Mol Genet Genomics (2007) 278:273–282
DOI 10.1007/s00438-007-0247-8

ORIGINAL PAPER

PpRab1, a Rab GTPase from maritime pine is differentially expressed during embryogenesis

Sónia Gonçalves · John Cairney · María Pérez Rodríguez · Francisco Cánovas · Margarida Oliveira · Célia Miguel

Received: 31 October 2006 / Accepted: 7 May 2007 / Published online: 12 June 2007

Abstract Rab-related small GTP-binding proteins are known to be involved in the regulation of the vesicular transport system in eukaryotic cells. We report the characterization of a previously isolated full-length cDNA *PpRab1* from *Pinus pinaster*. Amino acid sequence analysis revealed the presence of G1–G5 conserved domains of the GTPase Ras superfamily and a double cysteine motif in the C-terminal, characteristic of Rab proteins. The PpRab1 protein shows high sequence similarity to several Rab1 GTP-binding proteins in plants. Phylogenetic analysis showed that, within the Ras superfamily, PpRab1 is more closely related to the Rab family and within this, PpRab1 protein was found to cluster with *Arabidopsis* subfamily AtRABE, whose members are known to regulate ER-to-Golgi membrane trafficking steps. *PpRab1* transcripts were expressed at constitutively high levels for the initial stages of zygotic embryo development, and then their relative abundance decreased as embryo matures. The *PpRab1* transcript is not embryo-specific as it was found in roots, cotyledons and hypocotyls. An increase in *PpRab1* expression level was observed when seeds are germinated and collected at successive time points of development. In situ RT-PCR analysis revealed an expression signal in early zygotic embryos. In view of the proposed roles of Rab1 GTP-binding protein, the possible function of the protein encoded by *PpRab1* in embryogenesis is discussed.

Keywords *Pinus pinaster* · Embryogenesis · GTP-binding protein · Rab family · Maritime pine

Communicated by H. Ronne.

The nucleotide sequence reported in this paper has been submitted to Genbank under accession number DQ372931.

S. Gonçalves · M. Oliveira · C. Miguel (✉)
Forest Biotech Lab, Instituto de Biologia Experimental e Tecnológica (IBET)/Instituto de Tecnologia Química e Biológica (ITQB), Quinta do Marquês, 2784-505 Oeiras, Portugal
e-mail: cmiguel@itqb.unl.pt

J. Cairney
School of Biology and Institute of Paper Science and Technology at Georgia Tech, Georgia Institute of Technology, 500, 10th Street, N.W, Atlanta, GA 30332-0620, USA

M. P. Rodríguez · F. Cánovas
Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, 29071 Málaga, Spain

M. Oliveira
Dep. Biologia Vegetal, Univ. Lisboa, Fac. Ciências, Campo Grande, 1749-016 Lisbon, Portugal

Introduction

The intracellular transport of macromolecules is one of the fundamental principles of eukaryotic cells. The molecular mechanisms and machinery of vesicular trafficking are very well conserved from yeast to higher eukaryotes, and membrane trafficking is required for a variety of cellular functions. In plants, such transport pathways are important for storage-protein accumulation, for cell growth and differentiation, for secretion of protein and polysaccharide components of the cell wall and cell plate, and for morphogenesis, which depends on spatial and quantitative control of cell expansion (Moore et al. 1997). The transport of molecules destined for the vacuole, plasma membrane, endoplasmic reticulum (ER), Golgi complex and cell wall is thought to occur largely in small membrane vesicles of the exocytotic and endocytotic pathways.

It has become evident that many organelles of the intracellular transport pathways have at least one member of the Rat sarcoma (Ras) superfamily of small guanosine triphosphatases (GTPases) on their cytoplasmic face that function as GDP/GTP-regulated molecular switches (Bourne et al. 1991; Pennington 1995). The conservation within various GTPase families is very strong, where orthologues even from distantly related groups generally share 65–85% sequence identity (Jékely 2003).

First described as Ras-like proteins in brain (Rab), Rab/yeast proteins (Ypt) comprise the largest branch of the superfamily. Rab GTPases are regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways (Zerial and McBride 2001). In conjunction with soluble *N*-ethyl maleimide sensitive factor attachment protein receptor (SNARES), Rab proteins provide specificity for membrane fusion events. Different Rabs are localized on distinct vesicles and organelles, and the function of each Rab protein depends on its localization (Chavrier and Goud 1999).

In higher plants, genes encoding 57 Rab isoforms have been identified in the *Arabidopsis thaliana* genome (Pereira-Leal and Seabra 2001; Vernoud et al. 2003), which are grouped into eight families. The correlation between sequence similarity and regulation of membrane trafficking through related compartments appears to be a conserved feature in the Rab GTPase family (Vernoud et al. 2003). A number of Rab-homologous GTP-binding proteins have been isolated from higher plants (review by Bischoff et al. 1999), but the functions for most Rab GTPases have not yet been established. Nevertheless, both their high degree of homology to mammalian Rab proteins and their ability to complement ypt-deficient yeast mutants suggest a cellular function comparable to that of mammalian Rab proteins. Indeed, when plant Rab GTPase function is known, these isoforms also cosegregate in subfamilies containing their mammalian and yeast counterparts (Vernoud et al. 2003). In the absence of effective in vivo trafficking assays, information on plant Rab GTPase function has been derived from complementation and expression studies in yeast (Ueda et al. 2000) and on expression and subcellular localization studies (reviewed by Bischoff et al. 1999; Ueda et al. 2001; Cheung et al. 2002; Inaba et al. 2002; Schiene et al. 2004). Disruption of Rab function produces various developmental and cellular anomalies (Kamada et al. 1992; Cheon et al. 1993; Aspuria et al. 1995), but the in vivo trafficking role has not been identified for any plant Rab GTPase.

In mammals, Rab1 GTPase isoforms localize to ER, ER Golgi intermediate compartment, and Golgi compartments and regulate ER-to-Golgi membrane trafficking steps (Tisdale et al. 1992; Nuoffer et al. 1994). In yeast, *YPT1* is an essential gene required for ER-to-Golgi trafficking events (Segev et al. 1988). In plants, a large number of Rab1-related genes have been cloned and some expression and functional studies have been reported (reviewed by Bishoff et al. 1999). Batoko et al. (2000) concluded that the *Arabidopsis* Rab1b (AtRab1b) function is required for transport from the endoplasmic reticulum to the Golgi apparatus and suggest that this process may be coupled to the control of Golgi movement.

