Plant Cell Rep (2007) 26:1373–1382
DOI 10.1007/s00299-007-0367-y

PHYSIOLOGY AND BIOCHEMISTRY

Perinuclear and nuclear envelope localizations of *Arabidopsis* Ran proteins

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Received: 30 March 2007 / Accepted: 19 April 2007 / Published online: 26 May 2007

Abstract Using phragmoplastin-interacting protein 1 (PhrIP1) as bait, we isolated an *Arabidopsis* cDNA encoding Ran2, a small Ras-like GTP-binding protein. The interaction between PhrIP1 and Ran2 was confirmed by an in vitro protein–protein interaction assay with purified Ran2 and PhrIP1. The plant Ran2 shares high sequence homology, 78 and 86% at the amino acid level, with human Ran/TC4 and *C. elegans* Ran, respectively. Our results obtained from enzyme assays and Western blot analysis show that Ran2 has intrinsic GTPase activity and is present in the soluble fraction of *Arabidopsis* seedling extract. Fluorescent microscopy using anti-Ran2 antibody revealed that the Ran protein is localized in the perinuclear region with the highest concentration at the nuclear envelope. In contrast to its animal counterparts that are present in the nucleoplasm, the Ran protein is absent inside the nucleus. These results suggest that plant Ran proteins may be involved in mediation of nucleocytoplasmic transport and assembly of the nuclear envelope after karyokinesis in plant cells.

Communicated by K. Kamo.

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Keywords *Arabidopsis thaliana* · GTPase · Phragmoplastin · Nuclear envelope · Cell division · Cytokinesis

Abbreviations

At	*Arabidopsis thaliana*
Phr	Phragmoplastin
PhrIP1	Phragmoplastin-interacting protein 1
GST	Glutathione *S*-transferase
CBD	Chitin binding domain
FITC	Fluorescein isothiocyanate
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
DAPI	4′,6-Diamidine-2′-phenylindole

Introduction

The small GTP-binding protein Ran is required for the trafficking of proteins and RNAs into and out of the nucleus (Gorlich and Kutay 1999). It forms complexes with the nuclear transport receptors and their substrates, and assists their directional passage through the nuclear pores. Like all small GTP-binding proteins, animal and yeast Ran proteins have a very low intrinsic GTPase activity that requires stimulation by the Ran GTPase activating protein (RanGAP) and its accessory factor RanBP1 (Takai et al. 2001). Exchange of Ran-bound GDP by GTP is accomplished by the Ran guanine nucleotide exchange factor RCC1. The GTP-bound Ran (RanGTP) and GDP-bound Ran (RanGDP) have different roles in nuclear transport, and their respective abundance is regulated by the spatial separation of RanGAP and RanBP1, which are present at the outside of the nucleus, and RCC1, which is found

within the nucleus (Gorlich and Kutay 1999; Kalab et al. 2006). The nucleotide binding state of Ran thus serves as a marker for compartment identification.

In contrast to other small signaling GTPases like Ras and Rho, Ran itself is not membrane bound. It is unique in that the sequestering of its accessory proteins provides the spatial information for its activities (Kahana and Cleveland 1999). In mammalian cells, RanGAP is anchored to the outer basket of the nuclear pore by interaction with nucleoporin Nup358, whereas RCC1 is sequestered in the nucleus through binding to chromatin (Ohtsubo et al. 1989). Animal RanGAP contains a C-terminal Nup358-binding domain. Modification of this domain by the small ubiquitin-like protein SUMO causes a conformational change of RanGAP that allows Nup358 binding (Matunis et al. 1998). However, Rna1p, the RanGAP homolog in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, is localized in the cytoplasm (Hopper et al. 1990; Melchior et al. 1993) and without the Nup358-binding domain (Hillig et al. 1999). It has been well documented that mammalian and yeast Ran plays an important role in the nucleocytoplasmic transport of proteins and RNA. Several recent reports point toward a wider function of Ran in cellular signaling in animal cells (Sazer and Dasso 2000; Dasso 2001). Animal Ran GTPases play key roles in controlling the nuclear processes and mediating the assembly of the nuclear envelope and spindle through the cell mitotic cycle (Zhang and Clarke 2000, 2001; Wilde and Zheng 1999; Moor and Blobel 1993; Zhang et al. 2002).

Four Ran GTPases have been identified in *Arabidopsis*. AtRan1, AtRan2, and AtRan3 are highly homologous (95–96% of identity) differing only in their C-terminal regions (Haizel et al. 1997). The highly conserved RanGAP effector-binding motif (KKYEPTIGVEV) is present in Ran1–3 of *Arabidopsis*, and in Ran proteins of tomato and tobacco. Over expression of these plant Ran GTPases in fission yeast mutants can suppress cell cycle defects, indicating that these plant genes may have a function similar to their yeast counterparts (Ach and Gruissem 1994; Merkle et al. 1994; Haizel et al. 1997). The fourth *Arabidopsis* Ran, AtRan4, is only 65% identity to the other three Ran sequences, and its biological function remains unknown (Vernoud et al. 2003).

Mitosis in plant cells differs significantly from that of animal and yeast cells. Cell division in yeast follows a process known as closed mitosis, i.e., the nuclear envelope stays intact during mitosis (Rose et al. 2004). Although plant and animal cells undergo similar open mitosis, they do not undergo cytokinesis in the same way. A cell plate is established between the two daughter cells during cytokinesis in plants. The cell plate is formed by a well-regulated process of vesicle fusion at the equatorial plane (Samuels et al. 1995; Verma 2001; Bednarek and Falbel 2002; Verma and Hong 2005). Phragmoplastin, the first protein marker of the cell plate (Gu and Verma 1996, 1997), is a large GTPase and belongs to the dynamin superfamily of proteins (Hong et al. 2003; Verma and Hong 2005). It assembles into helical arrays that wrap around and squeeze vesicles into vesicle–tubule–vesicle structures during the formation of the cell plate (Zhang et al. 2000).

Using phragmoplastin as bait to screen an *Arabidopsis* cDNA library has led to the identification of two phragmoplastin-interacting proteins, UDP-glucose transferase 1 (AtUGT1, Hong et al. 2001b) and PhrIP1 (our unpublished data). UGT1 may transfer UDP-glucose from sucrose synthase to the callose synthase during the formation of cell plate (Hong et al. 2001a, b). PhrIP1, the phragmoplastin-interacting protein 1, contains several functional domains that have been implicated in binding to proteins and RNAs, and may play a role in cell plate formation (our unpublished data).

