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Plant RanGAPs are localized at the nuclear envelope in interphase and associated with microtubules in mitotic cells

Aniko Pay[1], Katja Resch[2], Hanns Frohnmeyer[2], Erzsebet Fejes[1], Ferenc Nagy[1] and Peter Nick[2,*]
[1]*Plant Biology Institute, Biological Research Center, H-6701 Szeged, PO Box 521, Hungary, and*
[2]*Institut für Biologie II/Botanik, Schänzlestrasse 1, Universität Freiburg, D-79104 Freiburg, Germany*

Received 17 December 2001; revised 7 March 2002; accepted 15 March 2002.
[*]For correspondence (fax +49 761 203 2612; e-mail pnick@uni-freiburg.de).

Summary

In animals and yeast, the small GTP-binding protein Ran has multiple functions – it is involved in mediating (i) the directional passage of proteins and RNA through the nuclear pores in interphase cells; and (ii) the formation of spindle asters, the polymerization of microtubules, and the re-assembly of the nuclear envelope in mitotic cells. Nucleotide binding of Ran is modulated by a series of accessory proteins. For instance, the hydrolysis of RanGTP requires stimulation by the RanGTPase protein RanGAP. Here we report the complementation of the yeast RanGAP mutant rna1 with *Medicago sativa* and *Arabidopsis thaliana* cDNAs encoding RanGAP-like proteins. Confocal laser microscopy of *Arabidopsis* plants overexpressing chimeric constructs of GFP with AtRanGAP1 and 2 demonstrated that the fusion protein is localized to patchy areas at the nuclear envelope of interphase cells. In contrast, the cellular distribution of RanGAPs in synchronized tobacco cells undergoing mitosis is characteristically different. Double-immunofluorescence shows that RanGAPs are co-localized with spindle microtubules during anaphase, with the microtubular phragmoplast and the surface of the daughter nuclei during telophase. Co-assembly of RanGAPs with tubulin correlates with these *in vivo* observations. The detected localization pattern is consistent with the postulated function of plant RanGAPs in the regulation of nuclear transport during interphase, and suggests a role for these proteins in the organization of the microtubular mitotic structures.

Keywords: *Arabidopsis thaliana* Wassilewskija, microtubules, nuclear envelope, nuclear transport, RanGAP1, small GTPases.

Introduction

Ran, a highly conserved small GTP-binding protein of the Ras superfamily, was originally identified as an essential component of the machinery that transports macromolecules into and out of the nucleus. The directionality of these processes, i.e. the assembly of the import complex in the cytosol, the release of the cargo protein in the nucleus, and the assembly of the export complex in the nucleus, is maintained by a sharp gradient in the concentration of RanGTP between the nucleus and cytoplasm. As Ran has only a low intrinsic GTPase activity, the conversion of RanGTP into RanGDP requires the RanGTPase-activating protein RanGAP1 and its associated factor RanBP1. Conversely, the exchange of RanGDP for RanGTP is promoted by the guanine nucleotide exchange factor RanGEF, also called RCC1. The gradient in the concentration of RanGTP, and thus the directionality of export and import, is maintained by a strict compartmentalization of these modulatory proteins, with RCC1 being confined to the nucleus whereas RanGAP1 decorates the outer part of the nuclear envelope (for a review see Görlich and Kutay, 1999).

Our knowledge about the molecular mechanisms that mediate the bidirectional transport of macromolecules through the nuclear membranes in higher plants is limited as compared to other eukaryotic systems, but it is rapidly expanding. Structural components of plant nuclear pore complexes (Heese and Raikhel, 1998), through which the import occurs, have been identified, as well as proteins

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required to mediate the import process. Genes have been identified in higher plants that encode these molecular components of the import process, such as importin α which recognizes nuclear localization signals of cargo proteins, and importin β which forms the active import complex in the cytosol together with RanGDP, importin α and the bound cargo protein (Smith and Raikhel, 1999). A gene encoding the export receptor XPO1 (Haasen *et al.*, 2000), which recognizes nuclear export signals on the cargo protein and forms the active export complex in the nucleus together with RanGTP, as well as genes coding for Ran and the RanGTP-binding protein RanBP1, have also been isolated (Merkle and Nagy, 1997). *In vitro* sytems have been developed to study import into (Merkle and Nagy, 1997) and export out of the nucleus (Haasen *et al.*, 2000) in plant cells. However, to date the characterization of genes coding for other key elements postulated to be involved in these processes, such as (i) the Ran accessory proteins RanGAP and RanGEF (RCC1); and (ii) proteins defining the structure of the nuclear pore complex, have remained elusive in plants.

During the past two years it became evident that, in animal cells, in addition to its role in nuclear import/export in interphase cells, the Ran protein participates in the organization of the division spindle during mitosis. A couple of recent publications (reviewed by Kahana and Cleveland, 2001) have demonstrated that spontaneous microtubule asters are formed in *Xenopus* egg extracts, when the conversion of Ran-GTP to Ran-GDP (triggered by RanGAP1) is blocked by expressing appropriate mutant versions of Ran. This effect can be counteracted by addition of importin α and β (Wiese *et al.*, 2001). These results suggest that, on breakdown of the nuclear envelope, which is followed by an immediate spindle formation, Ran-GTP promotes the release of microtubule-nucleating factors such as NuMa or TPX2 from the importins which then triggers formation of microtubule asters. In other words, in the absence of the nuclear envelope RanGAP will be distributed equally, whereas the concentration of the exchange factor RCC1 is highest in the closest vicinity to the chromosomes. Thus Ran-GTP-promoted release of NuMa and TPX2 can occur, and is indeed confined to these areas. Conversely, the assembly of a new nuclear envelope requires the presence of Ran-GDP provided by the activity of RanGAP1 (Hetzer *et al.*, 2000; Zhang and Clarke, 2000).

However, it is not known whether, and to what extent, Ran and the mechanism described above are involved in regulating spindle formation during mitosis in plant cells. When plant cells prepare for mitosis, this is heralded by a migration of the nucleus to the site where the prospective cell plate will be formed. This movement is driven by the phragmosome, a specialized array of actin microfilaments which is tethered to the nuclear envelope and defines the symmetry of the ensuing cell division (for a review see Lloyd, 1991). The nucleus will then organize the preprophase band, a microtubular structure that defines the axis of cell division (Murata and Wada, 1991). Later, when the daughter nuclei have been reformed, it is again the nuclear envelope that organizes the new microtubular cytoskeleton. Thus the nuclear envelope is functionally equivalent to the centrosomes that have been lost during the evolution of seed plants, and appears to be an important regulator for cell shape (for review see Lambert, 1993).

