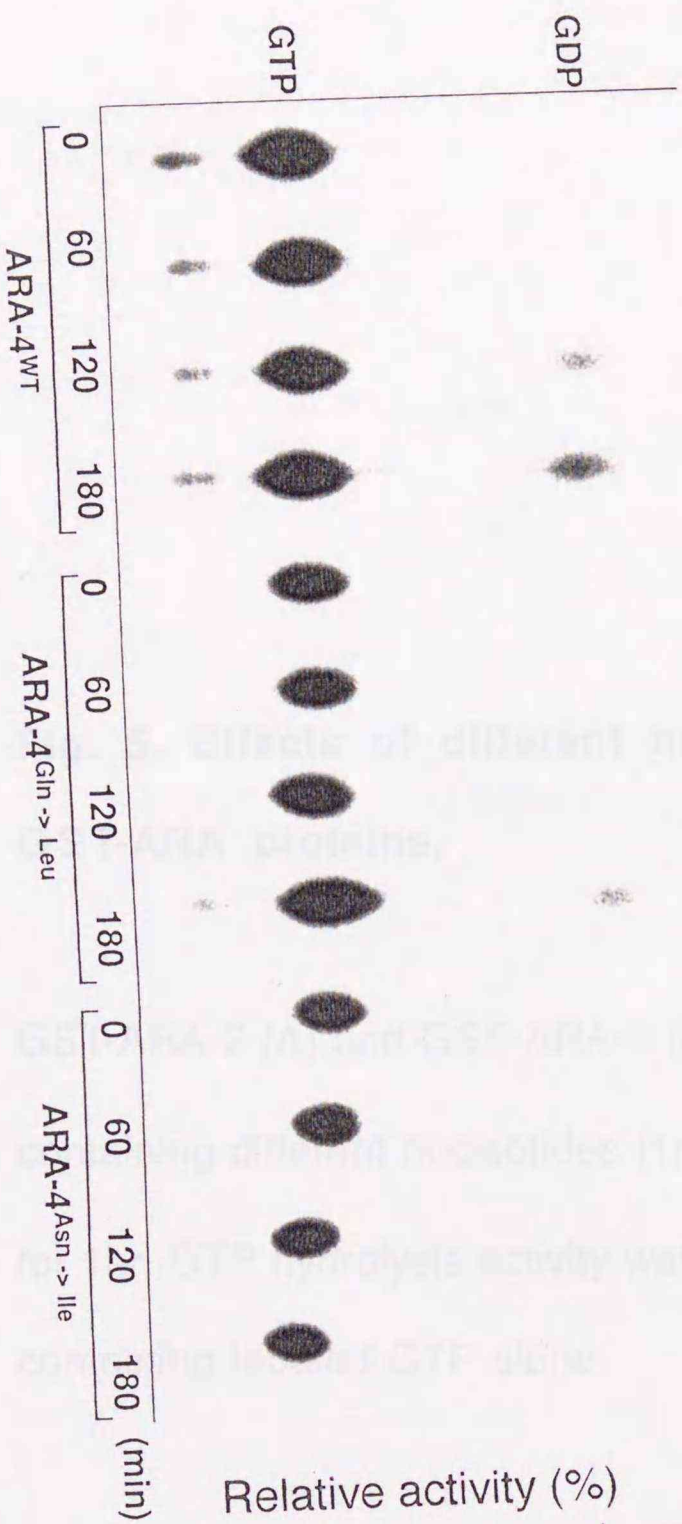
(A)



(C)



(B)



(D)