In this work, we describe the isolation and characterization of a cDNA clone that codes for a Rab1-related GTP binding protein which is predominantly expressed in early stages of embryogenesis of *Pinus pinaster*. To our knowledge this is the first report on expression patterns of a Rab GTPase in gymnosperms. The findings of this work may prove of particular importance in the improvement of the somatic embryogenesis process. In gymnosperms the greatest interest of somatic embryos is based on its practical application for large-scale vegetative propagation. Clonal propagation through somatic embryogenesis is one of the major biotechnology tools due to the potential to meet the need for raw material and the possibility of added benefit of ensuring consistent seedling quality. Although in commercial use in a few countries worldwide, mass production of conifers through somatic embryogenesis is relatively new and there are numerous biological unknowns regarding this complex developmental pathway.

Materials and methods

Plant material

Maritime pine immature cones were collected from clonal individuals of open-pollinated (OP) 'plus' trees (numbers 13, 32, 49, 54, 68 and 82) growing in a seed orchard at Mata Nacional do Escaroupim, Salvaterra de Magos, Portugal. The collection was performed weekly during the period of June 23rd to July 30th, 2002 and June 28th to July 12th, 2003. Embryos were isolated into nine developmental stages according to the staging system of Pullman and Webb (1994). Maritime pine mature seeds were provided by Banco de Germoplasma Vegetal Andaluz (BGVA, Spain). After two days in aerated water, seeds were transferred to aluminium containers with vermiculite. Seed germination and growth of pine seedlings were carried out at 25°C under continuous fluorescent white light (240 µmol photons m^{-2} s^{-1}) during 15 days. After germination, roots, hypocotyls and cotyledons were collected. To collect germinating seedlings at different developmental stages, seeds were also germinated in vitro. After seed coat removal, embryos inside the megagametophytes were inoculated on a semi-solid support of 3% agarose and germinated in the dark at room temperature. Whole seedlings were collected at days 3 (S3), 5 (S5), 7 (S7) and 9 (S9).

RNA extraction and cDNA preparation

Total RNA was extracted from zygotic embryos at different developmental stages using RNeasy extraction kit (Qiagen, Valencia, CA, USA) and treated with RNAse-free DNAse I (Qiagen) according to the manufacturer's instructions. Total RNA was extracted from roots, hypocotyls and cotyledons from ex vitro germinated seedlings and from in vitro germinating seedlings at different developmental stages as described in Chang et al. (1993). RNA concentration from zygotic embryos was then determined with RiboGreen® RNA quantitation reagent (Molecular Probes, Eugene, OR, USA), with the quantification performed at 480/520 nm (excitation/emission). Five hundred nanograms to one microgram of total RNA were reverse transcribed using the 1st Strand cDNA Synthesis Kit (AMV) (Roche, Manheim, Germany), according to manufacturer's instructions.

Oligonucleotide design

Primers for *PpRab1 cDNA* (forward 5′AGGACAGGA GCGATTCAGG3′ and reverse 5′CTCTTTACTTCCCCA GCCA3′) were designed to have a size of 18–30 bp, a GC content of 40–60% and a melting temperature (Tm) of 55–65°C using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA). Other criteria such as likelihood of primer self-annealing were also taken into account. All oligonucleotides were synthesized by TIB Molbiol Inc (Berlin, Germany).

Amplification of PpRab1

The *PpRab1* transcript was previously identified and the full-length sequence (A# DQ372931) determined as described by Gonçalves et al. (2005a). Stage T1 of embryo development was used as source of total RNA for the construction of an uncloned RACE cDNA library, following the manufacturer's instructions. Amplification of *PpRab1* was performed by an initial denaturation step of 94°C for 4 min, followed by 35 cycles with 45 s denaturation at 94°C, 45 s annealing at 55°C and 90 s elongation at 72°C. The reaction was terminated by an elongation step of 4 min at 72°C. PCR products were cloned and sequenced for confirmation of specific amplification. Measurements of plasmid concentration were performed in triplicate with a fluorometer using PicoGreen® RNA quantitation reagent (Molecular Probes).

Phylogenetic analysis

To compare *PpRab1* with putative homologues from humans, yeast and plant species, multiple alignments of the deduced protein sequences were conducted using CLUSTAL W (Thompson et al. 1994). Genetic distances between predicted peptide sequences were calculated using various parameters provided by the software and phylogenetic trees were constructed using the Ward method (Ward 1963).

Analysis of gene expression

Real-time RT-PCR

Reverse transcribed cDNA samples from the nine stages of zygotic embryo development were amplified by PCR using a LightCycler™ Instrument (Roche). Amplification and quantification of *PpRab1* was performed as described in Gonçalves et al. (2005b), with some modifications: following 10 min of denaturation at 95°C, 45 cycles were performed with 10 s denaturation at 95°C, 5 s annealing at 55°C and 13 s extension at 72°C. For this experiment, a range of six dilutions (1.9×10^7 to 10^2 copies) of the plasmid containing *PpRab1* cDNA (with four replicates) was tested in separate experiments under the same experimental conditions as those used for amplification of the embryo cDNA.

Calculations and statistical data analysis

Absolute quantification of the number of *PpRab1* fragments amplified in each sample was automatically calculated by the LightCycler™ software. Data in Fig. 4 are the average ± SE (Standard Error Mean) of transcript copy number from three replicates of each sample. Statistically significant differences were evaluated by a one-way ANOVA for $\alpha = 0.05$ and statistically significant mean differences between developmental stages were identified with Tukey's HSD using SPSS v. 12 (SPSS Inc.). Data subjected to ANOVA and Tukey's HSD were Ln transformed to meet the requirements of normality and homogeneity of variances.