This dynamic interaction of cytoskeleton and nuclear envelope during the cell cycle indicates intense signalling events from the nuclear envelope to molecular targets associated with the cytoskeleton. This is supported by the observation that the transition between cell growth and cell division is flexible in plants, and is regulated by exogenous and developmental signals (Cdc2/cyclin, Hemerly *et al.*, 1993; auxin-binding protein, Jones *et al.*, 1998; blue light, Wada and Furuya, 1970). The components of these signalling events are expected to reside in the nuclear envelope and to interfere with the organization of microtubules during spindle formation.

It is accepted that the RanGTPase-activating proteins (RanGAPs) are essential accessory factors in regulating the RanGTP gradient that determines the sequence of molecular events underlying nuclear transport and spindle formation in animal cells. The aim of the present study was therefore to isolate genes encoding plant RanGAP-like proteins and to characterize their cellular distribution during interphase and mitosis. In this paper we describe (i) the cloning of RanGAP cDNAs from *Medicago sativa* and *Arabidopsis thaliana* via functional complementation of a temperature-sensitive yeast RanGAP mutant; (ii) demonstrate the localization of the AtRanGAP1 and AtRanGAP2 proteins at the nuclear envelope of interphase cells in transgenic plants; and (iii) demonstrate the association of tobacco RanGAPs with spindle microtubules and phragmoplast during mitosis in cultured cells. Co-assembly of RanGAPs with tubulin from mitotic but not from interphase cells correlates well with the results of localization studies.

Results

Cloning of a Medicago *RanGAP1 by complementation of a yeast RanGAP mutant*

The PSY714 *Saccharomyces cerevisiae* strain carrying the temperature sensitive *rna1* mutation was transformed with the alfalfa λ-Max 1 cDNA library. After screening approximately 630 000 colonies, one transformant was obtained (MsRanGAP) which complemented the RanGAP-deficient phenotype of the *rna1* yeast mutant (Bischoff *et al.*, 1995). To verify that the complementation was due to

the expression of the plasmid containing the plant cDNA, we amplified the isolated MsRanGAP plasmid in *Escherichia coli* XL1-Blue cells and retransformed it into the *PSY714* yeast mutant. The number of transformant colonies was similar at both the permissive (23°C) and the restrictive temperature (37°C), confirming that the plant cDNA was responsible for the rescue of the mutant. The determined DNA sequence and the predicted protein sequence of the MsRanGAP cDNA are available from the GenBank database (accession number AF215731).

Identification of Arabidopsis AtRanGAP1 and AtRanGAP2 cDNA clones

When we were performing the complementation experiments, we did not have an *Arabidopsis* cDNA library equivalent of the alfalfa λ-Max 1 cDNA library. Thus we used the full-length alfalfa MsRanGAP cDNA as a probe to screen a CD4-15 *Arabidopsis* cDNA library and to isolate cDNAs encoding MsRanGAP homologues. Screening of 3×10^5 recombinant phages yielded six positive clones. The two longest inserts, 1.9 and 2.1 kb, were sequenced. These *Arabidopsis* cDNAs were closely related but not identical, and showed about 62 and 59% similarity to the MsRanGAP sequence (GenBank accession numbers AF214559and AF214560, respectively). To examine whether the two *Arabidopsis* RanGAP genes can provide the functions of the budding-yeast *rna1* gene, we tried to complement the temperature-sensitive *S. cerevisiae* PSY714mutant that has been used to identify the alfalfa RanGAP homologue. In contrast to the empty vector, the yeast/*E. coli* shuttle vector (pYES2) carrying either the AtRanGAP1 or AtRanGAP2 cDNA was able to complement the *rna1* yeast mutant at the restrictive temperature. To determine the number of AtRanGAP-related genes in *Arabidopsis*, Southern analyses were performed using the radiolabelled coding region of the cDNAs (AtRanGAP1 or AtRanGAP2) as probes. In both cases, after digestion of genomic DNA with three different restriction endonucleases, only a single hybridizing band was found under stringent hybridization (data not shown). These results were confirmed by searching the *Arabidopsis* whole-genome database for the positions and putative homologues of these two genes. First, BLAST searches revealed that the *Arabidopsis* genome does not contain any other genes encoding proteins that exhibit significant homology to AtRanGAP1 and AtRanGAP2. Second, BLAST searches showed that the AtRanGAP1 and AtRanGAP2 are present as single-copy genes. AtRanGAP1 is localized at chromosome 3 (BAC clone T20010, accession no. AL16381, start codon 67994, stop codon 69601) and contains one intron. AtRanGAP2 is localized at chromosome 5 (BAC clone F7K24, accession no. AF296837, start codon 34063, stop codon 32426) and contains four introns.

Structure of plant RanGAP proteins

Deduced amino acid sequences of the AtRanGAP1, AtRanGAP2, MsRanGAP1, human and yeast RanGAP are shown in Figure 1. Sequence comparison indicates that the plant RanGAP proteins display approximately 25% identity and 45% similarity to their yeast and human counterparts. Sequence conservation between *Medicago* and *Arabidopsis* RanGAP proteins is more significant, with about 65% identity. The predicted molecular weight of RanGAP proteins is between 58 and 60 kDa. Figure 1 depicts those amino acid residues that are evolutionarily conserved, and also demonstrates that the plant RanGAP proteins contain the conserved arginine residue shown to be necessary for Ran binding and GTPase activation in the yeast rna1 protein. These data, together with the fact that all the plant RanGAP proteins tested were able to complement the yeast *rna1* RanGAP mutant, indicate that the plant RanGAPs are functional orthologues of other eukaryotic RanGAPs. Figure 1 also illustrates that the N-terminal domain of plant RanGAPs displays only a low level of homology to that of yeast and human RanGAPs. However, the N-terminal domain of plant RanGAPs shows significant homology to MAF1 proteins which are found only in higher plants, as reported by Meier (2000). MAF1-like proteins are localized at the nuclear rim and interact with the MFP1 protein (Gindullis *et al.*, 1999) which is also associated with the nuclear envelope. MFP1 was found to bind DNA of the matrix attachment region, and was therefore proposed to play a role in attaching chromatin, through matrix attachment regions, to the nuclear envelope (Gindullis and Meier, 1999).

Expression of the AtRanGAP1/GFP and AtRanGAP2/GFP fusion proteins in transgenic plants

To monitor the cellular distribution of AtRanGAP proteins, we generated transgenic *A. thaliana* (ecotype Wassilewskija) plants that expressed the AtRanGAP1::GFP or AtRanGAP2::GFP fusion proteins. Expression level of the fusion proteins was determined by Western analysis using total protein extracts which were probed with a polyclonal antibody raised against full-length, recombinant AtRanGAP1. Figure 2(a) shows that this antibody detected a band at ≈60 kDa in protein extracts derived from untransformed plants consistent with the predicted molecular weight (58.8 kDa). This figure also shows that in extracts from transgenic plants expressing the AtRanGAP1::GFP, in addition to the 60 kDa band, the antibody detected a more abundant 100 kDa band which was clearly absent in the untransformed wild type. The abundance of this 100 kDa band varied among the different transgenic plants analysed, but showed good correlation with the GFP fluorescence detected by microscopy.