In the present work, we used PhrIP1 as bait to screen an *Arabidopsis* cDNA library, and identified Ran2 as a protein that interacts with PhrIP1. We assayed the intrinsic GTPase activity of purified recombinant AtRan2. Using polyclonal antibodies raised against AtRan2, we demonstrate that the Ran proteins are present in the perinuclear region with the highest concentration at the nuclear envelope in plant cells at the interphase of cell cycle. These results together with previous reports on the nuclear envelope localization of RanGAP (Rose and Meier 2001; Patel et al. 2004; Jeong et al. 2005) suggest an important role of Ran proteins in regulation of nucleocytoplasmic transport and nuclear envelope reassembly after mitosis in plant cells.

Materials and methods

Isolation of *Arabidopsis Ran2* cDNA

The *Arabidopsis* cDNA library, made in pJG4-5 from mRNAs of 6 to 8-day-old seedlings, was provided by Dr. H. Goodman (Massachusetts General Hospital, Boston, USA). The library plasmids were amplified in *E. coli* Top10F' cells and purified by CsCl gradient. The bait plasmid pEG-PhrIP1 was constructed by inserting the coding region of the *Arabidopsis* PhrIP1 cDNA at the *Bam*HI–*Xho*I sites of pEG202 (Golemis et al. 1996). Yeast EGY48 cells containing the reporter gene plasmid pSH18-34 were transformed with the bait plasmid using LiAc (Gietz et al. 1995). The cells were selected in liquid synthetic complete (SC) medium without uracil and histidine for 12 h and then propagated in yeast–peptone–dextrose (YPD) medium to an OD_{600} of 2.0. Yeast cells (approximately 10^8 cells) were transformed with the pooled *Arabidopsis* cDNA library plasmids. Yeast colonies were selected on SC-galactose medium without uracil, histidine,

tryptophan, and leucine. Colonies were lifted on 3 MM filter discs, permeabilized by liquid nitrogen, and incubated in a Z buffer containing X-Gal (120 mmol/l sodium phosphate, pH 7.0, 10 mmol/l KCl, 1 mmol/l $MgCl_2$, 0.2 mmol/l β-mercaptoethanol, and 300 mg/l X-Gal) at 28°C. Plasmids were isolated from colonies showing a positive (blue colored) reaction and reintroduced into EGY48 for confirmation of protein interaction. Inserts of the plasmids were sequenced by Beijing Sunbiotech Co., Ltd.

The plasmid p33 contained a full coding region of *Arabidopsis Ran*2. The full-length cDNA, p*Ran*2, was used for protein expression in *E. coli*.

In vitro protein binding assay

Ran2 was expressed as a fusion protein (CBD-Ran2) with the chitin-binding domain (CBD) in *E. coli* and purified with chitin beads according to the instruction manual of IMPACT (intein mediated purification with an affinity chitin-binding tag; New England BioLabs, Massachusetts, USA). GST–PhrIP1 fusion protein expressed in *E. coli* was purified with gluthatione-sepharose beads and eluted after cleavage by thrombin as described by Guan and Dixon (1991). The CBD-Ran2 bound to beads were incubated with the purified PhrIP1 for 1 h on ice with slight shaking, added to 3× SDS loading buffer (6% SDS, 87.5 mmol/l Tris, pH 6.8, 30% glycerol, and 0.03% bromophenol blue), and resolved on 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with purified PhrIP1 rabbit IgG (see below). Horseradish peroxidase-labeled goat antibody against rabbit IgG was used as the secondary antibody. Signals were visualized on X-ray films using the enhanced chemiluminescence protein detection system (Amersham).

GTPase assay

The assay of GTPase activity of Ran2 was performed in 150 µl of reaction mixture containing 100 mmol/l Tris–HCl (pH 8.0), 10 mmol/L $MgCl_2$, 0.4 mmol/l dithiothreitol (DTT), 650 µmol/l GTP and 0.45 µg of purified proteins (concentration 10 µg/ml). Controls were the same reaction mix but without the Ran2 protein or GTP. The reactions were incubated at 37°C for 0, 30, 40, 50, 60, 75, 90, 105, 115 min and 10 µl aliquots of the reaction were quenched with 5 µl 20% trichloroacetic acid (TAA). They were then mixed with 35 µl distilled H_2O and centrifuged for 15 min at 12,800 rpm in a microcentrifuge. The clear solution (50 µl) was added 200 µl $FeSO_4$-$(NH_4)_2MoO_4$ solution containing 0.7% $FeSO_4$, 0.14% $(NH_4)_2MoO_4$ and 5 mmol/l H_2SO_4. The amounts of hydrolyzed inorganic phosphate (P) were measured at $OD_{660\ nm}$ with a Beckman DU800 spectrophotometer. The phosphates generated in both control samples were used to deduce the amount of phosphate hydrolyzed by Ran2. The mean values of the phosphate contents from three independent experiments were plotted.

To determine the amount of protein needed to hydrolyze 5 nmol GTP, reaction mixtures (25 µl) containing 100 mmol/l Tris–HCl (pH 8.0), 10 mmol/l $MgCl_2$, 0.4 mmol/l dithiothreitol (DTT), 5 nmol/l GTP and purified protein of 0, 1.5, 2.0, 2.5, 3.5 µl (concentration 10 µg/ml) were incubated at 37°C for 60 min. Controls were the same reaction mix but without the Ran2 protein or GTP. The reactions were quenched with 5 µl of 20% trichloroacetic acid, mixed with 20 µl distilled H_2O, and centrifuged for 15 min at 12,800 rpm. The phosphate content was determined and plotted as described above.

Antibody preparation, purification and labeling with FITC

CBD–Ran2 fusion protein was cleaved from the chitin beads using DTT (50 mmol/l). The cleaved products were resolved on 10% SDS-PAGE. The Ran2 protein band was excised from 10 gels, eluted with 0.85% NaCl, lyophilized, and used to raise antibodies in rabbits.

To isolate anti-Ran2 IgG from rabbit serum, 10 ml serum was diluted with 10 ml of 0.85% NaCl solution and precipitated by adding 4.1 g Na_2SO_4 at 28°C with stir for 2 h. After centrifuging for 30 min at 3,000 rpm in a Sorvall centrifuge, the supernatant was removed and the precipitate was dissolved with 13.4 ml sterilized H_2O. The resulting solution was precipitated again by adding 3.86 g Na_2SO_4 and centrifuged until the solution became colorless. The final precipitate was dissolved with 4.0 ml sterilized H_2O and transferred into a dialysis bag to dialyze against flowing H_2O for 4 h, and then with a 20-fold volume of 0.01 mol/l phosphate buffer pH 7.2 (0.075% Na_2HPO_4, 0.010% KH_2PO_4, 0.9% NaCl) at 4°C overnight.