Reverse transcription PCR (RT-PCR)

A two-step reverse transcription PCR (RT-PCR) was performed. Total RNA from megagametophytes, seedlings and zygotic embryos were reverse transcribed as described above. PCR was performed in a total of 20 µl containing 0.5 mM dNTPs, 0.5 µM of each primer, 2.5 U of Taq DNA polymerase (Invitrogen, CA, USA) in a buffer consisting of 20 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.1 mM DTT, 5% (v/v) glycerol, 0.25 mM $MgCl_2$ and 2.5 µl of reverse transcribed RNA. Samples were first denatured at 94°C, for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 90 s. A final extension step of 4 min at 72°C completed the program. As a control of RNA quality an ubiquitin transcript (Gonçalves et al. 2005b) was amplified in the same conditions as described above.

In situ reverse transcription PCR (RT-PCR)

Megagametophytes containing embryos and isolated zygotic embryos in different developmental stages were fixed and paraffin-embedded as described in Silva et al. (2003), with the following modification: tissues were fixed in freshly prepared 4% paraformaldehyde and 0.25% glutaraldehyde pH 7.4 sodium phosphate buffer (PBS). In situ reverse-transcription PCR was generally performed as described in Silva et al. (2003), with some modifications. After sectioning, samples were immersed in Histo-Clear for 10 min and then rehydrated through a graded ethanol series. Sections were refixed in 4% paraformaldehyde for 5 min and dried at room temperature. In order to allow RT-PCR reagents to gain access to the target sequences, the slides were treated with 0.01 mg/ml pectinase Macerozyme R10 (Duchefa, Haarlem, The Netherlands) and 15 μg/ml pepsin (Sigma, MO, USA). DNase treatment with 1,000 U/ml RNase-free DNase (Roche) proceeded overnight. For reverse transcription reaction, slides were incubated with 20 mM Tris–HCl, 50 mMKCl, 5 mM $MgCl_2$, 0.8 mM each dNTP, 5 μM dithiothreitol (DTT), 0.8 μM *PpRab1* antisense primer, 0.8 U/μl of RNase Inhibitor (Invitrogen, CA, USA) and 1 U/μl of *Murine Leukemia virus* reverse transcriptase (Applied Biosystems), for 1 h, at 37°C. Amplification of *PpRab1* cDNA was performed in a PCR reaction containing 20 mM Tris–HCl (pH8.0), 10 mM EDTA, 0.1 mM DTT, 5% (v/v) glycerol, 5 mM $MgCl_2$, 0.8 mM each dNTP, 10 μM (DIG)-11-dUTP (Roche), 0.06% bovine serum albumin (BSA), 1 μM each primer and 0.06 U/μl of Taq polymerase (Invitrogen). Samples were first denatured at 94°C, for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. A final extension step of 7 min at 72°C completed the program. A positive control was performed by omitting the DNase treatment and two negative controls were also performed (reaction without reverse transcription—Negative 1, and without primers in the PCR reaction—Negative 2).

Results

PpRab1 is a member of the Ras superfamily

A differential display experiment, designed to identify genes expressed during zygotic embryogenesis in maritime pine, led to the isolation of a cDNA clone for a transcript encoding a putative GTP-binding protein named PpRab1 (*Pinus pinaster* Rab1) (Gonçalves et al. 2005a). As described, a 666-bp fragment corresponding to this transcript was obtained from cDNA of maritime pine zygotic embryos and specific primers were then designed and used to isolate the 5′ end of the transcript by RACE experiments. The full-length *PpRab1* cDNA is 910 bp long, with a putative open reading frame (ORF) of 609 bp (A#DQ372931). The ORF is preceded by a 5′-untranslated region of 74 bp and followed by a 3′ untranslated region of 227 bp. The *PpRab1* open reading frame encodes a predicted protein of 202 amino acids with a calculated molecular mass of 33.23 kDa and an isoelectric point (pI) of 8.64. A sequence similarity search using BLAST (Altschul et al. 1990) revealed that the PpRab1 polypeptide is closer (86% similarities and 77% identities) to a *Pisum sativum* GTP-binding protein related to Rab1 (Nagano et al. 1993). A comparison of the PpRab1 protein with the most similar Rab GTP-binding proteins, one from yeast, one from humans and seven from angiosperm plants is presented in Fig. 1. Analysis of the deduced amino acid sequence revealed that PpRab1 protein contains the conserved regions of the GTPase Ras superfamily involved in guanine and phosphate/Mg^{2+} binding [GDSGVGKS ($GX_4GK(S/T)$); GSYIST (D-$(X)_n$-T); DTAG (DX_2G); NKCD (N/T)(K/Q)XD and FLETSAK (OOE(A/C/S/T)SA(K/L)], according to Bourne et al. (1991) (highlighted in Fig. 1). These will be referred as to G1–G5. Further analysis of the deduced amino acid sequence revealed that PpRab1 contains the double cysteine motif (GGCC) in the C-terminal, target of the post-translational modification prenylation, required for membrane association and characteristic of the Rab family of GTP-binding proteins (Fig. 1).

PpRab1 contains Rab-specific sequences

To determine the evolutionary relationships among the proteins encoded by the isolated pine cDNA and other GTP-binding proteins of humans, nematode worm, yeast and plants, a phylogenetic analysis was performed using complete predicted protein sequences. The analysis revealed that the pine PpRab1 was found to fall inside the Rab family of GTP-binding proteins, confirming previously reported sequence similarities (Gonçalves et al. 2005a). In addition, PpRab1 protein exhibits five conserved short stretches of residues that seem to be diagnostic for the Rab family (RabF1–RabF5) as proposed by Pereira-Leal and Seabra (2000) (Fig. 2).