Similar results were obtained by analysing extracts derived from AtRanGAP2::GFP transgenic plants (data not shown). It follows that the antibody recognizes both AtRanGAP proteins: the 60 kDa band represent the endogenous AtRanGAP1, AtRanGAP2, and the 100 kDa band, the GFP fusion proteins, respectively.

AtRanGAP1 is localized to the nuclear envelope in interphase cells

The presence of the GFP fusion protein in the Western analysis was always correlated with a strong green fluorescence of nuclei in the transformants (Figure 2c,d). This fluorescence, observed in nuclei of both AtRanGAP1::GFP (Figure 2c) and AtRanGAP2::GFP (Figure 2d) overexpressors, was never detected in nuclei of wild-type seedlings (Figure 2b), excluding the possibility that it was caused by unspecific autofluorescence. The nuclei were ubiquitously labelled in all cells of the seedlings (Figure 2e). A detailed analysis of the GFP fluorescence by confocal laser scanning microscopy revealed that the fusion proteins were strictly localized at the nuclear envelope for both AtRanGAP1 (Figure 2f) and AtRanGAP2 (Figure 2g). At high magnification, the association of the fusion protein with the nuclear envelope was found to be discontinuous. It appeared as patchy areas on the surface of the nucleus that were interconnected by broad filamentous connections. This was observed for both AtRanGAP1::GFP (Figure 2h) and AtRanGAP2::GFP (Figure 2i). The fluorescent signals that occasionally were observed inside the nucleus (for example Figure 2h, iii, white arrow) could be traced through subsequent confocal sections (Figure 2h, i–iii, white arrow) to the nuclear surface, suggesting that they represent protrusions of the envelope towards the centre of the nucleus. This is consistent with the recently published finding that plant nuclei are often not spherical, but irregular in shape with numerous protrusions and lacunae extending into and sometimes even through the nucleus (Collings *et al.*, 2000). The phenotype of transgenic plants overexpressing AtRanGAP1::GFP or AtRanGAP2::GFP was not obviously different from wild-type plants that were grown in parallel under exactly the same conditions. To test whether the observed patterns correspond to the localization of endogenous RanGAP-proteins, RanGAP was detected in seedlings of the untransformed wild type by immunofluorescence using the antibody raised against AtRanGAP1 (Figure 2j,k). As a negative control, the primary antibody was replaced by the respective pre-immune serum (data not shown). This antibody specifically stained the nuclear envelope (Figure 2j), and high-resolution confocal microscopy again revealed patchy staining (Figure 2k) similar to that observed in the AtRanGAP1::GFP plants (Figure 2h,i).

RanGAPs are associated with spindle and phragmoplast during mitosis

We determined the localization of the tobacco RanGAP protein during cell division in a partially synchronized tobacco cell line (VBI-O) by using double-immunofluorescence analysis of RanGAP and microtubules. Our data show that the tobacco RanGAP protein is closely associated with spindle microtubules (Figure 3a). Merges of both signals reveal that the majority of RanGAP decorates the spindle microtubules. However, a smaller subpopulation of RanGAP is localized between microtubule bundles forming minute, interconnected side branches (Figure 3a). During telophase, when the daughter nuclei had already been reformed (Figure 3c), RanGAP was concentrated in the phragmoplast, a microtubular structure associated with the growing cell plate. When the two images obtained for RanGAP and microtubules are merged (Figure 3c), it becomes evident that, similar to the situation in the spindle (Figure 3a), a second subpopulation of RanGAP is observed that is not associated with phragmoplast microtubules, but forms a reticulate-like mesh inside the newly formed daughter nuclei. We assume that this type of RanGAP signal again represents the numerous protrusions of nuclear envelope (Collings *et al.*, 2000) that are observable during this stage.

The RanGAP signal overlaps with the tubulin signal at the nuclear envelope (Figure 3c, white arrow), at the site where the new microtubular interphase array is formed. In addition to staining of the nuclear envelope, in some cases interphase cells of the tobacco suspension cultures showed small vesicular signals subjacent to the cell wall (data not shown). In experiments where this tobacco cell line was transiently transformed with the AtRanGAP1::GFP and AtRanGAP2::GFP chimeric gene, the distribution of GFP fluorescence was very similar (data not shown).

Figure 1. Sequence alignment of five putative RanGAP protein sequences and of the *Arabidopsis* MAF1.
Arabidopsis AtRanGAP1; *Medicago sativa* MsRanGAP; *Arabidopsis* AtRanGAP2; *Arabidopsis* AtMAF1; *Saccharomyces cerevisiae* ScRNA1; human HsRanGAP (GenBank accession nos AF214559, AF215731, AF214560, AB008267, X17376and NP_002874, respectively). Black shading, amino acids identical in at least three sequences; grey shading, functionally conserved amino acids in at least three sequences; ◆, amino acids fully conserved in putative MAF1protein sequences from higher plants (Meier, 2000); *, conserved arginine residue necessary for Ran binding and GTPase activation in the yeast RNA1protein.

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AtRanGAP1    1 ---------------------------MDHSAKTTQNRVLSVKMWPPSKSTRLMLVERMTKNITTPSIFS
MsRanGAP     1 ---------------------------MDSSVPSYQHRTLSIKLWPPSQSTRLMLVERMVRNLTTPSIFS
AtRanGAP2    1 ----------------------------MADILDSRPHAFSIKLWPPSLPTRKALIERITNNFSSKTIFT
AtMAF1       1 MAETETESITTSSPPPISETENSTTLPTTETEKNPNPVTISLRIWPPTQKTRDAVINRLIETLSTESILS
ScRNA1       1 ----------------------MATLHFVPQHEEEQVYSISGKALKLTTSDDIKPYLEELAALETCTKLD
HsRanGAP     1 ---------------MASEDIAKLAETLAKTQVAGGQLSFKGKSLKLNTAEDAKDVIKEIEDFDSLEALR