To label the antibody with fluorescein, 10 ml purified IgG (150 mg) was mixed with 2.75 ml of 0.01 mol/l phosphate buffer pH 7.2, 2.25 ml carbonate buffer pH 9.3 (1.73% $NaHCO_3$, 0.86% Na_2CO_3) and 7.5 mg fluorescein isothiocyanate (FITC) and stirred overnight at 4°C. The FITC-conjugated antibody was purified with a Sephadex G-25 column.

Soluble protein isolation and Western blot analysis

Two-week-old *Arabidopsis* seedlings frozen in liquid nitrogen were ground with mortar and pestle into a powder, which was then resuspended in an extraction buffer made

of 25 mmol/l Tris–HCl, pH 8.0, 20 mmol/l NaCl, 1 mmol/l EDTA, 2 mmol/l β-mercaptoethanol, 1 mmol/l PMSF, and 1 mmol/l Leupeptin. The homogenate was centrifuged at 14,000 rpm in a Sorvall centrifuge for 30 min. The supernatant (soluble protein extract) was added to 3× SDS loading buffer. Western blot was performed as described elsewhere (Hong et al. 2001b) with the purified anti-Ran2 IgG as the first antibody. Negative control of Western blot was treated as described above using the preimmune serum as the first antibody.

Subcellular location of the Ran proteins

Immunofluorescence microscopy was used to determine the subcellular location of Ran proteins following the method of Gu and Verma (1996) with some modifications. Briefly, 3-day-old onion root tips or 2-day-old garlic root tips were fixed in Canoys Fluid containing ethanol/acetic acid (3:1) for 24 h at room temperature and washed with EB buffer pH 4.8 (0.08% citric acid and 0.18% tribasic sodium citrate) for 20 min with stirring at 200 rpm. The plant tissues were digested in a solution containing 0.4% cellulase and 0.4% pectolyase in EB buffer for 65 min at 37°C and washed with EB buffer for 20 min. The digested plant tissue was crushed with the dissecting needle to free individual cells. The cells were permeabilized with 45% acetic acid, fixed with Canoys Fluid, and air dried for 10 min. Cells were blocked with 0.05% Tween-20, 1% BSA in PBS at 37°C for 1 h, incubated with FITC-labeled antibody for 1 h, and washed with PBS for 10 min. DNA was stained with DAPI for 10 min at room temperature and washed with PBS for 10 min. Photographs were taken on a fluorescence microscope (Olympus) with appropriate filters for FITC and DAPI staining. Two negative controls of immuno fluorescent localization were performed, one incubated with unconjugated anti-Ran2 antibodies and the other with free FITC.

Results

Identification of *Arabidopsis* Ran2 as a PhrIP1-interacting protein

PhrIP1 is an interacting protein of the cell plate-specific phragmoplastin that may play an important role in cell plate formation during cytokinesis in plants (our unpublished data). As part of our research to isolate the proteins needed to initiate and complete the positioning of the cell plate at this time in the cell cycle, we used PhrIP1 as bait to identify proteins that can interact with it. The PhrIP1 cDNA was cloned in pEG202 in frame with the DNA binding domain of LexA (Golemis et al. 1996; Hong et al. 2001b) and used to screen an *Arabidopsis* cDNA library constructed in vector pJG4-5. Approximately five million yeast colonies were screened, and all positive clones were subjected to reconfirmation to eliminate any false candidates. One cDNA clone, p33 (for clone No. 33), was isolated. Colonies containing pEG202/p33 or pJG4-5/PhrIP1 served as negative controls and did not turn blue in the X-Gal assay (Fig. 1a), suggesting that the presence of both PhrIP1 and the protein encoded in p33 were required for the observed interactions.

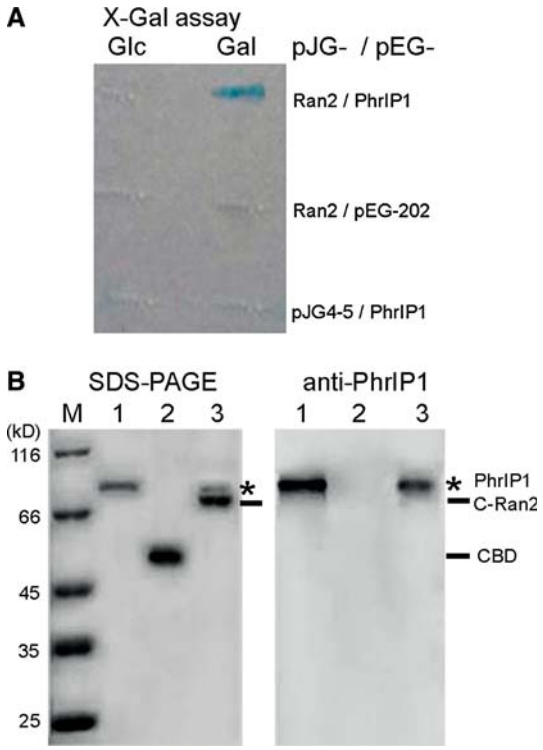

Fig. 1 Interaction between PhrIP1 and Ran2. **a** Interaction of PhrIP1 with Ran2 in the yeast two-hybrid system. Cloning vectors pEG202 (pEG-) and pJG4-5 (pJG-) were used as negative controls. Yeast colonies containing the plasmids were grown on SC-Ura-Trp-His + Leu medium in the presence of glucose (*Glc*) or galactose (*Gal*). The yeast cells on the filter were permeabilized with liquid nitrogen, and incubated in a Z buffer containing X-Gal. **b** Pull-down assay of soluble PhrIP1 by immobilized Ran2. Lane 1, soluble PhrIP1 that was purified as GST-tagged protein and cleaved from the glutathione-beads by thrombin. Lane 2, peptide of the chitin binding domain (*CBD*) tag that was immobilized on chitin beads. The beads were incubated with soluble PhrIP1, and served as a negative control in the pull-down assay. Lane 3, CBD-Ran2 bound to the chitin beads was incubated with soluble PhrIP1, a significant amount of which (indicated by *) was pulled down by Ran2. The pull-down products were separated on 10% SDS-PAGE and stained with Coomassie blue G-250. Molecular masses (kD) of standard protein markers (*lane M*) are indicated. **c** Detection of PhrIP1 in the pull-down products of Ran2. A replica of the gel shown on **b** was used for Western blot analysis using anti-PhrIP1 IgG as the first antibody. The bands corresponding to PhrIP1 were visualized on X-ray film