PpRab1 putative role in ER-Golgi traffic

Given the specific distributions of Rab GTPases to different cellular membranes, it was reported that, in *Arabidopsis*, this GTPase family could be further divided into eight subfamilies based on sequence similarity and segregation with yeast and mammalian orthologs (Vernoud et al. 2003). By phylogenetic analysis, the PpRab1 protein was found to cluster with *Arabidopsis* subfamily AtRABE (after classification of Vernoud et al. 2003), which members are

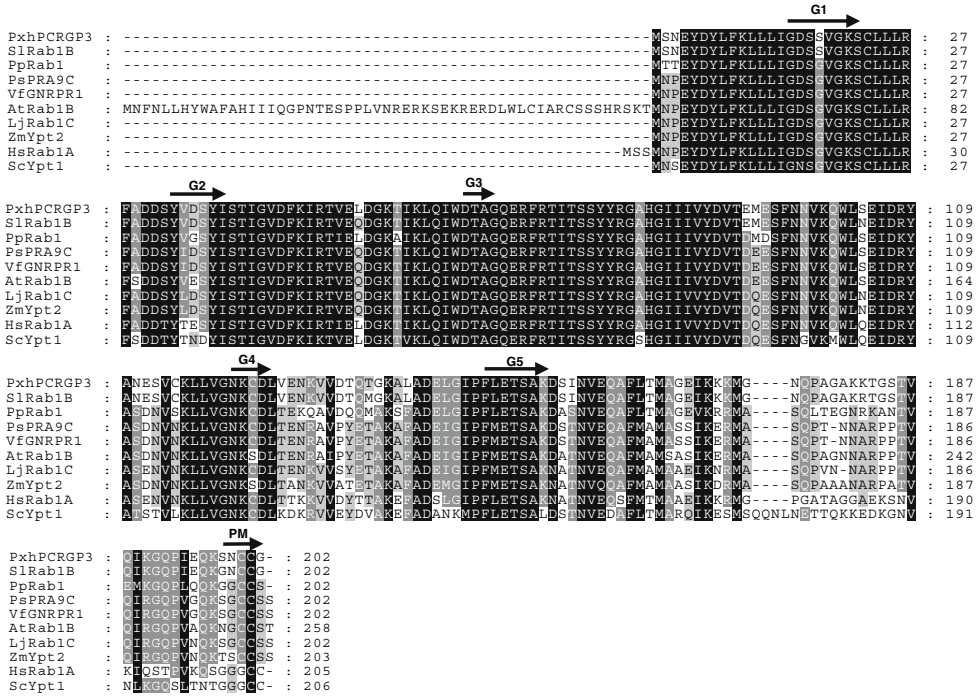

Fig. 1 Alignment of the PpRab1 protein with the most similar GTP-binding proteins from yeast, humans and angiosperm plants. The deduced amino acid sequence of PpRab1 is compared with Rab1B from *Arabidopsis thaliana* (Accession # AAC24370), PhPCRGP3 from *Petunia* × *hibrida* (Accession # AAD10389), Rab1B from *Solanum lycopersicum* (Accession # AAA80679), PRA9C from *Pisum sativum* (Accession # BAA02118), GNRPR1 from *Vicia faba* (Accession # CAA82707), Rab1C from *Lotus japonica* (Accession # CAA98160), Ypt2 from *Zea mays* (Accession # CAA44919), Rab1a from *Homo sapiens* (Accession # CAG38727) and Ypt1 from *Saccharomyces cerevisiae* (Accession # BAA09201). The alignment was made using CLUSTAL W (Thompson et al. 1997) and displayed using GeneDoc (Nicholas and Nicholas 1997). Gaps (−) are introduced to maximize alignment. White letters in a *black box* indicate identical or similar amino acids; white letters in a *grey box* indicate 11 out of 14 matches or better. Conserved regions (G1–G5) and prenylation motif (PM) are highlighted

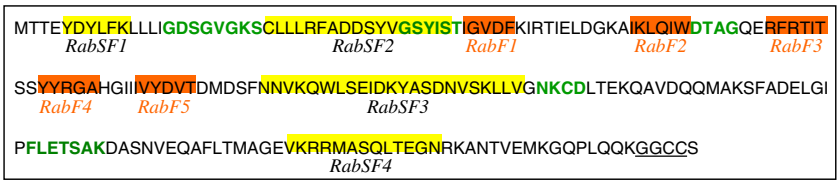

Fig. 2 Conserved sequence motifs in the primary structure of PpRab1 protein. *Orange* residues found to be Rab specific (RabF1–F5); *green* highlights the conserved nucleotide binding (G1–G5) motifs; *yellow* Rab subfamily defining sequence SF1–SF4; *underlined* prenylation signal

localized to ER, ER–Golgi intermediate compartment and Golgi compartments and regulate ER-to-Golgi membrane trafficking steps (Tisdale et al. 1992; Nuoffer et al. 1994).

PpRab1 gene expression analysis

PpRab1 is highly expressed in pine zygotic embryos

Real-time reverse transcription PCR (real-time RT-PCR) was used to quantitatively determine the expression pattern of the *PpRab1* gene during embryo development. Total RNA was isolated from zygotic embryos divided into nine developmental stages. The primers used hybridize within the open reading frame, (numbering from ATG), from 195–214 and 493–513 nt, to generate an amplicon of 318 nucleotides. The 5′-primer binds just downstream of G3 (Fig. 1) and include part of RabF5 encoding region, while the 3′-primer includes part of RabSF4. The primers thus target Rab-specific and Rab subfamily sequences within PpRab1. For determination of *PpRab1* expression level an absolute quantification method was used as described by Gonçalves et al. (2005b). The results presented in Fig. 3 reveal that expression of the *PpRab1* gene is upregulated in the early stages of embryo development. Expression of the *PpRab1*

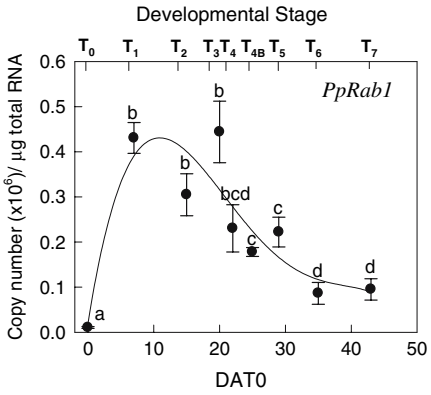

Fig. 3 Expression of *PpRab1* in pine embryos. Average ± SE of *PpRab1* transcript copy number per developmental stage as evaluated by real-time RT-PCR. DAT0—days after T0 stage of embryo development. Means followed by different letter suffixes are statistically significantly different as evaluated by ANOVA [F (8,25) = 35.10; $P < 0.001$], followed by Tukey's HSD

gene is higher in stages T1, T2 and T3 than in the other stages of embryo development. Compared to the average of T1–T3 stages, the amount of transcript is reduced by a factor of 1.8 in stages T4, T4B and T5 and by a factor of 2.3 in stages T6 and T7. However, the greatest difference is observed when comparing stage T0 to stages T1–T3, where the *PpRab1* transcript level increases by a factor of 37.