AtRanGAP1   44 RKYGLLSVEEAEQDAKRIEDLAFATANKHFQNEPDGDGTSAVHVYAKESSKLMLDVIKRGPQEESE----
MsRanGAP    44 RKYGLLNKDEAEKDAKEIEDAAFVTASQHFEKEPDGDGSSAVQIYAKESSKLMLEVLKRGPRGKEENGEL
AtRanGAP2   43 EKYGSLTKDQATENAKRIEDIAFSTANQQFEREPDGDGGSAVQLYAKECSKLILEVLKKGPVAKVAAREL
AtMAF1      71 KRFGSLESEEAASSVAKSIEDEAVAIASATVFG--DDDGIEILKAYSKEISKRMLESVKAKSNVASP----
ScRNA1      49 LSGNTIGTEASEALAKCIAENTQVRESLVEVNFADLYTSRLVDEVVDSLKFLLPVLLKCPHLEIVN----
HsRanGAP    56 LEGNTVGVEAARVIAKALEKKSELKRCHWSDMFTGRLRTEIPPALISLGEGLITAGAQLVELDLSDN---

AtRanGAP1  110 --VEVSKDGDVFFDISGGSRAFIEEEEARDLLRPLADPRNSYTKIRFSNRSFGSEAAKFAASVLSSIKDQ
MsRanGAP   114 ISEKGDAAVETVFDISGGRRAFIDGQEASELLKPLMGP-NSFTKICFSNRSFGLDAAHVVEPMLISIKDQ
AtRanGAP2  113 ISEDSVSPRETFFDISKGKRAFIEAEEAEELLKPLKEPGNAYTKICFSNRSFGLGAARVAEPILASLKDQ
AtMAF1     135 -------PPKDGDGIESAVDSKIDSSEA------------------------------------------
ScRNA1     115 ----LSDNAFGLRTIELLEDYIAH---AVNLKHLIISN--------------------------------
HsRanGAP   123 --AFGPDGVQGFEALLKSSACFTLQELKLNNCGMGIGG--------------GKILAAALTECHRKSSAQ

AtRanGAP1  178 LTEVDLSDFVAGRPEAEALEVMNMFSSALEGSKLRYLNLSDNALGEKGIRAFASLINSQHDLEELYLMND
MsRanGAP   183 LKEVDLSDFIAGRPEAEALEVMNIFSSALERAVLRYLNLSNNAMGEKGVRAFRALLKSQNDLEELYLMND
AtRanGAP2  183 LKEVDLSDFVAGRPELEALEVMNIFSDALQGSILSSLNLSDNALGEKGVRAFGALLKSLSSLEELYLMND
AtMAF1         ----------------------------------------------------------------------
ScRNA1     146 ---NGVGPFAGERIGKALFHLAQNKKAASKPFLETFICGRNRVREWIRSLLSSGFEKPLRSLKVVKLYQN
HsRanGAP   177 GKPLALKVFVAGRNRLENDGATALAEAFRVIGTLEEVHMPQNGINHPGITALAQAFAVNPLLRVINLNDN

AtRanGAP1  248 GISEDAARAVRELLPSTDKIRVLQFHNNMTGDEGATAIAEIVRE-CPSLEDFRCSSTRIGSEGGVALAEA
MsRanGAP   253 GISEEAAKAVAELIPSTEKLKVLHFHNNMTGDEGAFAIAEVVKR-SPALEDFRCSSTRVGSEGGVALAEA
AtRanGAP2  253 GISKEAAQAVSELIPSTENLRVLHFHNNMTGDEGALAIAEVVKR-SPLLENFRCSSTRVGSKGGIALSEA
AtMAF1         ----------------------------------------------------------------------
ScRNA1     213 GIRPKGVATLIHYG--------------------------------------------------------
HsRanGAP   247 TFTEKGAVAMAETLKTLRQVEVINFGDCLVRSKGAVAIADAIRGGLPKLKELNLSFCEIKRDAALAVAEA

AtRanGAP1  317 LEHCSHLKKLDLRDNMFGVEGGIALAKTLSVLTHLTEIYMSYLNLEDEGTEALSEALLKSAPSLEVLELA
MsRanGAP   322 LGACTHLKKLDLRDNMFGVEAGVALSKVIPVFADLTEIYLSYLNLEDDGAEALANALKESAPSLETLDMA
AtRanGAP2  322 LEHCTHMEKLDLRDNMFGTEAGVSLSKTLSSFKHMTELYLSYLNLEDEGAIAIVNALKESASPIEVLEMA
AtMAF1         ----------------------------------------------------------------------
ScRNA1     227 LQYLKNLEILDLQDNTFTKHASLILAKALPTWKDS---------------------LFELNLNDCLLKTA
HsRanGAP   317 MADKAELEKLDLNGNTLGEEGCEQLQEVLEGFNMAKVLASLSDDEDEEEEEEGEEEEEEAEEEEEEDEEE

AtRanGAP1  387 GNDI------------------------------------------TVKSTGNLAACIASKQSLAKLNLS
MsRanGAP   392 GNDI------------------------------------------TAKATVSVAECISSKQFLTKLNLS
AtRanGAP2  392 GNDI------------------------------------------TVEAASALAACVAAKQDLNKLNLS
AtMAF1         ----------------------------------------------------------------------
ScRNA1     276 GSDE------------------------------------------VFKVFTEVKFPN---LHVLKFEYN
HsRanGAP   387 EEEEEEEEEEEPQQRGQGEKSATPSRKILDPNTGEPAPVLSSPPPADVSTFLAFPSPEKLLRLGPKSSVL

AtRanGAP1  415 ENELKDEGTILIAKAVEGH-DQLVEVDLSTNMIRRAGARALAQTVVKKNTFKLLNINGNFISEEGIDEVN
MsRanGAP   420 ENELKDEGAGLISKALEGR-GQLSEVDLSTNLITWSGAKLLAEAVVQKPGFKLLNINANFISDEGIDELK
AtRanGAP2  420 ENELKDEGCVQIANCIEEVNSKLQYIDMSTNYIRRAGARALAHVVVKKEAFKLLNIDGNIISEEGIEELK
AtMAF1         ----------------------------------------------------------------------
ScRNA1     301 EMAQETIEVSFLPAMEKGNLPELEKLEINGNRLDED-SDALDLLQSKFDDLEVDDFEEVDSEDEEGEDEE
HsRanGAP   457 IAQQTDTSDPEKVVSAFLKVSSVFKDEATVRMAVQDAVDALMQKAFNSSSFNSNTFLTRLLVHMGLLKSE

AtRanGAP1  484 DMFKDCLDKLVPLDDNDPEGEDFEDEDEEE-----GEDGNELESKLGSLKIKQGEE-----
MsRanGAP   489 DIFKNSPDMLGPLDENDPEGEDVDEEAEDD-------SDNDELESKLKGLEI---------
AtRanGAP2  490 EIFKKSPELLGALDENDPDGEEDDDDEEDEEDEENEGNGNGELESKLKNLEVNQED-----
AtMAF1         -------------------------------------------------------------
ScRNA1     370 DEDEDEKLEEIETERLEKELLEVQVDDLAER--------LAETEIK---------------
HsRanGAP   527 DKVKAIANLYGPIMALNHMVQQDYFPKALAPLLLAFVTKPNSALESCSFARHSLLQTLYKV
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RanGAPs co-assemble with microtubules in extracts from mitotic cells, but not from stationary cells