The insert in p33 (0.66 kb long) encodes AtRan2, a small Ras-like GTP-binding protein. To verify the protein–protein interaction in vitro, Ran2 protein was expressed and purified as a fusion protein (CBD–Ran2) with a chitin binding (CBD) tag. PhrIP1 was purified as a GST-tagged protein and eluted from the glutathione–agarose beads with 10 mmol/l glutathione. The beads-bound Ran2 was incubated with soluble PhrIP1. The pull-down products by the beads containing CBD–Ran2 were assayed for the presence of PhrIP1 by Western blot analysis. As shown in Fig. 1b, a significant amount of PhrIP1 was pull-down by Ran2-containing beads (lane 3), suggesting that the interaction between the PhrIP1 and Ran2 occurs under in vitro conditions and that no additional protein is required for this interaction.

Ran2 is a member of the conserved Ran protein family

AtRan2 (At5g20020) is a 221 amino acid-long member of the Ran GTPase family that belongs to the superfamily of small GTPases including Ras, Rho, Rab, Arf/SAR1, and Ran (Vernoud et al. 2003; Jiang and Ramachandran 2006). The Ran family in *Arabidopsis* contains four members (Ran1–4), of which Ran1–3 share high sequence homology (~96%), and are distantly related (67% homology) to the fourth member, AtRan4 (Fig. 2b). AtRan1–3 contain an N-terminal, highly conserved effector-binding motif for binding with RanGAP, and a C-terminal, acidic domain for GDP binding and protein-protein interaction (Fig. 2a). Removal of the C-terminal acidic domain of human Ran promotes GDP release (Richards et al. 1995), localizes the protein to the cytoplasm (Lounsbury et al. 1996) and abrogate the cell cycle arrest (Ren et al. 1993). This acidic domain is also required for interaction with Ran-binding proteins in animals (Richards et al. 1995) and in plants (Haizel et al. 1997).

These two domains are present in most Ran proteins of different organisms, but are surprisingly absent in AtRan4 (Fig. 2a), suggesting that this unique member may have a

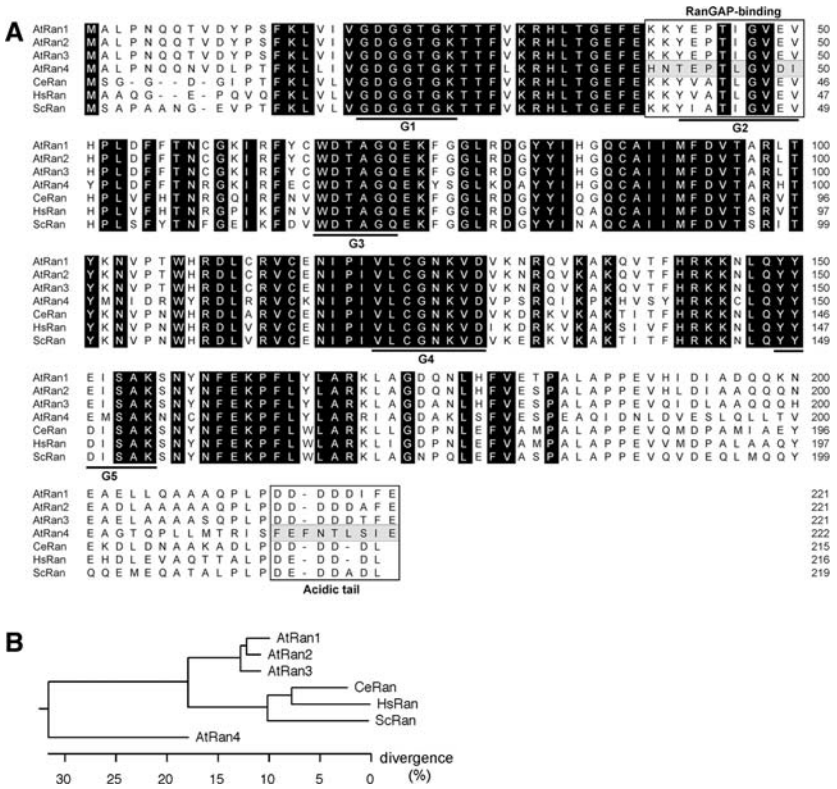

Fig. 2 The Ran family of proteins. **a** Multiple sequence alignment of Ran peptides from *Arabidopsis* (AtRan1–4; At5g20010, At5g20020, At5g55190, and At5g55080; Haizel et al. 1997; Vernoud et al. 2003), human (HsRan; GenBank Acc. P62826; Drivas et al. 1990), *C. elegans* (CeRan; NP_499369; *C. elegans* Sequencing Consortium 1998), and yeast (ScRan, P32835; Kadowaki et al. 1993). Underlined are the conserved GTP binding/hydrolysis motifs (G1–G5). Boxed are the effector-binding domain (RanGAP-binding) involved in protein–protein interaction with RanGAP, and the acidic C-terminal region (acidic tail) implicated in nucleus targeting. *Highlighted in the two boxes* are the amino acid sequences of AtRan4, indicating its difference from the consensus motifs. **b** Phylogenetic tree of the Ran family of proteins. Note that AtRan1–3 are more closely related to animal and yeast Ran proteins than to AtRan4, a distinct member of the Ran family with an unknown function

distinct role or subcellular localization. The expression patterns of *Ran*1–3 genes (At5g20010, At5g20020 and At5g55190) in *Arabidopsis* are very similar (Haizel et al. 1997) and may partially overlap in their function. A recent study on AtRan3 revealed that it is localized like its human and *C. elegans* counterparts (Yano et al. 2006) in the nucleoplasm.

AtRan2 shares high sequence homology with human Ran/TC4 and *C. elegans* Ran, 78 and 86% similarity, respectively (Fig. 2a). Over-expression of plant *Ran* genes have been found to suppress the phenotype of the cell cycle regulatory homologue *pim*46-1 mutant in fission yeast (Ach and Gruissem 1994; Merkle et al. 1994; Haizel et al. 1997). These data indicate that Ran2 may be functionally similar to its mammalian/yeast counterparts and involved in regulating multiple cellular functions, including nucleocytoplasmic transport, nuclear membrane assembly, spindle assembly, and microtubule organization throughout the cell cycle (Trieselmann and Wilde 2002; Bamba et al. 2002; Wilde and Zheng 1999).

