AtSGP1, AtSGP2 and MAP4Kα are nucleolar plant proteins that can complement fission yeast mutants lacking a functional SIN pathway

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Accepted 8 March 2004
Journal of Cell Science 117, 4265-4275 Published by The Company of Biologists 2004
doi:10.1242/jcs.01200

Summary

In the fission yeast *Schizosaccharomyces pombe*, the onset of septum formation is signalled via the septation initiation network (SIN) involving several protein kinases and a GTPase. *Arabidopsis thaliana* and *Brassica napus* proteins homologous to fission yeast spg1p (AtSGP1, AtSGP2), cdc7p (AtMAP3Kε1, AtMAP3Kε2, BnMAP3Kε1) and sid1p (AtMAP4Kα1, AtMAP4Kα2, BnMAP4Kα2) exhibit a significant similarity. The plant proteins AtSGP1/2 and BnMAP4Kα2 are able to complement the *S. pombe* mutant proteins spg1-B8 and sid1-239, respectively and to induce mutisepta when overexpressed in wild-type yeast. Yeast two-hybrid assays demonstrated interactions both between plant proteins and between plant and yeast proteins of the SIN pathway. However, the primary structure of the proteins as well as the partial complementation of yeast mutants indicates that plant homologous proteins and their yeast counterparts have diverged during evolution. Real-time RT-PCR studies demonstrated plant SIN-related gene expression in all organs tested and a co-expression pattern during the cell cycle, with a higher accumulation at G_2-M. During interphase, the plant SIN-related proteins were found to co-localise predominantly in the nucleolus of the plant cells, as shown by fusions to green fluorescent protein. These data suggest the existence of a plant SIN-related pathway.

Key words: Cell cycle, Cytokinesis, Kinase, Plant, Signalling

Introduction

Cytokinesis is the final event of the cell cycle that divides the mother cell into two daughter cells, after the completion of mitosis. In contrast to animal cytokinesis, where a contractile ring tightens the cytoplasm, plant somatic cytokinesis is characterised by the formation of a transient membrane compartment called the cell plate, which matures into a new cell wall flanked by cytoplasmic membranes. The cell plate is built de novo by material mobilised from Golgi-derived vesicles and delivered by a dynamic cytokinesis-specific cytoskeletal array, the phragmoplast. The cell plate is generally initiated in the centre of the cell division plane, defined by a preprophase band (PPB) of microtubules, and fuses with the parental plasma membrane (Staehelin and Hepler, 1996).

Several studies demonstrate that mitogen-activated protein kinases (MAPK) are involved in the formation of the cell plate. Two orthologous MAPK from tobacco and alfalfa (NtNTF6 and MsMMK3), as well as the tobacco MAP kinase kinase kinase (MAP3K) NtNPK1 have been shown to be activated in late mitosis. Furthermore, these proteins were shown to localise to the midplane during late anaphase and to the phragmoplast (Bögre et al., 1999; Calderini et al., 1998; Nishihama et al., 2001). Overexpression of a dominant negative form of NtNPK1 in tobacco cells inhibits expansion of both the phragmoplast and the cell plate, resulting in multinucleate cells with incomplete cell plates (Nishihama et al., 2001). Krysan et al. have shown that the three *A. thaliana* orthologues of NtNPK1 (ANP1, ANP2, ANP3) are also involved in the formation of the cell plate (Krysan et al., 2002).

Despite recent advances in our understanding of the formation of the cell plate in plant cells, little is known about the mechanism(s) responsible for initiating cytokinesis and coordinating its timing with mitosis. The phenotype of *titan* and *pilz* mutants, affected in genes encoding tubulin-folding cofactors (Liu and Meinke, 1998; Steinborn et al., 2002), illustrate that spatial and temporal coordination between mitosis and cytokinesis is mediated in part by cytoskeletal components. The co-localisation of the cdc2 kinase and three types of microtubular structures, namely the PPB, the spindle, and the cell plate, suggests that the latter kinase also plays an important role in this coordination (Weingartner et al., 2001).