Co-localization of RanGAP and microtubules in mitotic cells suggests that these proteins are components of the same complex formed during mitosis. To test this hypothesis, cytosolic extracts from cycling and stationary tobacco cells were subjected to a microtubule co-assembly assay (Nick *et al.*, 1995). The assembly of microtubules is induced by addition of Mg^{2+}, GTP and taxol at 30°C in the presence of low concentrations of KCl (to inhibit unspecific binding of proteins to tubulin) in this assay. The assembled microtubules are collected by ultracentrifugation and the co-assembled proteins are then detached from this microtubule sediment by resuspension at high ionic strength, and subsequently separated by a second ultracentrifugation. In extracts from cycling cells (Figure 3b, left panel), RanGAPs co-assemble with microtubules and are depleted from the supernatant. Subsequently, they can be detached from the microtubule sediment at high ionic strength. This figure also shows that RanGAPs do not co-assemble with microtubules, but remain in the supernatant in extracts derived from stationary cells.

Discussion

Localization of plant RanGAPs in interphase cells

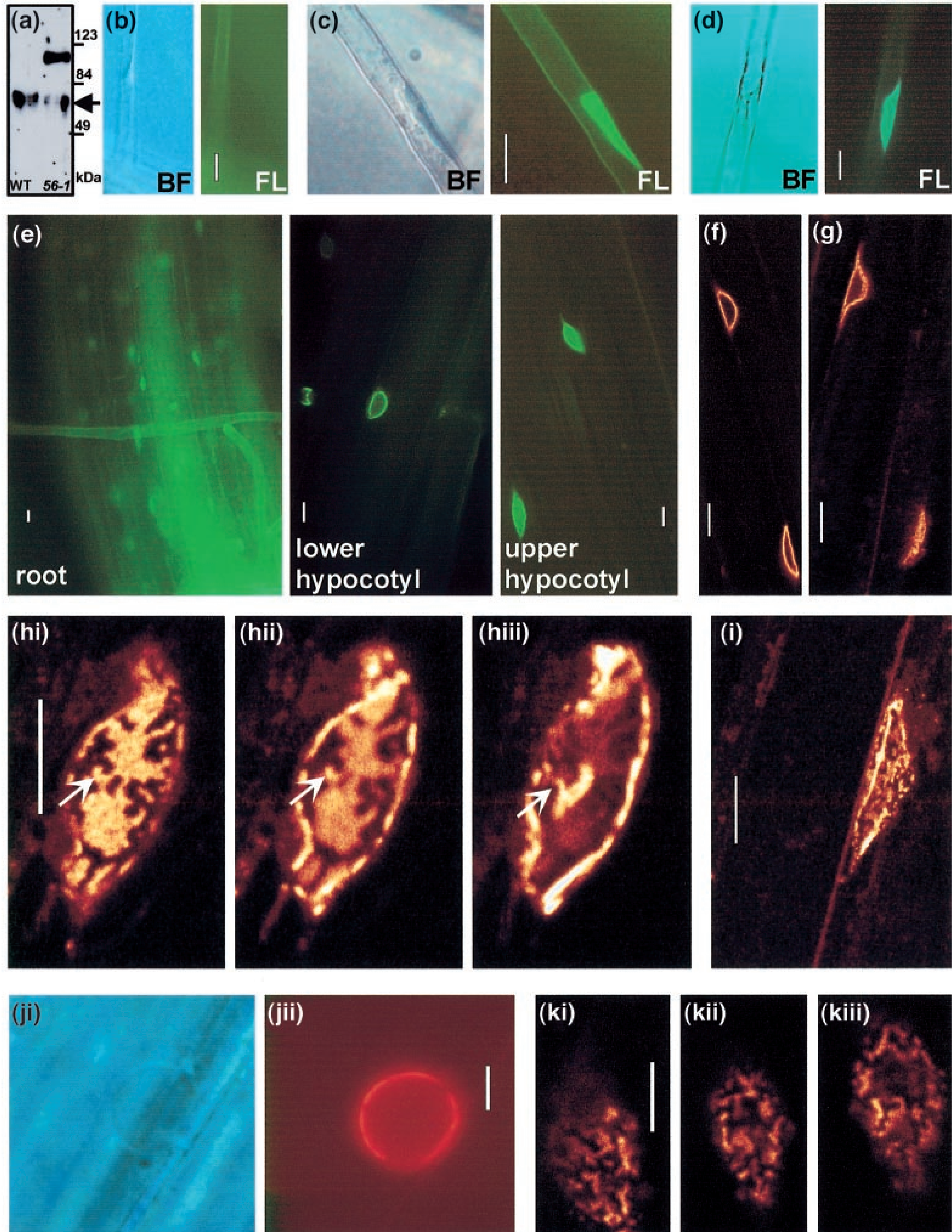
Yeast and human RanGAP proteins hydrolyse RanGTP into RanGDP and, together with other Ran-binding proteins such as RCC1 and RanBP1, mediate the completion of the RanGTP–GDP cycle. They therefore play a key role in regulating nuclear import/export processes. The sequence homology with the human and yeast RanGAP, together with the successful complementation of the *rna1* mutant that possesses a non-functional RanGAP protein, suggest that plant RanGAPs are functional orthologues. Moreover, the AtRanGAP1::GFP and AtRanGAP2::GFP fusion proteins decorate the nuclear envelope of all cell types monitored in transgenic *Arabidopsis* plants. This localization pattern is not an overexpression artefact, as visualization of endogenous AtRanGAPs by immunofluorescence in the wild type revealed a very similar localization. Taken together, our results strongly suggest that these plant proteins, like their animal and yeast counterparts, are involved in mediating nuclear import of proteins in plant cells.

However, some features of the plant RanGAP genes and proteins differ sharply from their mammalian or yeast counterparts. First, the *Arabidopsis* genome contains two genes coding for RanGAP proteins. The AtRanGAP1 and AtRanGAP2 proteins display about 60% identity to each other and about 25% identity to the yeast or mammalian RanGAP proteins. The N-terminal domain of plant RanGAP proteins, as pointed out by Meier (2000), exhibits significant homology to a group of plant-specific proteins designated as MAF1, described by Gindullis *et al.* (1999). This so-called WPP motif has recently been shown to be necessary and sufficient for targeting to the nuclear rim (Rose and Meier, 2001). However, decoration of the nuclear envelope both by MAF1 and AtRanGAP1 and AtRanGAP2 appears to be discontinuous and exhibits patchy patterns. A hypothesis, based on localization patterns and sequence homology of these proteins, and assuming a novel link between Ran signal transduction and proteins located in the nuclear envelope (possibly at or around the nuclear pores), was recently outlined by Meier (2000). Our results do not contradict this hypothesis, but it remains to be tested (i) whether these proteins indeed interact with each other; and (ii) whether the MAF1/MPF1 proteins play a role in targeting RanGAP to the nuclear envelope.