Ran2 contains intrinsic GTPase activity

Like many other small GTP-binding proteins, mammalian Ran has been demonstrated to contain a low level of intrinsic GTP hydrolysis activity. To verify the GTPase activity of AtRan2, we purified recombinant CBD–Ran2 with chitin beads and released soluble Ran2 protein with DTT (50 mmol/l). The purified soluble Ran2 had a molecular mass of 26 kDa (Fig. 3a), and contained low but measurable levels of GTPase activity (Fig. 3b, c). These results demonstrate that, like other Ran proteins, Ran2 can hydrolyze GTP without the requirement for other protein factors and thus has intrinsic GTPase activity.

Ran2 protein is in the soluble fraction of plant cells

We prepared cell extracts of *Arabidopsis* seedlings as described previously (Hong et al. 2001b) and analyzed the expression levels of the Ran proteins on Western blots. The pre-immune serum did not react with any visible band (Fig. 4c). The anti-Ran2 antibody reacted strongly with a 26 kDa polypeptide band (Fig. 4 lane 1), which is in good agreement with the recombinant AtRan2 purified from *E. coli* (lane 2). Because Ran1 and Ran3 share high peptide sequence similarity with Ran2, it is likely that the 26-kDa band detected by the anti-Ran2 polyclonal antibodies may include all three Ran proteins. These data show that the Ran proteins are soluble in plant cells, consistent with the report by Haizel et al. (1997).

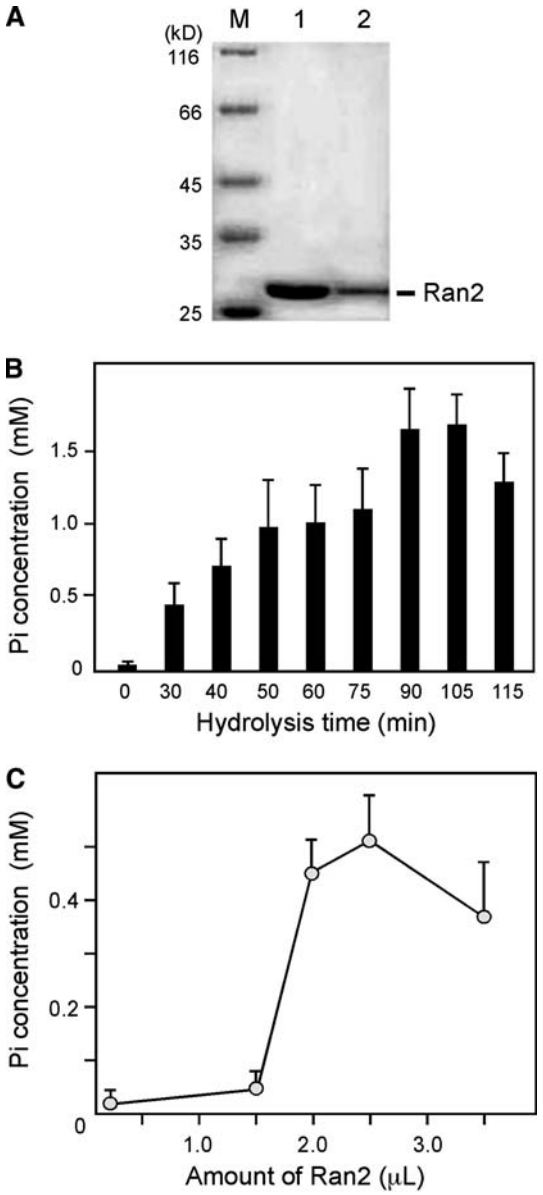

Fig. 3 Intrinsic GTPase activity of recombinant Ran2. **a** Purified Ran2 on 10% SDS-PAGE. *Lane 1*, the soluble product (Ran2) of the recombinant CBD-Ran2 protein after the cleavage of the CBD domain with dithiothreitol (*DTT*). Lane 2, the soluble product (Ran2) of the cleavage after dialysis. Molecular masses (kD) of standard protein markers (lane M) are indicated. **b** Time course of GTP hydrolysis by Ran2. Purified Ran2 was added to the reaction mixtures containing 100 mmol/l Tris–HCl (pH 8.0), 10 mmol/l $MgCl_2$, 0.4 mmol/l DTT, 650 μmol/l GTP. The reactions were allowed to proceed for the time (min) indicated. The concentrations (mM) of hydrolyzed inorganic phosphate (Pi) were determined and the mean values with standard deviations calculated from three independent experiments were plotted. **c** Dependence of GTP hydrolysis on different concentrations of Ran2. Different amounts (μl) of Ran2 protein was added to the reaction mixtures containing 5 nmol GTP and incubated at 37 °C for 60 min. The concentrations (mM) of hydrolyzed inorganic phosphate (Pi) were determined and the mean values with standard deviations calculated from three independent experiments were plotted

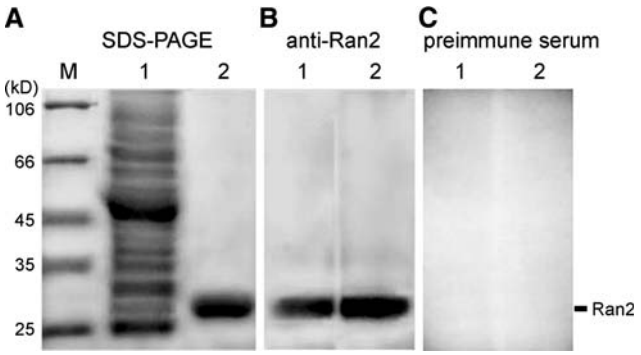

Fig. 4 Western blot analysis of Ran2 in *Arabidopsis* seedlings. **a** Soluble protein extract resolved on 10% SDS-PAGE. *Lane 1*, soluble protein extract from two-week-old *Arabidopsis* seedlings. Lane 2, Positive control of purified Ran2. Molecular masses (kD) of standard protein markers (*lane M*) are indicated. **b** Western blot of the same samples as in **a**. The proteins were transferred to the nitrocellulose membrane and probed with purified Ran2 IgG as the first antibody. The Ran2 protein bands were visualized on X-ray film after incubation with HRP-conjugated second antibody (goat antibody against rabbit IgG). **c** Western blot of the same membrane as in b, except that the pre-immune serum was used as the first antibody