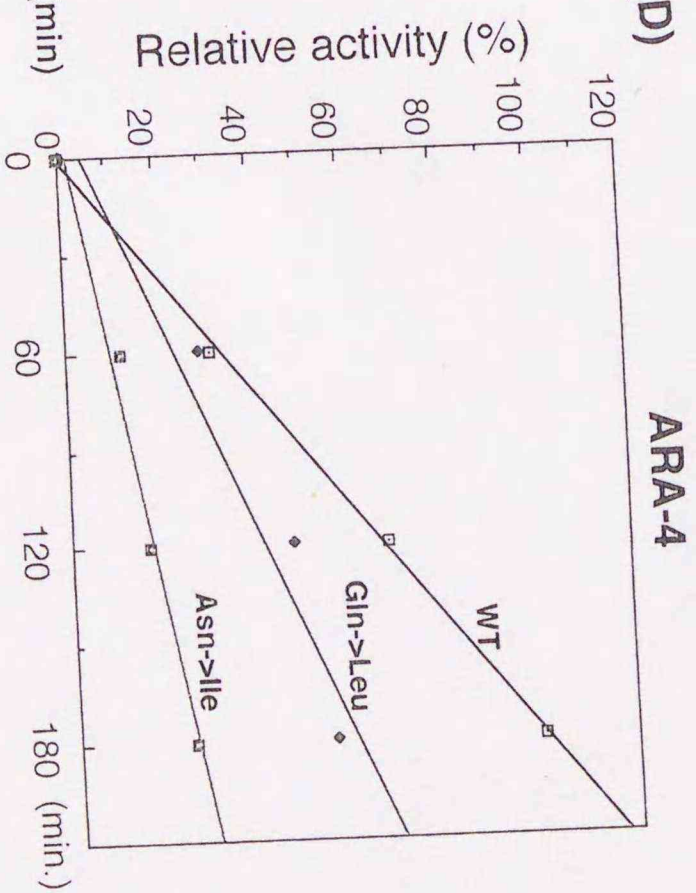


Fig. 5. Effects of different nucleotides on GTP hydrolysis reaction of GST-ARA proteins.

GST-ARA-2 (A) and GST-ARA-4 (B) proteins were incubated in the reaction buffer containing different nucleotides (1mM ATP, 1mM CTP, 1mM UTP and 1mM GTP- γ -S) for 1hr. GTP hydrolysis activity was shown as to be relative to the level of the buffer containing labeled GTP alone.