Localization of plant RanGAPs in mitotic cells

RanGAP is localized in the phragmoplast. The phragmoplast organizes the initiation and extension of the new cell plate that, in higher plants, extends centrifugally. This requires factors that mediate the fusion of the numerous vesicles that are transported along microtubules to the cell plate. RanGAP1 has been shown to promote vesicle

Figure 2. Localization of AtRanGAP1::GFP and AtRanGAP2::GFP fusion proteins in transgenic *Arabidopsis thaliana* seedlings.
(a) Western blot analysis of total extracts from wild type (cv. Wassilewskija A.th.) and a line overexpressing AtRanGAP1::GFP that were challenged with the antibody raised against AtRanGAP1. The antibody recognizes a band of the predicted size (58.8 kDa, arrow) for AtRanGAP1 in both wild-type and overexpressor lines. In addition it recognizes an upshifted band in the overexpressing line that is absent in extracts from the wild type. 10 μg of total protein are loaded per lane.
(b–e) Nuclear localization of the AtRanGAP::GFP fusion proteins in root hairs. BF, bright field images; FL, epifluorescence images. (b) Non-transformed Wassilewskija wild type; (c) AtRanGAP1::GFP; (d) AtRanGAP2::GFP; (e) ubiquitous expression of the RanGAP1::GFP fusion protein in root cells, the lower hypocotyl and the upper hypocotyl of an overexpressor plant.
(f,g) Confocal images of RanGAP1::GFP (f) and RanGAP2::GFP (g) in nuclei of etiolated hypocotyls.
(Hi–iii) show three consecutive sections of a confocal stack to detect localization of AtRanGAP1::GFP at high magnification. White arrow, protrusion of nuclear envelope into centre of nucleus.
(i) Individual section of a confocal stack for AtRanGAP2::GFP at high magnification.
(j,k) Visualization of AtRanGAPs in hypocotyl cells of non-transformed Wassilwskija wild-type seedlings by immunofluorescence using the antiserum mentioned in (a), with conventional epifluorescence (j; i, bright field; ii, rhodamin filter set) and high-resolution confocal images (k; i–iii show three consecutive sections of a confocal stack). Scale bars, 7.5 μm.


(a)
123
84
49
kDa
WT 56-1
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root
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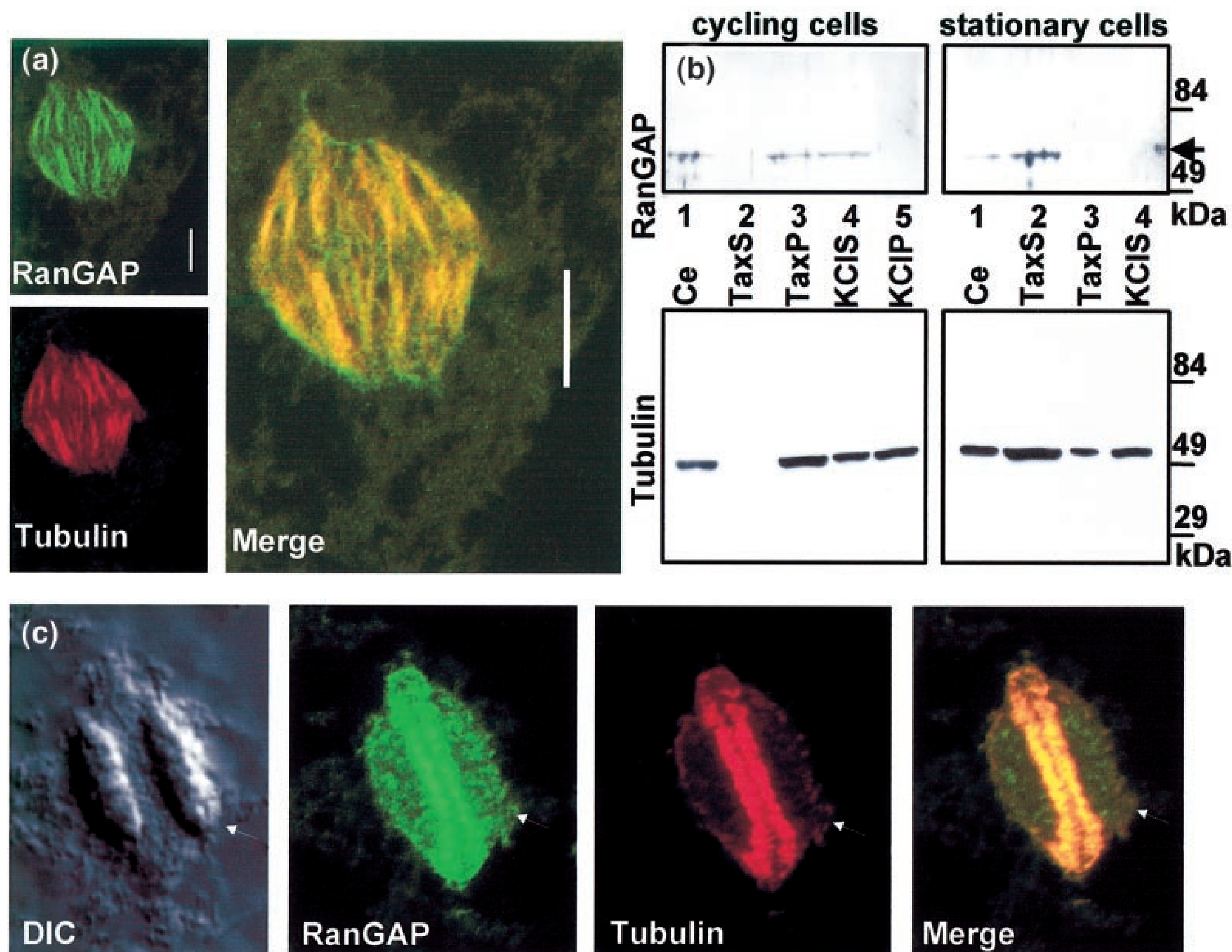