The Ran protein is localized in the perinuclear region and the nuclear envelope in cells at the interphase

To investigate the subcellular localization of plant Ran proteins, we stained 3-day-old onion root tip cells with FITC-labeled anti-Ran2 antibody and DNA-specific dye DAPI. Given the high sequence similarity among Ran proteins, there is a probability that the anti-Ran2 polyclonal antibodies may cross-react with Ran1 and Ran3. As shown in Fig. 5a–d, Ran proteins were localized in the perinuclear region and highly concentrated at the nuclear envelope in cells at interphase. We also used garlic root tip cells for subcellular localization and obtained the same results (Fig. 5e–h). The control root tip cells that were treated with unconjugated anti-Ran2 antibody or free FITC did not show any detectable green fluorescence background (data not shown). The location pattern of plant Ran proteins is almost identical to that of AtRanGAP1 (Rose and Meier 2001; Pay et al. 2002; Jeong et al. 2005), and is similar to that of *C. elegans* Ran except that a small amount of CeRan was also found inside the nucleus (Bamba et al. 2002). Together, our data showed that the *Arabidopsis* Ran proteins have a localization pattern very similar to both *C. elegans* Ran and *Arabidopsis* RanGAP1 at interphase.

Discussion

We demonstrated that *Arabidopsis* Ran2 has a relatively low level of intrinsic GTPase activity just like its yeast and human counterparts. In vivo, this GTPase activity of Ran converts Ran-GTP to Ran-GDP and is stimulated by RanGAPs and RCC1. The exchange of Ran-GDP to Ran-GTP, which is assisted by RanBP1, completes the Ran-GTP to Ran-GDP to Ran-GTP cycle. The conversion between Ran-GTP and Ran-GDP states plays a vital role in regulating transport of proteins across the nuclear envelope (Mattaj and Englmeier 1998; Gorlich and Kutay 1999). The high sequence homology between plant Ran2 and human/yeast Ran and the facts that over-expression of Ran2 suppresses the cell cycle defects in fission yeast mutant (Ach and Gruissem 1994; Merkle et al. 1994; Haizel et al. 1997) suggest that plant Ran proteins may have a similar function as their mammalian/yeast homologs. The subcellular localization of plant Ran proteins in the perinuclear region and at the nuclear envelope correlates with their putative function. These results strongly suggest that plant Ran proteins may be involved in mediating nucleocytoplasmic transport and assembly of the nuclear envelope in plant cells.

Plant Ran proteins also exhibit some features different from those of their mammalian and yeast counterparts. First, multiple Ran proteins are present in plants (Haizel et al. 1997; Vernoud et al. 2003), whereas human and *C. elegans* contain a single *Ran* gene in each genome. Second, *Arabidopsis* Ran proteins, as shown in Fig. 5, are localized in the perinuclear region and at the nuclear envelope, which is different from the nuclear localization of human Ran (Moor and Blobel 1993; Ren et al. 1993). Recently, GFP-tagged AtRan3 has been localized to the nucleus (Yano et al. 2006), which is not consistent with our subcellular localization data (Fig. 5). It is unknown whether or not this discrepancy in subcellular localization is caused by the presence of the GFP tag, which contains a weak nuclear-targeting signal. On the other hand, the difference in subcellular localization between Ran1 and Ran3 may implicate that these *Arabidopsis* Ran paralogs have distinct functions for each of them. Based on the localization patterns and sequence homology of Ran proteins, a possible link between the Ran signal transduction and proteins located in the nuclear envelope, possibly at or around the nuclear pores, has been proposed (Meier 2000).

The subcellular localization results (Fig. 5) are consistent with the Western blot data (Fig. 4), which indicate the predominant presence of the Ran proteins in the soluble fraction (cytosol). Ran proteins appear to exist in a gradient from the nuclear envelope (high concentration) to the perinuclear cytoplasmic region (low concentration). It is unknown how the Ran proteins are shuttled between the nuclear envelope and the surrounding cytoplasm. It is possible that Ran proteins are targeted to the nuclear envelope by forming a complex with their activating protein, AtRanGAP, which contains a WPP domain at the N-terminus (Rose and Meier 2001). This speculation is

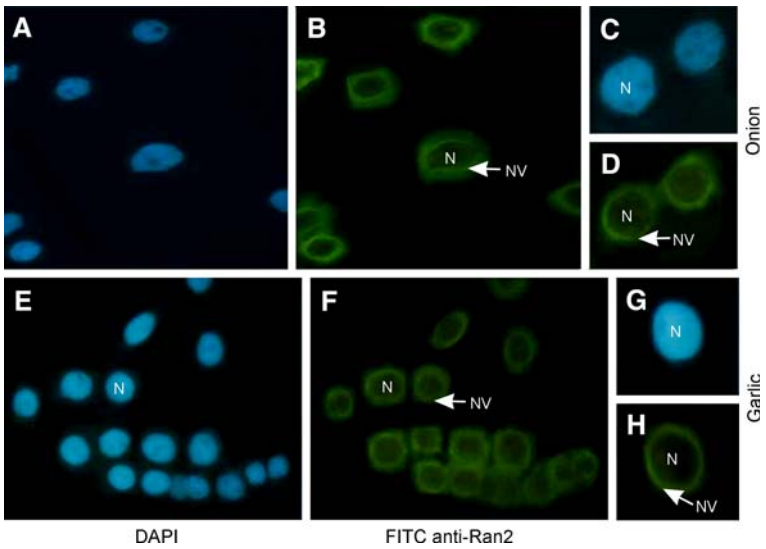

Fig. 5 Subcellular localization of Ran proteins in onion and garlic root tips by direct immunofluorescence labeling with anti-Ran2 antibody. **a–d** Subcellular localization of Ran proteins in onion root tips. **e–h** Subcellular localization of Ran proteins in garlic root tips. Three-day-old root tip cells of onion or garlic were stained with DAPI to indicate the position of the nuclei (*N*), and anti-Ran2 antibody labeled with FITC. Fluorescent images were taken using a fluorescence microscope equipped with appropriate filters for DAPI and FITC. Note that Ran2 (in *green*) is mainly localized to the nuclear envelope (*NV*) at interphase

supported by the fact that the Ran proteins have a very similar subcellular localization pattern to that of AtRanGAP (Rose and Meier 2001). The unique WPP domain of AtRanGAP has been shown to be necessary and sufficient to target RanGAP to the nuclear rim, and the WP amino acid pair is critical for this function (Rose and Meier 2001; Patel et al. 2004; Jeong et al. 2005). Animal RanGAPs adopt a different mechanism for protein targeting to the nuclear envelope. In vertebrates, SUMOylation of the C-terminal domain of RanGAP leads to a conformational change that allows the protein to bind to Nup358 (Matunis et al. 1996, 1998). Plants and animals have apparently evolved different mechanisms to regulate and anchor RanGAP and Ran proteins to the outer surface of the nuclear envelope.