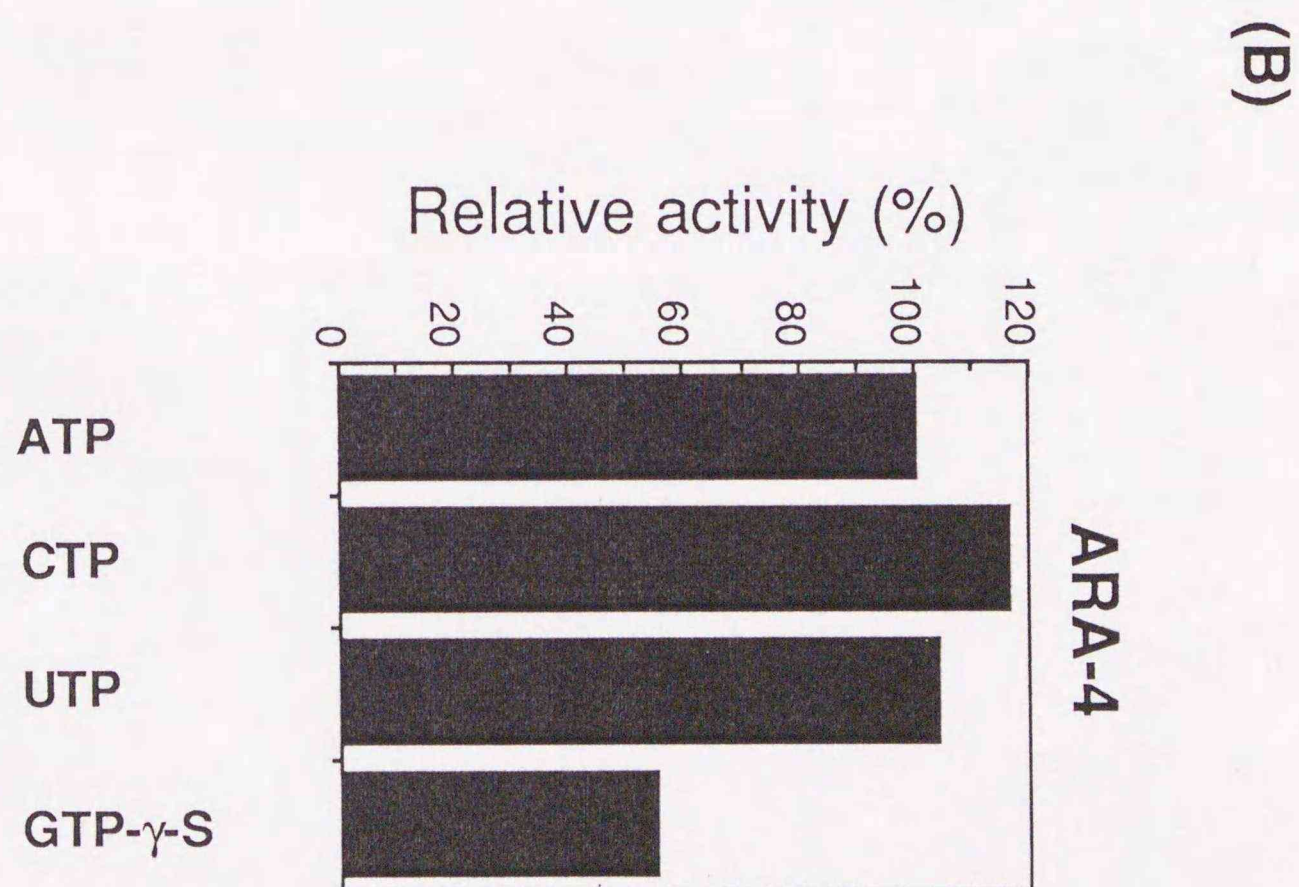
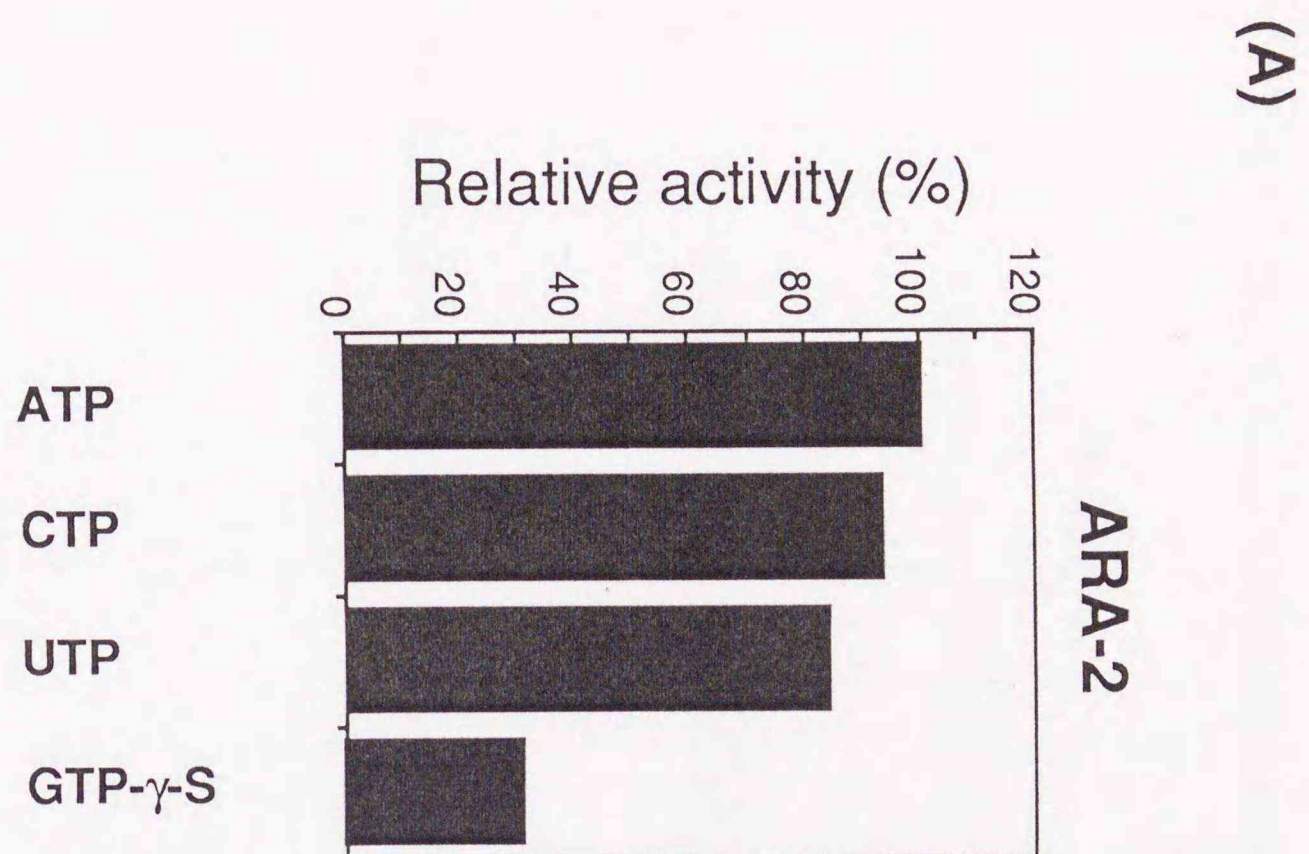