PpRab1 expression is not embryo-specific

To investigate whether or not gene expression was embryo-specific the abundance of *PpRab1* transcripts in other pine tissues was analyzed by RT-PCR (Fig. 4) using the same *PpRab1* primers. Megagametophytes isolated at different

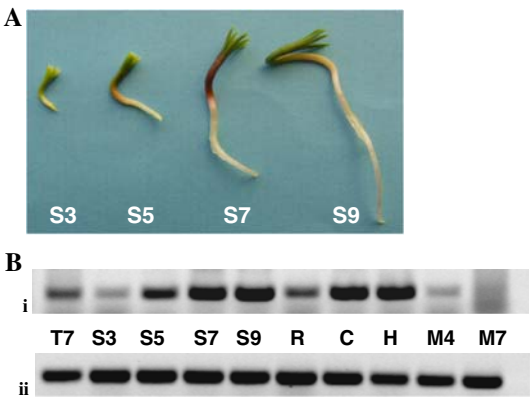

Fig. 4 RT-PCR analysis of *PpRab1* transcript in different tissues **a**, stages of seedling development used for gene expression studies. *Left to right Bar* cm. **b**, RT-PCR analysis of *PpRab1* transcript in different tissues: T7–T7 stage of embryo development; *S3–S9* germinating seedling with 3–9 days, respectively; *R* root; *C* cotyledons; *H* hypocotyls; *M4*, *M7* megagametophyte of seeds containing T4 and T7 staged embryos, respectively. *i PpRab1* PCR product (318 bp) *ii* ubiquitin transcript

embryo developmental stages were tested as well as germinating seedlings in four developmental stages (Fig. 4a). Other tissues, such as roots, hypocotyls and cotyledons were also tested for the presence of *PpRab1* transcript. By analysis of Fig. 4b it is clear that the transcript *PpRab1* is found ubiquitously in different plant tissues, ruling out the possibility of *PpRab1* being embryo-specific. When seeds are germinated and collected at different time points of development (S3, S5, S7 and S9) an increase of *PpRab1* level of expression is observed. Transcript *PpRab1* appears to be expressed at a lower level in roots when compared to hypocotyls and cotyledons and a faint band was observed in the megagametophyte of seeds containing T4 staged embryos. The transcript *PpRab1* was absent from megagametophyte of seeds containing T7 staged embryos.

Distribution of PpRab1 expression in pine embryos

In situ RT-PCR was carried out to spatially localize the *PpRab1* transcript expression in zygotic embryos at different developmental stages (Fig. 5). The positive control, performed by omitting the DNase treatment, resulted in a strong signal in the cells nuclei all over the embryonic region (Fig. 5a). The omission of reverse transcriptase from the reverse transcription reaction (negative control) resulted in megagametophytes and embryos that did not stain purple (Fig. 5b). Also, the nuclear region was clearly unstained (Fig. 5b, detail). The amplification of *PpRab1* transcript revealed an overall purple staining in the zygotic embryos at the tested developmental stages. In stage T1, the embryo showed some weak signal (Fig. 5c) and in stage T4, the sub-apical region and suspensor cells appeared stained (Fig. 5d, e).

Discussion

The predicted 202 amino acid protein encoded by *PpRab1* transcript contains significant sequence similarity (86%) with GTP-binding proteins belonging to the Rab family. All well conserved domains characteristic of the Ras GTPases superfamily, G1–G5 (Bourne et al. 1991) are present in PpRab1 and the protein clusters with the Rab family of the Ras GTPases superfamily. The G1–G5 conserved sequence motifs in the Rab proteins are required for guanine nucleotide binding. In addition, several non-conserved regions are present that confer unique functions for the regulation of distinct transport (Steele-Mortimer et al. 1994). The N-terminal domain of a Rab protein is involved in membrane–membrane recognition and/or fusion. The hypervariable region composed of the C-terminal 30 amino acids has been thought to determine the targeting of Rab proteins to specific endo- and exocytic compartments

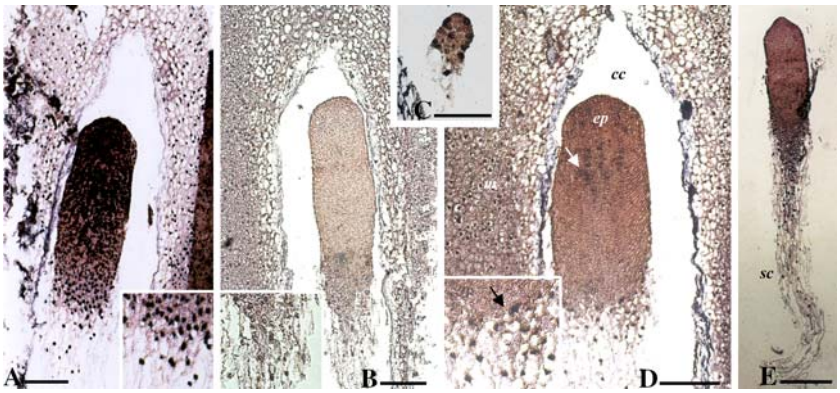

Fig. 5 Spatial localization of *PpRab1* transcript in maritime pine tissues by in situ RT-PCR. **a** Positive control. Notice the signal in all cells, particularly at nuclear level, of the embryo and suspensor cells (detail). **b** Negative control. Note the absence of any specific signal (detail). **c** Longitudinal section of a T1 zygotic embryo after in situ RT-PCR for amplification of *PpRab1* transcript. Some staining is visible in the cells of the embryo. **d**, **e** Zygotic embryo in stage T4, with specific amplification signal in the sub-apical region and suspensor cells (*arrow*). *cc* corrosion cavity; *ep* embryo proper; *m* megagametophyte; *sc* suspensor cells. *Bars* 600 μm (**a**, **b**, **d**, **e**); 300 μm (**c**)

(Seabra 1998), but recent findings suggest that multiple sequence regions contribute to membrane targeting of Rab GTPases (Bassam et al. 2004). Of particular importance is the post-translational processing at the C terminus resulting in addition of geranylgeranyl lipids. The residues GGCC in the C-terminal of PpRab1 fit the consensus for plant Ypt/Rab proteins (Crowell 2000). Furthermore, PpRab1 contains the F1–F5 regions that distinguish Rabs from other small GTPases (Pereira-Leal and Seabra 2000). Therefore, we propose that PpRab1 is a functional Rab-related GTP binding protein.