A subset of the mammalian nuclear pore complex proteins, including Nup358, is among the first proteins to assemble on the decondensing chromosomes in early telophase (Haraguchi et al. 2000; Bodoor et al. 1999a, b; Wiese et al. 1997). Although none of them has been shown to be functionally required for nuclear envelope reassembly during cell division, it is conceivable that the DNA-binding Nups may play a role in the association of nuclear envelope components with the decondensing chromatin (Rose et al. 2004).

Evolution of different proteins into roles in nuclear envelope and nuclear pore dynamics might explain why RanGAP utilizes different anchor surfaces for nuclear-envelope attachment. The finding that Ran-GTP hydrolysis is required for the fusion of nuclear vesicles points to a role for the RanGAP-anchoring factor and RanGAP at an early stage of nuclear envelope reformation. The fundamental differences in nuclear envelope targeting of RanGAP in plants and animals lead us to speculate that there might be other different players in the spatial organization of Ran signaling in the plant and animal kingdoms.

In addition, plant Ran may have diverse functions in mitosis in plant cells. Base on the interaction of Ran2, PhrIP1 and phragmoplastin, we propose that Ran2 may be targeted to the cell plate at cytokinesis and play a role in mediation of vesicle fusion or membrane recycling. Ran2 could be re-directed to the forming cell plate either by the interaction with PhrIP1, which is part of the cell plate-specific phragmoplastin complex (our unpublished data), or by the association with RanGAP, which is re-distributed to the cell plate during cytokinesis (Pay et al. 2002; Jeong et al. 2005). Therefore, it would be of intriguing to investigate the dynamic distribution of Ran2 during the cell cycle.

Given the high sequence homology (95–96% identical at the amino acid level) shared by Ran2 with Ran1 and Ran3, it is likely that the polyclonal anti-Ran2 antibodies used in this study may cross-react with the other two Ran proteins. The three genes are also known to be expressed in a very similar pattern in *Arabidopsis* (Haizel et al. 1997). Whether or not these three Ran proteins have different subcellular localization remains to be studied. It would be of interest to further investigate if the three Ran proteins in *Arabidopsis* differ in subcellular localizations and more importantly in biological functions, especially during the progression of cell cycle.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China to Z.Z. (No. 30070370) and from the National Science Foundation to Z.H. (NSF-MCB 0548525, and NSF-IOB 0543923). We thank Drs. Zaiyun Li and Xianhong Ge for help in the preparation of plant specimens and subcellular localization of Ran2. We also thank Professor Junchu Zhou for advice on immunofluorescence microscopy, and Dr. Allan Caplan for critical reading of the manuscript.

References

Ach RA, Gruissem W (1994) A small nuclear GTP-binding protein from tomato suppresses a *Schizosaccharomyces pombe* cell-cycle mutant. Proc Natl Acad Sci USA 91:5863–5867

Bamba C, Bobinnec Y, Fukuda M, Nishida E (2002) The GTPase Ran regulates chromosome positioning and nuclear envelope assembly in vivo. Curr Biol 12:503–507

Bednarek SY, Falbel TG (2002) Membrane trafficking during plant cytokinesis. Traffic 3:621–629

Bodoor K, Shaikh S, Enarson P, Chowdhury S, Salina D, Raharjo WH, Burke B (1999a) Function and assembly of nuclear pore complex proteins. Biochem Cell Biol 77:21–329

Bodoor K, Shaikh S, Salina D, Raharjo WH, Bastos R, Lohka M, Burke B (1999b) Sequential recruitment of NPC proteins to the nuclear periphery at the end of mitosis. J Cell Sci 112:2253–2264

C. elegans Sequencing Consortium (1998) Genome sequence of the nematode *C. e*legans: a platform for investigating biology. Science 282:2012–2018

Dasso M (2001) Running on Ran: nuclear transport and the mototic sindle. Cell 104:321–324

Drivas GT, Shih A, Coutavas E, Rush MG, D'Eustachio P (1990) Characterization of four novel ras-like genes expressed in a human teratocarcinoma cell line. Mol Cell Biol 10:1793–1798

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360

Golemis EA, Gyuris H, Brent R (1996) Interaction trap/two hybrid system to identify interacting proteins. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Wiley, New York, pp 20.1.1–20.1.28

Gorlich D, Kutay U (1999) Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol 15:607–660

Gu X, Verma DPS (1996) Phragmoplastin, a dynamin-like protein associated with cell plate formation in plants. EMBO J 15:695–704

Gu X, Verma DPS (1997) Dynamics of phragmoplastin in living cells during cell plate formation and uncoupling of cell elongation from the plane of cell division. Plant Cell 9:157–169

Guan K, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione *S*-transferase. Anal Biochem 192:262–267

Haizel T, Merkle T, Pay A, Fejes E, Nagy F (1997) Characterization of proteins that interact with the GTP-bound form of the regulatory GTPase Ran in *Arabidopsis*. Plant J 11:93–103

Haraguchi T, Koujin T, Hayakawa T, Kaneda T, Tsutsumi C, Imamoto N, Akazawa C, Sukegawa J, Yoneda Y, Hiraoka Y (2000) Live fluorescence imaging reveals early recruitment of emerin, LBR, RanBP2, and Nup153 to reforming functional nuclear envelopes. J Cell Sci 113:779–794

Hillig RC, Renault L, Vetter IR, Drell TT, Wittinghofer A, Becker J (1999) The crystal structure of rna1p: a new fold for a GTPase-activating protein. Mol Cell 3:781–791

Hong Z, Delauney AJ, Verma DPS (2001a) A cell plate-specific callose synthase and its interaction with phragmoplastin and UDP-glucose transferase. Plant Cell 13:755–768