Fig. 6. The method illustrating immuno-precipitation of GST-ARA and nucleotide complexes.

Binding nucleotides assay

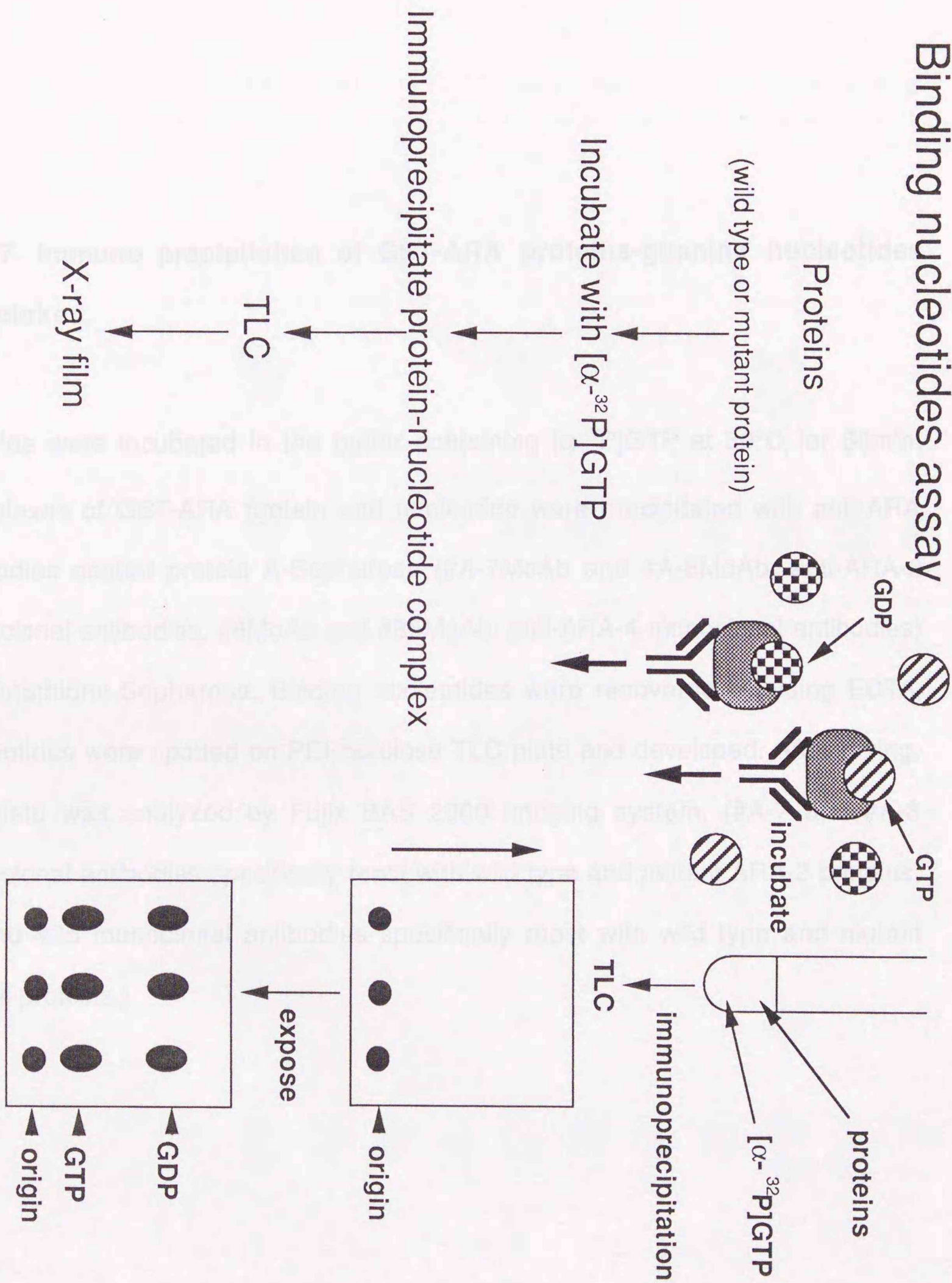


Fig. 7. Immuno precipitation of GST-ARA proteins-guanine nucleotides complexes.

Proteins were incubated in the buffer containing [α - 32 P]GTP at 30°C for 60min. Complexes of GST-ARA protein and nucleotide were precipitated with anti-ARA antibodies 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 nucleotides were recovered by using EDTA. Nucleotides were spotted on PEI-cellulose TLC plate and developed. After drying, the plate was analyzed by 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.)

Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins

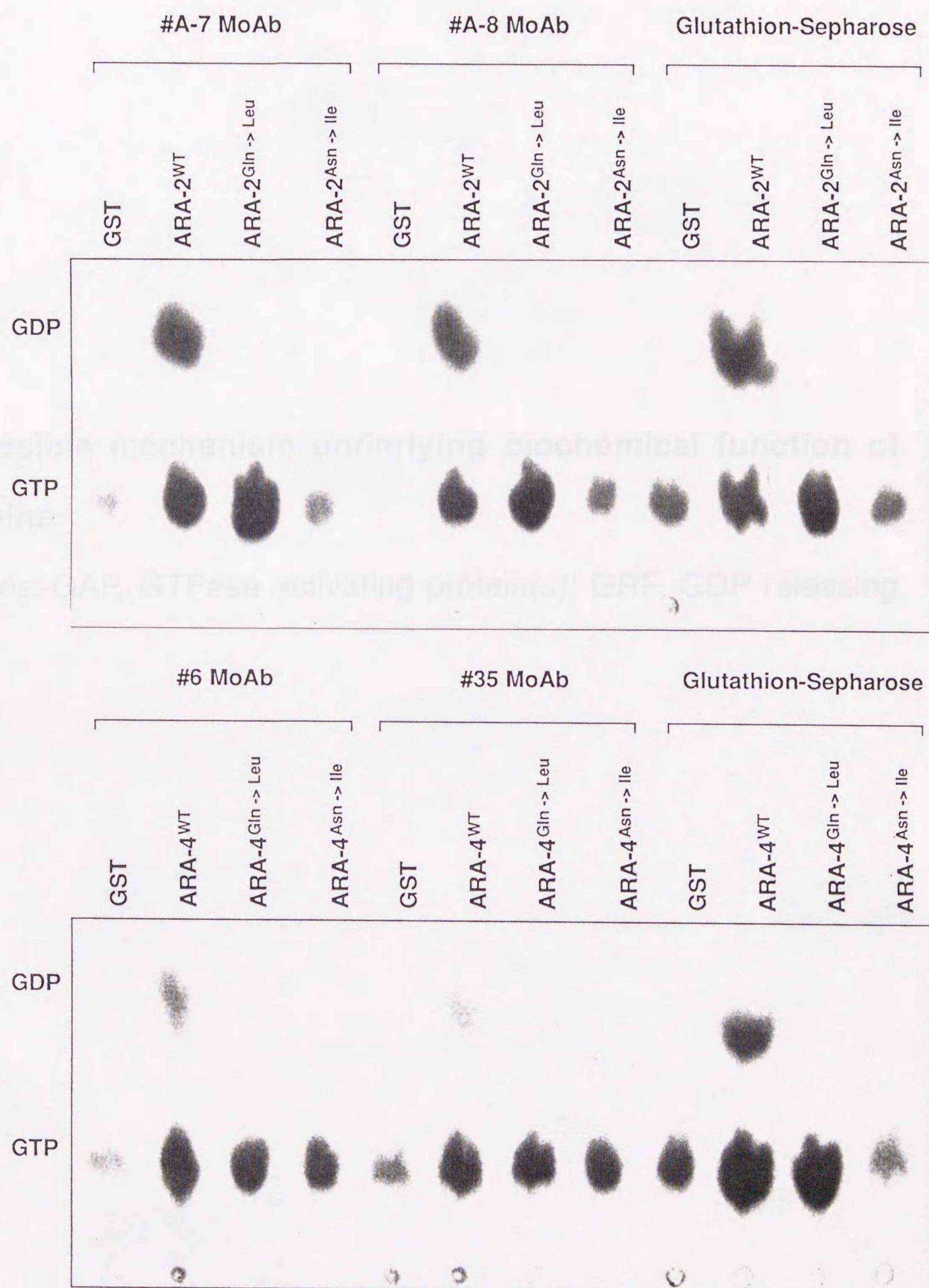
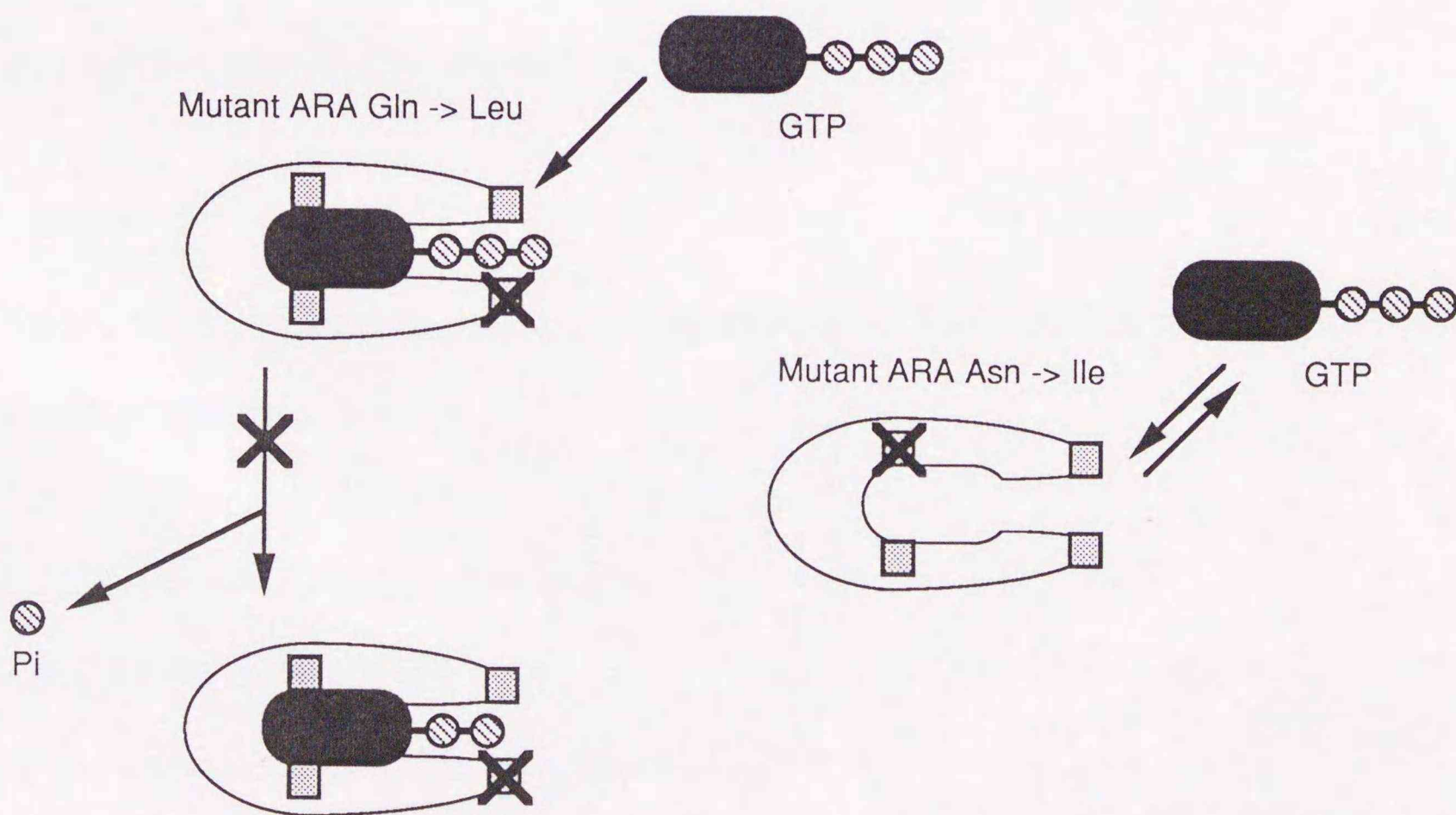