The need shared by all eucaryotes to coordinate mitosis and cytokinesis, in order to ensure an even distribution of chromosomes over the daughter cells, suggests that mechanisms promoting communication between the regulation of the cell cycle and the organisation of the cytoskeleton probably involve some common features across kingdoms. The mechanisms that regulate the onset of mitosis are relatively

well understood in contrast to the mechanisms ensuring the control of signal transduction during late mitosis and cytokinesis (Heese et al., 1998). Mitotic progression relies on the activity of the cyclinB-CDK complex during M phase. Before anaphase, the latter complex activates the anaphase promoting complex/cyclosome (APC/C), and promotes exit from mitosis by inactivating the cyclinB-cdc2 complex itself. Several studies in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* support the idea that the latter mechanism is not sufficient for the initiation of cytokinesis, but rather, that an additional protein kinase cascade triggering a cytokinetic pathway is required (Nigg, 2001). Indeed, genetic and molecular analyses in *S. pombe* provided evidence for the role of the septum initiation network (SIN) involving numerous protein kinases (Bardin and Amon, 2001; McCollum and Gould, 2001) (see Fig. 1). Localisation studies have revealed that the SIN cascade functions by transducing septum-promoting signal(s) from a single spindle pole body (SPB) to the division site. Upon entry into mitosis, a yeast monomeric G protein, named spg1p, initially activated on both SPB, is inactivated on one SPB at anaphase onset, by the byr4p and cdc16p proteins, which form a two-component GTPase activating protein (GAP). This allows spg1p to recruit cdc7p, a MAP3K-related protein, to a single SPB. Cdc7p determines the localisation of two other SIN components to the SPB, i.e. sid1p, a MAP kinase kinase kinase kinase-related (MAP4K) protein, and cdc14p. The sid1p-cdc14p complex then promotes activation of the SPB-localised sid2p-mob1p protein kinase complex, which translocates to the cell-division site to trigger initiation of septum formation. Coordination between mitotic exit and septum assembly occurs through the action of the APC, which degrades B-type cyclins and possibly other proteins, resulting in cdc2p inactivation, necessary for proper SPB localisation of SIN proteins (Bardin and Amon, 2001).

Jouannic et al. have previously reported cdc7p-related MAP3Kε1 proteins from *Brassica napus* and *Arabidopsis thaliana* which can complement an *S. pombe cdc7* mutant, suggesting their involvement in cell division in plants (Jouannic et al., 2001). We report on the identification and characterisation of new SIN-related components in plants (Fig. 1). The AtSGP1/2 and BnMAP4Kα2 plant proteins are able to complement cytokinesis deficiency in fission yeast lacking spg1p or sid1p, respectively. Moreover, in the yeast two-hybrid system, interaction was detected between the AtSGP1/2 proteins and the fission yeast SIN pathway proteins cdc7p and byr4p, and between the plant proteins AtSGP1 and BnMAP3Kε1. Localisation studies using green fluorescent protein (GFP) fusion proteins suggest that the plant SIN-related proteins are nucleolar in higher plants. Using real-time PCR and a synchronised cell suspension cultures of *Arabidopsis*, we demonstrated that the plant SIN-related genes are co-expressed in a cell-cycle dependent manner, with a higher accumulation at G_2-M. The data are compatible with a role of the plant SGP1/2, MAP3Kε1/2 and MAP4Kα2 proteins in a SIN-related network regulating cell division in plants.