Figure 3. Interaction of tobacco RanGAP and microtubules in synchronized cells of the tobacco line VBI-O.
(a) Confocal section of a stack through a division spindle. Tobacco RanGAPs were visualized with FITC; microtubules in the same cells with Texas Red. Merging of both signals reveals a close association of RanGAPs with spindle microtubules. The green transverse interconnections between the spindle microtubules indicate RanGAP that is not co-localized with microtubules.
(b) Tobacco RanGAPs co-assemble with tubulin in extracts from cycling, but not in extracts from stationary cells. Soluble extracts (lane 1, Ce) were complemented with taxol, Mg^{2+} and GTP and incubated at 30°C to induce assembly of microtubules. These were collected by ultracentrifugation yielding a taxol pellet (lane 3, TaxP). RanGAPs are completely depleted from the remaining supernatant (lane 2, TaxS) in cycling cells, but not in stationary cells. The microtubule sediment (lane 3, TaxP) is then resuspended at high ionic strength, and detached proteins (lane 4, KClS) are separated from the washed microtubule sediment (lane 5, KClP) by ultracentrifugation. Total protein loaded, 10 μg per lane. Arrow, RanGAP band (predicted MW of AtRanGAP1 58.8 kDa).
(c) Confocal section through a phragmoplast. RanGAPs are visualized with FITC, microtubules with Texas Red; the yellow signal in the merge highlights co-localization. White arrow marks nuclear envelope of daughter nucleus; DIC, differential-interference contrast images. Scale bar, 7.5 μm.

recruitment by stimulating GTP hydrolysis by Ran (Zhang and Clarke, 2000). The localization of RanGAP in the phragmoplast and in the nuclear envelope of the two daughter nuclei would be consistent with such a function.

RanGAP is localized in sites of microtubule nucleation. In higher plants that lack centrosomes, the major microtubule-organizing centre (MTOC) is the nuclear envelope (for review see Lambert, 1993). The breakdown of the nuclear envelope and the formation of a spindle occur almost instantaneously, suggesting that microtubule-nucleating components of the nuclear envelope are used for the nucleation of spindle microtubules (for review see Nick, 1998). A further nucleation centre, which appears to be responsible for the cortical interphase array of microtubules, is associated with the phragmoplast, a structure that organizes the centrifugal extension of the growing cell plate. In tobacco cells, all three structures (nuclear envelope, spindle and phragmoplast) were visualized using an antibody recognizing AtRanGAP1 and AtRanGAP2. In dividing tobacco VBI-O cells transiently expressing either the AtRanGAP1::GFP or AtRanGAP2::GFP fusion protein, we also detected GFP fluorescence in the nuclear envelope

and in cortical vesicular structures during interphase, and at phragmoplast-like structures in mitotic cells (data not shown). Therefore, to determine the extent to which different subtypes of tobacco and AtRanGAPs are localized to these MTOCs will be feasible only by using antibodies differentially recognizing these RanGAP proteins. The association of plant RanGAP with microtubule-nucleating centres contrasts with the situation in animal cells, where ectopic Ran-GTP can induce microtubule asters (Kahana and Cleveland, 2001), and the proper organization of the spindle requires the activity of the RanGAP1 antagonist RCC1. In the *Xenopus* oocyte, it appears that release of nucleating factors such as NuMa or TPX2 from importin α and β initiates the formation of microtubule asters. The situation in higher plants is fundamentally different because the spindle is not initiated from centrosomes, but probably from factors originating from the nuclear envelope that are masked during interphase. Therefore, in contrast to mammalian systems, plant RanGAP could function as sequestering factors that are involved in this masking process. Evolutionary precursors of plant mitosis in protists such as the intranuclear mitosis in *Euglena* or the finestral mitosis in *Chlamydomonas* indicate that microtubule-nucleating factors located in the nuclear envelope are 'unmasked' at the onset of mitosis and induce the division spindle (Heath, 1981).

Tobacco RanGAP co-assemble with microtubules in extracts from cycling, but not from stationary cells. RanGAP co-assembles with polymerizing tubulin dimers into microtubules and can be completely depleted from the supernatant (Figure 3B). The RanGAP that has been co-sedimented with microtubules can subsequently be detached completely by washing the microtubule sediment at high ionic strength, demonstrating that the co-sedimentation of RanGAP is not caused by unspecific aggregation. In contrast, RanGAP was unable to co-sediment with microtubules in extracts from stationary cells, but remained completely in the first supernatant. These results lead to the following conclusions. The co-localization of RanGAP with spindle and phragmoplast microtubules in mitotic cells corresponds to a biochemical interaction between RanGAP and microtubules. Although RanGAP and microtubules are present in extracts of stationary cells (where RanGAP is not observed along microtubules), they cannot interact biochemically, indicating that this interaction is indirect through a protein that is present only in cycling cells.

Are there functions for RanGAPs that are specific for plants? Our findings are consistent with the classical function of RanGAPs as regulators of nuclear transport. More recently, additional functions of eukaryotic Ran and RanGAP proteins have been uncovered in the re-establishment of the nuclear envelope (Hetzer *et al.*, 2000; Zhang and Clarke, 2000) and in the organization of microtubules (Kahana and Cleveland, 2001). Our results from cycling tobacco cells suggest that these functions are also preserved in plants. This raises the question: what aspects of plant RanGAPs are specific for plants? The targeting of RanGAP to the nuclear envelope appears to involve protein domains that are not known from animal RanGAPs (Rose and Meier, 2001). The present study demonstrates a fundamental difference in the relationship between RanGAP and microtubules, and indicates a function as a factor that masks microtubule-nucleating sites on the nuclear envelope. The molecular mechanism by which plant RanGAP proteins contribute to these processes appears to be partially different. The importance of this function is supported by the recent finding that antisense suppression of RanBP1 leads to mitotic arrest (Kim *et al.*, 2001). The *Arabidopsis* genome contains three genes encoding Ran and two genes encoding RanGAP proteins. The sequence of these AtRan (Merkle and Nagy, 1997), as well as that of the AtRanGAP proteins, is closely related, and they appear to be expressed in a nearly identical fashion. To determine whether they function differentially or represent a simple redundancy for regulating nuclear transport, microtubule nucleation and vesicle fusion, we have isolated null mutants for these genes. In addition, we have already isolated potential downstream targets of RanGAPs using the yeast two-hybrid approach. Functional studies of these putative RanGAP-interacting proteins will focus on defining their role in mediating dynamics of cytoskeleton, as the function of plant RanGAPs appears to be distinct from their animal counterparts in this respect.

Experimental procedures

Yeast strains and cDNA libraries

The *Saccharomyces cerevisiae* PSY714 (rna1-1, ura3-52, leu2Δ1, trp1, gal$^+$, MATa) temperature-sensitive mutant strain was a generous gift from Dr Lieu Anh Nguyen; Dana Farber Cancer Institute, Boston, MA, USA (Corbett *et al.*, 1995). The CD4-15 *Arabidopsis thaliana* L. (Columbia) seedling λ cDNA expression library was obtained from the Arabidopsis Biological Resource Center, OH, USA (Kieber *et al.*, 1993).