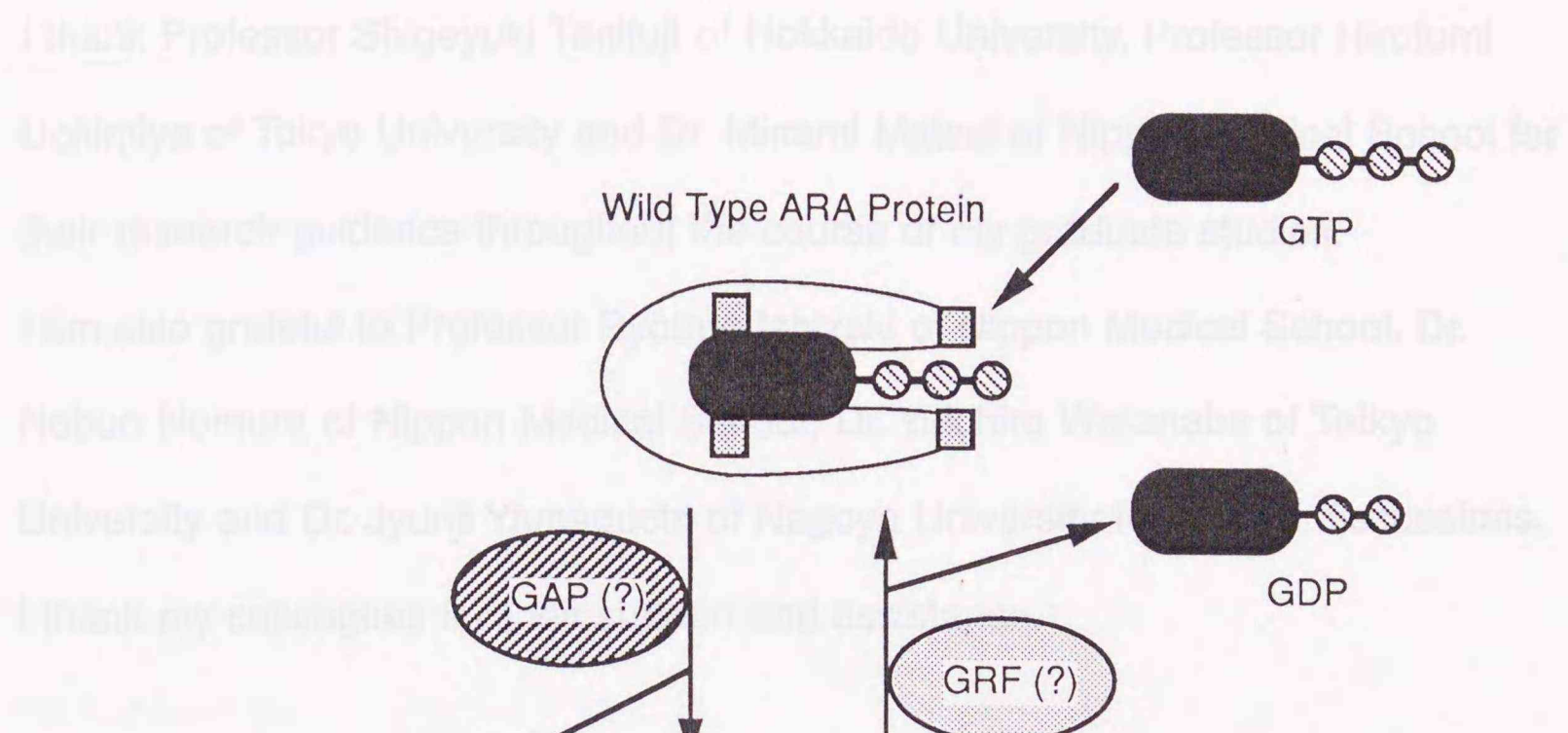