The molecular machinery that governs vesicular transport has been intensely studied, with an emphasis on the macromolecular complex that ensures matching of vesicle to correct target membrane (reviewed in Jahn and Sudhof 1999). Part of this complex is the small Ras-like GTPase Rabs, that plays an essential, although not completely understood, function in the docking process. A sequence comparison with different members of the Rab family revealed that the amino acid sequences of PpRab1, *Arabidopsis* and human Rab1 and yeast Ypt1 share the four conserved regions specific of Rab sub-family. In agreement with these findings, a phylogenetic analysis with different members of the Rab family from yeast, nematode worm, humans and *Arabidopsis* revealed that PpRab1 groups with isoforms of Rab1 protein. In plants, Rab1-related genes have been identified in tomato, pea, soybean, maize and *Arabidopsis*, among others (reviewed by Bischoff 1999). Newly synthesized proteins enter the secretory pathway by translocation through ER membranes. Subsequent transport from the ER to Golgi complexes involves recruitment of cargo proteins into vesicle transport intermediates. The small GTPase Rab1 has been assigned to an ER–Golgi intermediate compartment in *Drosophila* (Satoh et al. 1997) and in mammals (Plutner et al. 1991; Tisdale et al. 1992; Nuoffer et al. 1994). The docking process of ER-derived vesicles with the *cis*-Golgi has been shown to require Rab1 or YPT1 GTPase (Segev 1991; Soogard et al. 1994). In *Arabidopsis*, transient expression of a dominant-negative mutant of *AtRABD2a* (former *AtRab1b*) resulted in accumulation of a secreted GFP marker in an intracellular compartment reminiscent of the ER and inhibited movement of Golgi complexes along cytoskeletal elements (Batoko et al. 2000). When functions of mammalian Rab GTPases sharing significant similarity to yeast counterparts were studied, they were found to regulate membrane trafficking through compartments with related function (Vernoud et al. 2003). Attending to this, the results obtained in *Arabidopsis* support the hypothesis that RABD2 performs the same role as in its yeast and human counterparts, even though the cellular function of Rab1-related proteins in plant cells is not well documented.

Initial evidence from the preferential expression of *PpRab1* gene in early stages of zygotic embryogenesis in maritime pine came from the differential display data (Gonçalves et al. 2005a). To verify this and also to detect low levels of *PpRab1* in other stages of zygotic embryogenesis, the highly sensitive method of real-time PCR was used. The *PpRab1* specific mRNA was found to accumulate mainly in stages T1, T2 and T3 increasing drastically from T0 to these stages. The amount of transcript is then progressively reduced in stages T4, T4B, T5, T6 and T7. These results suggest that *PpRab1* may be performing an important role in the beginning of maturation. However, the transcript *PpRab1* is also found in different plant tissues, ruling out the possibility of *PpRab1* being embryo-specific. The expression pattern of *PpRab1* suggests that it may be involved in a process that to some extent is required by most cells but that has particular significance in developing embryos and germinating seedlings. According

to Dodeman et al. (1997), important genes in embryogenesis are often expressed in vegetative tissues. Cairney et al. (2006) demonstrated that of 68.721 EST sequences generated from embryos and megagametophytes of loblolly pine, approximately 71.5% of sequences were present in *Pinus taeda* EST collections derived from root, xylem, stem, needles, pollen cone, and shoot. The study performed by in situ RT-PCR to localize the spatial expression of *PpRab1* confirmed the detection of the transcript and revealed a clear transcript-specific signal in T1 embryos and in the sub-apical region and suspensor cells of T4 embryos. Embryogenesis can usually be separated into initial morphogenetic phase characterized by cell division and the onset of cell differentiation, followed by a maturation phase that involves accumulation of major storage products and preparation for seed desiccation, dormancy, and germination (Meinke 1995). The most clear morphogenetic event occurring in gymnosperm embryogenesis becomes visible at stage T4 where there is formation of cotyledons. It may be that *PpRab1* plays an important role in this phase, due to its increased expression in stages that precede this formation both in zygotic and somatic embryos. Germinating seedlings show pronounced *PpRab1* expression. Transcript *PpRab1* appears to be expressed in roots, hypocotyls and cotyledons. We must point out that these samples also originate from seedlings, supporting the pattern of high expression in seedlings. Even though, *PpRab1* expression appears to be lower in roots when compared to hypocotyls and cotyledons. To confirm these results, the study of *PpRab1* expression patterns in adult tissues must be conducted. Together with the strong homology to mammalian Rab proteins, the available data obtained from plants suggest a specific involvement of Rab1 and Rab2 in ER–Golgi traffic. It may be that *PpRab1* performs a similar function. The ER-to-Golgi pathway is thought to be particularly important to cells that must undergo rapid growth and/or membrane biogenesis (Driouich et al. 1993; Hawes et al. 1995). Elevated expression levels of Rab1 and Rab2 were found in germinating pollen, seedlings or flower tissues where, *ZmRab1a,b,c*, *AtRab2a* and *PhRab1* genes were highly expressed (Palme et al. 1992, 1993; Jako et al. 1996).