Isolation of plant cDNAs that complement/suppress the yeast rna1 *mutation*

Competent yeast cells (strain PSY714) were prepared from cultures grown in YPGal media at 23°C and transformed with the λ-Max 1 alfalfa cDNA expression library, as described (Gietz *et al.*, 1992). Transformed yeast cells were plated on SD plates lacking uracil and supplemented with 2% galactose at 23°C to allow cells to recover and to express the cDNAs; after 24 h plates were transferred to the restrictive temperature (37°C) for selection. After 5 days, colonies growing only on SDGal$^+$Ura$^-$ at 37°C were analysed further. DNA was isolated and the rescued cDNA fragments were used for the isolation of full-length *A. thaliana* cDNA homologues by plaque hybridization.

Cloning and sequence analysis

Full-length AtRanGAP1 and AtRanGAP2 cDNAs first were modified by PCR using synthetic oligonucleotide primers to remove the TAG stop codon and generate *Bam*HI and *Sma*I cloning sites. The amplification products obtained were then subcloned as *Bam*HI–*Sma*I fragments into pKS plasmids. These AtRanGAP fragments were then transferred into a modified pPCV812 binary plasmid (Koncz *et al.*, 1999). This plasmid contained an expression casette consisting of the CaMV 35S-promoter, a linker region including *Bam*HI and *Sma*I sites, and the coding sequence of smGFP (green fluorescence protein, Haseloff *et al.*, 1997) followed by the NOS transcription termination signal. Junction regions of the final construct were then sequenced, and the binary vector containing the 35S promoter-driven AtRanGAP1/GFP/NOS chimeric gene was transformed into the GV3101 *Agrobacterium tumefaciens* strain as described. All DNA and RNA manipulations were performed as described by Sambrook *et al.* (1989). The DNA sequences were determined by using the dideoxy chain termination method and a Perkin-Elmer automated DNA sequencer (ABI373 model). Both protein and nucleotide sequences were compared to the NCBI database using the BLAST network service.

Plant material and production of transgenic Arabidopsis *expressing the AtRanGAP::GFP fusion proteins*

Transgenic *Arabidopsis* plants (ecotype Wassilewkija) expressing AtRanGAP1::GFP or AtRanGAP2::GFP fusions were generated by *A. tumefaciens*-mediated transformation according to a protocol described by Kircher *et al.* (1999). Twenty-five primary transgenic plants were grown to maturation in a greenhouse, and self-pollinated. The seeds were germinated in the presence of hygromycin to select hygromycin-resistant, homozygous seedlings or plants that could be used for analysis.

The tobacco cell line VBI-O was cultivated at 3-week subcultivation intervals, as described by Nick *et al.* (2000). It was synchronized by a protocol using hydroxyurea and oryzalin (Opatrný and co-workers, Charles University, Prague, Czech Republic, unpublished results).

Microscopy

The AtRanGAP::GFP fusion constructs were visualized in etiolated seedlings of *Arabidopsis* either under a conventional epifluorescence microscope (Zeiss, Axioskop, Oberkochen, Germany) using a specific GFP-filter set (filter set 13; Zeiss). Alternatively, experiments were performed with a confocal laser-scanning microscope (DM RBE; Leica, Bensheim, Germany) using the 488 nm line of an argon–krypton laser for excitation, a beam splitter at 510 nm, a bandpass filter at 515 nm, and a line algorithm averaging 16 individual scans. For immunofluorescence visualization the protocol by Wang and Nick (2001) was used with minor modifications, using the RanGAP1-antibody at a dilution of 1 : 100.

Production of AtRanGAP1 antibody

AtRanGAP1 was cloned into the pQE-vector and transformed into *Escherichia coli* M15[pRep] by heat shock using standard procedures (Sambrook *et al.*, 1989). The transformed cells were cultivated in 100 ml 2YT-medium in presence of 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin overnight at 37°C. 20 ml of this preculture were used to inoculate 1 L of 2YT-medium in presence of the antibiotics. Cells were induced by 2 mM IPTG when the optical density reached 0.4 and cultivated for additional 4 h. The recombinant AtRanGAP1 protein was purified on a Ni-NTA resin (Qiagen, Hilden, Germany) following the protocol of the producer. The purified protein was separated by SDS–PAGE on a preparative 10% acrylamide slab gel (Protean II xi cell, Biorad, München, Germany). The recombinant AtRanGAP1 was visualized by incubating the gel for 10 min in ice-cold KCl (0.1% w/v) and excised from the gel. The protein was extracted from the acrylamide matrix by electroelusion (Biotrap, BT 1000, Schleicher & Schuell, Dassel, Germany) and concentrated (Centriprep-10, Amicon Inc., Beverly, USA) to a concentration of 1 μg μl^{-1} in Tris-buffered saline (TBS). This solution was used for the production of polyclonal mouse antisera according to standard protocols (Professor Dr Bessler, Institut für Immunbiologie, University of Freiburg, Germany). The antiserum was used at a dilution of 1 : 1000 for Western blotting and could detect recombinant AtRanGAP1 down to a concentration of 20 ng.

Western blot analysis and microtubule co-assembly assays

Individual leaves from wild-type and RanGAP1::GFP overexpressing *Arabidopsis* plants were shock-frozen and ground in a mortar in liquid nitrogen until a fine powder was obtained. The powder was mixed with two volumes of fresh, hot sample buffer (130 mM Tris–HCl pH 6.5, 4% w/v SDS, 10% w/v glycerol, 10% v/v 2-mercaptoethanol) complemented with 8 M urea and incubated at 95°C for 10 min. The samples were then ultrasonicated on ice for 30 sec and spun down for 10 min at 15 300 *g* at 4°C. The supernatant was transferred into a fresh reaction tube, frozen in liquid nitrogen and stored at –20°C until protein analysis. Protein concentrations were determined directly in the processed samples using the amido-black method (Popov *et al.*, 1975). The microtubule co-assembly assays were performed with the tobacco cell line VBI-O according to the protocol described in detail in Nick *et al.* (1995). Accordingly, the cells were either synchronized by a protocol using hydroxyurea and oryzalin (Opatrný and co-workers, unpublished results) yielding samples with up to 40% of mitotic cells, or cultivated for 14 days yielding samples consisting only of stationary cells. Protein extracts prepared were analysed by conventional SDS–PAGE and Western blotting as described by Nick *et al.* (1995), loading 10 μg of total protein per lane. AtRanGAP1 was visualized on the blots by the anti-RanGAP1 antibody at a dilution of 1 : 1000 and a secondary antibody raised against mouse IgG that was conjugated to horseradish peroxidase (Sigma, Neu-Ulm, Germany) using a bioluminescence system (ECL, Amersham-Pharmacia, Freiburg, Germany).

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