Fig. 8. Possible mechanism underlying biochemical function of ARA proteins

Abbreviations: GAP, GTPase activating protein(s); GRF, GDP releasing factor(s)



Part 3: *In vitro* mutation analysis of plant small GTP-binding proteins



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Abbreviations:

A)

ara, *Arabidopsis thaliana* gene homologous to the mammalian *ras* gene

ARA, protein product of *ara*

B)

BCIP, 5-bromo-4-chloro-3-indolyl phosphate

bp, base pair(s)

C)

CaMV, cauliflower mosaic virus

GAP, GTPase-activating protein

CBB, coomassie brilliant blue

CTAB, cetyl triethylammonium bromide

D)

Denhardt's solution, 0.02% bovine serum albumin/0.02% Ficoll-400/0.02%

polyvinyl pyrrolidone

G)

GST, glutathione S-transferase

GTP- γ -S, guanosine 5'-O-(3-thiotriphosphate)

Abbreviations

I)

IPTG, isopropyl-b-D-thiogalactopyranoside

K)

kb, kilo-base(s) or 1000 bp

N)

NBT, nitro blue tetrazolium

NP-40, Nonidet P-40

P)

PEI, polyethyleneimine

PMSF, phenylmethylsulfonyl fluoride

PVDF, polyvinylidene difluoride

R)

rab, small GTP-binding protein-encoding gene(s) isolated from rat brain

RAB, protein product of *rab*

ras, oncogene encoding RAS

RAS, GTP-binding protein(s) (product of *ras*)

RHO, small GTP-binding protein isolated from *Aplysia*

Abbreviations

S)

SAS, small GTP-binding protein-encoding gene(s), isolated from *Dictyostelium discoideum*

SAS, protein product of SAS

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate poly acrylamide gel electrophoresis

SEC4, *Saccharomyces cerevisiae* protein involved in secretion

SSC, 0.15M NaCl / 0.015M Na3citrate pH 7.6

T)

TLC, thin-layer chromatography

Triton X-100, polyethylene glycol mono-p-iso-octylphenyl ether

X)

Xaa, any amino acid

Y)

YPT, *Saccharomyces cerevisiae* gene(s) encoding small GTP-binding protein(s)

YPT, protein product of YPT

ypt, *Schizosaccharomyces pombe* gene(s) encoding small GTP-binding protein(s)

Ypt, protein product of ypt

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Bourne, H. R., Sanders, D. A. and McCormick, F.: The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349 (1991) 117-127

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