GTPases have been reported as implied in several aspects of development, including embryogenesis in several organisms such as chick (Kaarbø et al. 2003), *Drosophila*, *C. elegans* (Yochem et al. 1997) and sea urchin (Conner and Wessel 2001). In plants, small GTPases were proposed to be required for zygotic embryo viability in *Antirrhinum* (Ingram et al. 1998). In *Arabidopsis*, TTN5, a member of the Arl2 class of GTP binding proteins has been proposed to regulate microtubule function in seed development (Tzafrir et al. 2002). Members of the Rop family including Rop2 were proposed to participate in multiple distinct signalling pathways that control plant growth, development, and responses to the environment (Li et al. 2001). Khang and co-workers (2001) have reported ADL1Ap, a GTP binding protein, as critical for several stages of plant development, including embryogenesis, seedling development, and reproduction of *Arabidopsis*. Many *Arabidopsis* embryo-defective mutants are likely to be altered in basic or so-called housekeeping functions which first become essential during early stages of development (Dodeman et al. 1997). The *gnom* (*gn*) embryos can give rise to ball-shaped seedlings without any signs of apical–basal organization in the most extreme cases (Vroemen et al. 1996). GNOM (Shevell et al. 1994) encodes a guanine nucleotide exchange factor (GEF) for small GTP-binding proteins of the ARF family (Steinmann et al. 1999). GNOM is required for the transport of PIN1 and affects apical–basal embryo axis formation as an essential component of the vesicle transport machinery (Laux et al. 2004).

We suggest that *PpRab1* is transcribed in developing embryo in preparation for cotyledon development, but further studies are required to clarify PpRab1 protein role during embryogenesis.

Acknowledgments This research was supported by Fundação para a Ciência e a Tecnologia (FCT) and the III Framework Program of the EC through grant SFRH/BD/3135/2000 to Sónia Gonçalves. Dr. John Cairney acknowledges support from the National Science Foundation Plant Genome Program (Grant # 0217594). Dr João Maroco is acknowledged for assisting with the statistical analysis. Estação Florestal Nacional (EFN) is acknowledged for making plant material available.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Aspuria ET, Anai T, Fujii N, Ueda T, Miyoshi M, Matsui M, Uchimiya H (1995) Phenotypic instability of transgenic tobacco plants and their progenies expressing *Arabidopsis thaliana* small GTP-binding protein genes. Mol Gen Genet 246:509–513

Bassam RA, Wasmeier C, Lamoreux L, Strom M, Seabra MC (2004) Multiple regions contribute to membrane targeting of Rab GTPases. J Cell Sci 117:6401–6412

Batoko H, Zheng H-Q, Hawes C, Moore I (2000) A Rab1 GTPase is required for transport between the endoplasmic reticulum and Golgi apparatus and for normal Golgi movement in plants. Plant Cell 12:2201–2217

Bischoff F, Molendijk A, Rajendrakumar CVS, Palme K (1999) GTP-binding proteins in plants. Cell Mol Life Sci 55:233–256

Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanisms. Nature 349:117–127

Cairney J, Zheng L, Cowels A, Hsiao J, Zismann V, Liu J, Ouyang S, Thibaud-Nissen F, Hamilton J, Childs K, Pullman G, Zhang Y, Oh T, Buell R (2006) Expressed sequence tags from loblolly pine embryos reveal similarities with angiosperm embryogenesis. Plant Mol Biol 62:485–501

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Chavrier P, Goud B (1999) The role of ARF and Rab GTPases in membrane transport. Curr Opin Cell Biol 11:466–475

Cheon CI, Lee NG, Siddique ABM, Bal AK, Verma DPS (1993) Roles of plant homologs of Rab1p and Rab7p in the biogenesis of the peribacteroid membrane, a subcellular compartment formed de novo during root nodule symbiosis. EMBO J 12:4125–4135

Cheung AY, Chen CY-h, Glaven RH, de Graaf BHJ, Vidali L, Hepler PK, Hu W-m (2002) Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. Plant Cell 14:945–962

Conner SD, Wessel GM (2001) Syntaxin, VAMP, and Rab3 are selectively expressed during sea urchin embryogenesis. Mol Reprod Dev 58:22–29

Crowell DN (2000) Functional implications of protein isoprenylation in plants. Prog Lipid Res 39:393–408

Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48:1493–1509

Driouich A, Faye L, Staehelin LA (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends Biochem Sci 18:210–214

Gonçalves S, Cairney J, Oliveira MM, Miguel C (2005a) Identification of genes differentially expressed during embryogenesis in maritime pine (*Pinus pinaster*). Silva Lusitana 13(2):203–216

Gonçalves S, Cairney J, Maroco J, Oliveira MM, Miguel C (2005b) Evaluation of control transcripts in real-time RT-PCR expression analysis during maritime pine embryogenesis. Planta 222:556–563

Hawes C, Faye L, Satiat-Jeunemaitre B (1995) The Golgi apparatus and pathways of vesicle trafficking. In: Smallwood M, Knox JP, Bowles D (eds) Membranes: specialised functions in plants. BIOS, Oxford

Inaba T, Nagano Y, Nagasaki T, Sasaki Y (2002) Distinct localization of two closely related Ypt3/Rab11 proteins on the trafficking pathway in higher plants. J Biol Chem 277:9183–9188

Ingram GC, Simon R, Carpenter R, Coen ES (1998) The *Antirrhinum ERG* gene encodes a protein related to bacterial small GTPases and is required for embryonic viability. Curr Biol 8:1079–1082

Jahn R, Südhof TC (1999) Membrane fusion and exocytosis. Annu Rev Biochem 68:863–911

Jako C, Teyssendier De La Serve B (1996) Cloning and characterization of a cDNA encoding a Rab1-like small GTP-binding protein from *Petunia hybrida*. Plant Mol Biol 31:923–926

Jékely G (2003) Small GTPases and the evolution of the eukaryotic cell. BioEssays 25:1129–1138

Kaarbø M, Crane DI, Murrell WG (2003) RhoA is highly up-regulated in the process of early heart development of the chick and important for normal embryogenesis. Dev Dyn 227:35–47

Kamada I, Yamauchi S, Youssefian S, Sano H (1992) Transgenic tobacco plants expressing *rgp* 1, a gene encoding a ras-related GTP-binding protein from rice, show distinct morphological characteristics. Plant J 2:799–807

Kang B-H, Busse JS, Dickey C, Rancour DM, Bednarek SY (2001) The *Arabidopsis* cell plate-associated dynamin-like protein, ADL1Ap, is required for multiple stages of plant growth and development. Plant Physiol 126:47–68