Hong Z, Zhang ZM, Olson JM, Verma DPS (2001b) A novel UDP-glucose transferase is part of the callose synthase complex and interacts with phragmoplastin at the forming cell plate. Plant Cell 13:769–779

Hong Z, Bednarek S, Blumwald E, Hwang I, Jurgens G, Menzel D, Osteryoung K, Raikhel N, Shinozaki K, Tsutsumi N, Verma DPS (2003) A unified nomenclature for *Arabidopsis* dynamin-related large GTPases based on homology and possible functions. Plant Mol Biol 53:261–265

Hopper AK, Traglia HM, Dunst RW (1990) The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. J Cell Biol 111:309–321

Jeong SY, Rose A, Joseph J, Dasso M, Meier I (2005) Plant-specific mitotic targeting of RanGAP requires a functional WPP domain. Plant J 42:270–282

Jiang SY, Ramachandran S (2006) Comparative and evolutionary analysis of genes encoding small GTPases and their activating proteins in eukaryotic genomes. Physiol Genomics 24:235–251

Kadowaki T, Goldfarb D, Spitz LM, Tartakoff AM, Ohno M (1993) Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. EMBO J 12:2929–2937

Kahana JA, Cleveland DW (1999) Beyond nuclear transport. Ran-GTP as a determinant of spindle assembly. J Cell Biol 146:1205–1210

Kalab P, Pralle A, Isacoff EY, Heald R, Weis K (2006) Analysis of a RanGTP-regulated gradient in mitotic somatic cells. Nature 440:697–701

Lounsbury KM, Richards SA, Carey KL, Macara IG (1996) Mutations within the Ran/TC4 GTPase. Effects on regulatory factor interactions and subcellular localization. J Biol Chem 271:32834–32841

Mattaj IW, Englmeier L (1998) Nucleocytoplasmic transport: the soluble phase. Annu Rev Biochem 67:265–306

Matunis MJ, Coutavas E, Blobel G (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase activating protein RanGAP1 between the cytosol and the nuclear pore complex. J Cell Biol 135:1457–1470

Matunis MJ, Wu JA, Blobel G (1998) SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. J Cell Biol 140:499–509

Meier I (2000) A novel link between ran signal transduction and nuclear envelope proteins in plants. Plant Physiol 124:1507–1510

Melchior F, Weber K, Gerke V (1993) A functional homologue of the RNA1 gene product in *Schizosaccharomyces pombe*: Purification, biochemical characterization, and identification of a leucine-rich repeat motif. Mol Biol Cell 4:569–581

Merkle T, Haizel T, Matsumoto T, Harter K, Dallmann G, Nagy F (1994) Phenotype of the fission yeast cell cycle regulatory mutant *pim*-46 is suppressed by a tobacco cDNA encoding a small, Ran-like GTP-binding protein. Plant J 6:555–565

Moor MS, Blobel G (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature 365:661–663

Ohtsubo M, Okazaki H, Nishimoto T (1989) The RCCI protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. J Cell Biol 109:1389–1397

Patel S, Rose A, Meulia T, Dixit R, Cyr RJ, Meier I (2004) *Arabidopsis* WPP-domain proteins are developmentally associated with the nuclear envelope and promote cell division. Plant Cell 16:3260–3273

Pay A, Resch K, Frohnmeyer H, Fejes E, Nagy F, Nick P (2002) Plant RanGAPs are localized at the nuclear envelope in interphase and associated with microtubules in mitotic cells. Plant J 30:699–709

Ren M, Drivas G, D'Eustachio P, Rush MG (1993) Ran/TC4: a small nuclear GTP-binding protein that regulates DNA synthesis. J Cell Biol 120:313–323

Richards SA, Lounsbury KM, Macara IG (1995) The C terminus of the nuclear RAN/TC4 GTPase stabilizes the GDP-bound state and mediates interactions with RCC1, RAN-GAP, and HTF9A/RANBP1. J Biol Chem 270:14405–14411

Rose A, Meier I (2001) A domain unique to plant RanGAP is responsible for its targeting to the plant nuclear rim. Proc Natl Acad Sci USA 98:15377–15382

Rose A, Patel S, Meier I (2004) Plant nuclear envelope proteins. Symp Soc Exp Biol 56:69–88

Samuels AL, Giddings THjr, Staehelin LA (1995) Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. J Cell Biol 130:1345–1357

Sazer S, Dasso M (2000) The Ran decathlon: multiple roles of Ran. J Cell Sci 113:1111–1118

Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. Physiol Rev 81:153–208

Trieselmann N, Wilde A (2002) Ran localizes around the microtubule spindle in vivo during mitosis in *Drosophila* embryos. Curr Biol 12:1124–1129

Verma DPS (2001) Cytokinesis and building of the cell plate in plants. Annu Rev Plant Physiol Plant Mol Biol 52:751–784

Verma DPS, Hong Z (2005) The ins and outs in membrane dynamics: tubulation and vesiculation. Trends Plant Sci 10:159–165

Vernoud V, Horton A C, Yang Z, Nielsen E (2003) Analysis of the small GTPase gene superfamily of *Arabidopsis*. Plant Physiol 131:1191–1208

Wiese C, Goldberg MW, Allen TD, Wilson KL (1997) Nuclear envelope assembly in *Xenopus* extracts visualized by scanning EM reveals a transport-dependent 'envelope smoothing' event. J Cell Sci 110:1489–1502

Wilde A, Zheng Y (1999) Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. Science 284:1359–1362

Yano A, Kodama Y, Koike A, Shinya T, Kim HJ, Matsumoto M, Ogita S, Wada Y, Ohad N, Sano H (2006) Interaction between methyl CpG-binding protein and ran GTPase during cell division in tobacco cultured cells. Ann Bot 98:1179–1187

Zhang C, Clarke PR (2000) Chromatin-independent nuclear envelope assembly induced by Ran GTPase in *Xenopus* egg extracts. Science 288:1429–1432

Zhang C, Clarke PR (2001) Roles of Ran-GTP and Ran-GDP in precursor vesicle recruitment and fusion during nuclear envelope assembly in a human cell-free system. Curr Biol 11:208–212

Zhang Z, Hong Z, Verma DPS (2000) Phragmoplastin polymerizes into spiral coiled structures *via* intermolecular interaction of two self-assembly domains. J Biol Chem 275:8779–8784

Zhang C, Hutchins JR, Muhlhauser P, Kutay U, Clarke PR (2002) Role of importin-beta in the control of nuclear envelope assembly by Ran. Curr Biol 12:498–502