Laux T, Würschum T, Breuninger H (2004) Genetic regulation of embryonic pattern formation. Plant Cell 16: S190–S202

Li H, Shen J-J, Zheng Z-L, Lin Y, Yang Z (2001) The Rop GTPase switch controls multiple developmental processes in *Arabidopsis*. Plant Physiol 126:670–684

Meinke DW (1995) Molecular genetics of plant embryogenesis. Annu Rev Plant Physiol Plant Mol Biol 46:369–394

Moore I, Diefenthal T, Zarsky V, Schell J, Palme K (1997) A homolog of the mammalian GTPase Rab2 is present in *Arabidopsis* and is expressed predominantly in pollen grains and seedlings. Proc Natl Acad Sci 94:762–767

Nagano Y, Murai N, Matsuno R, Sasaki Y (1993) Isolation and characterization of cDNAs that encode eleven small GTP-binding proteins from *Pisum sativum*. Plant Cell Physiol 34:447–455

Nicholas KB, Nicholas HB (1997) Genedoc: a tool for editing and annotating multiple sequence alignments (http://www.psc.edu/biomed/genedoc)

Nuoffer C, Davidson HW, Matteson J, Meinkoth J, Balch WE (1994) A GDP-bound of rab1 inhibits protein export from the endoplasmic reticulum and transport between Golgi compartments. J Cell Biol 125:225–237

Palme K, Diefenthal T, Vingron M, Sander C, Schell J (1992) Molecular cloning and structural analysis of genes from *Zea mays* (L.) coding for members of the ras-related ypt gene family. Proc Natl Acad Sci USA 89:787–791

Palme K, Diefenthal T, Moore I (1993) The YPT gene family from maize and *Arabidopsis*: structural and functional analysis. J Exp Bot 44:183–195

Pennington SR (1995) GTP-binding proteins. 1 Heterotrimeric G Proteins. Protein Profile 2:167–177

Pereira-Leal JB, Seabra MC (2000) The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. J Mol Biol 301:1077–1087

Pereira-Leal JB, Seabra MC (2001) Evolution of the Rab family of small GTP-binding proteins. J Mol Biol 313:889–901

Plutner H, Cox AD, Pind S, Khosravi-Far R, Bourne JR, Schwaninger R, Der CJ, Balch WE (1991) Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. J Cell Biol 115:31–43

Pullman GS, Webb DT (1994) An embryo staging system for comparison of zygotic and somatic embryo development. In: Proceedings of the TAPPI R&D division biological sciences symposium, October 3–6, Minneapolis, MN, pp 31–34. Technical Association of the Pulp and Paper Industry Press, Atlanta, GA

Rodríguez-Concepción M, Yalovsky S, Gruissem W (1999) Protein prenylation in plants: old friends and new targets. Plant Mol Biol 39:865–870

Satoh AK, Tokunaga F, Kawamura S, Ozaki K (1997) In situ inhibition of vesicle transport and protein processing in the dominant negative Rab1 mutant of *Drosophila*. J Cell Sci 110:2943–2953

Schiene K, Donath S, Brecht M, Pühler A, Niehaus K (2004) A Rab-related small GTP binding protein is predominantly expressed in root nodules of *Medicago sativa*. Mol Gen Genomics 272:57–66

Seabra MC (1998) Membrane association and targeting of prenylated Ras-like GTPases. Cell Signal 10:167–172

Segev N (1991) Mediation of the attachment or fusion step in vesicular transport by the GTP-Binding Ypt1 protein. Science 252:1553–1556

Segev N, Mulholland J, Botstein D (1988) The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell 52:915–924

Shevell DE, Leu W-M, Gilimor CS, Xia G, Feldmann KA, Chua N-H (1994) EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec 7. Cell 77:1051–1062

Silva C, Tereso S, Nolasco G, Oliveira MM (2003) Cellular location of *Prune dwarf virus* in almond sections by in situ reverse transcription-polymerase chain reaction. Phytopatology 93:278–285

Soogard M, Tani K, Ruby YR, Geromanos S, Tempst P, Kirchhausen T, Rothman JE, Söllner T (1994) A Rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell 78:937–948

Steele-Mortimer O, Clague MJ, Huber LA, Chavrier P, Gruenberg J, Gorvel J-P (1994) The N-terminal domain of a rab protein is involved in membrane–membrane recognition and-or fusion. EMBO J 13:34–41

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, Galweiler L, Palme K, Jürgens G (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science 286:316–318

Thompson J, Higgins D, Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gaps penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X window interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Tisdale EJ, Bourne JR, Khosravi-Far R, Der CJ, Balch WE (1992) GTP binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. J Cell Biol 119:749–761

Tzafrir I, McElver JA, Liu C-h, Yang LJ, Wu JQ, Martinez A, Patton DA, Meinke DW (2002) Diversity of TITAN functions in *Arabidopsis* seed development. Plant Physio1 28:38–51

Ueda T, Matsuda N, Uchimya H, Nakano A (2000) Modes of interaction between the *Arabidopsis* Rab protein, Ara4, and its putative regulator molecules revealed by a yeast expression system. Plant J 21:341–349

Ueda T, Yamaguchi M, Uchimiya H, Nakano A (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis thaliana*. EMBO J 20:4730–4741

Vernoud V, Horton AC, Yang Z, Nielsen E (2003) Analysis of the Small GTPase Gene Superfamily of *Arabidopsis*. Plant Physiol 131:1191–1208

Vroemen CW, Langeveld S, Mayer U, Ripper G, Jurgens G, VanKammen A, DeVries SC (1996) Pattern formation in the *Arabidopsis* embryo revealed by position-specific lipid transfer protein gene expression. Plant Cell 8:783–791

Ward, JH Jr (1963) Hierarchical grouping to maximize payoff. (WADD-TN-61–29, AD-261 750). Lackland AFB, TX: Personnel Laboratory, July 1963

Yochem J, Sundaram M, Han M (1997) Ras is required for a limited number of cell fates and not for general proliferation in *Caenorhabditis elegans*. Mol Cell Biol 17:2716–2722

Zerial M, McBride H (2001) Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2:107–117