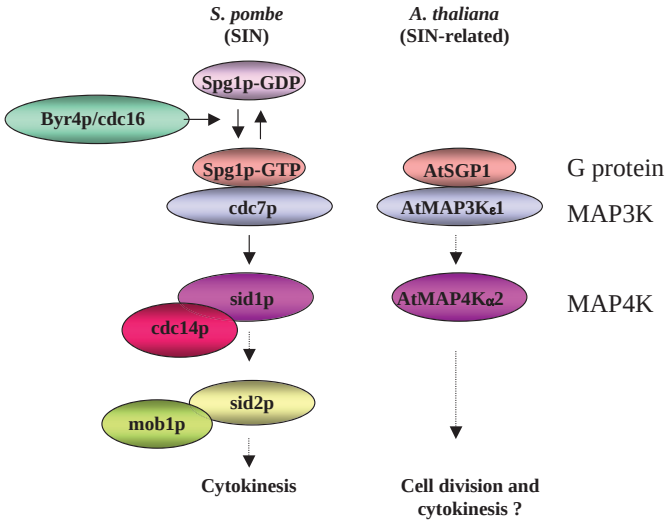

Fig. 1. Model of the SIN pathway in *S. pombe* and homologues from *A. thaliana*. A parallel is drawn between fission yeast and plants. See text for abbreviations and explanation.

Materials and Methods

Isolation of *AtSGP1, AtSGP2, AtMAP4Kα1* and *AtMAP4Kα2*

AtSGP1, *AtSGP2*, *AtMAP4Kα1* and *AtMAP4Kα2* coding regions were amplified from an *A. thaliana* root cDNA library by PCR using gene-specific primers flanking the corresponding sequences according to genomic DNA annotations: *AtSGP1* 5′-TCTCTGTTTCATCAATG-GCCG-3′, 5′-AATTAGGAATCGTAGTCG-3′; *AtSGP2* 5′-TTCT-CAATGGCTCAATCGTG-3′, 5′-CATATTCTAGAAGTCGATGA-3′; *AtMAP4Kα1* 5′-GTTGTGGAAATGGATG-3′, 5′-TCTGTGAGTTT-CTCAAC-3′; *AtMAP4Kα2* 5′-TAGTGGTTGGAGCGGGAGAGAA-GGTGG-3′, 5′-ATTCCATCTCTGTAGGTTGTCTCAGGG-3′. These PCR fragments were cloned into the pBluescript SK vector by blunt-end ligation, and their identities were confirmed by DNA sequencing.

Plant material

Arabidopsis thaliana ecotype Columbia seeds were sown in soil in a growth chamber (17°C, 65% humidity, light 250 υE/m^2/second) with a 16 hour photoperiod. Plantlets were harvested 17 days after sowing, at the four leaf stage. Roots, rosette leaves, cauline leaves, inflorescence stems, flower buds, mature flowers and 0.5 cm long green siliques were harvested from a pool of 50 plants, 60 days after sowing. While harvesting the primary inflorescence stems, stem nodes were discarded. The *A. thaliana* cell suspension culture was synchronised using aphidicoline, according to the method of Doutriaux et al. (Doutriaux et al., 1998). The T0 reference point corresponds to a sample collected immediately after aphidicoline release. After removal of the drug, an aliquot of the cell suspension culture was collected every 2 hours and frozen in liquid nitrogen.

Analysis of gene expression

RNA extractions, cDNA preparations and real-time quantitative RT-PCR analysis were performed according to the method of Charrier et al. (Charrier et al., 2002). PCR amplification was performed using specific oligonucleotides (Table 1). The oligonucleotides used for the amplification of *AtMAP3Kε1*, *AtMAP3Kε2*, *AtMAP4Kα1*, *AtMAP4Kα2* and *ACTIN 2/8* have been described previously (Charrier et al., 2002).

S. pombe strains, plasmids and transformants

DNA fragments corresponding to the full length *AtSGP1*, *AtSGP2* and *BnMAP4Kα2* coding regions were generated by digestion and cloned into the *S. pombe* expression vector pREP3ΔATG. Standard

Table 1. Genes and oligonucleotides used for the real-time RT-PCR experiments

Sequence of the oligonucleotides	Gene (accession no.)
5′-TCTTGTTCAAGTATCATCGAAAAACC-3′ 5′-GTAGTATCGTCGTCTCCGTCAAAG-3′	*AtSGP1*(AB005232)
5′-CGATTCACACGGTTGTTATGGAG-3′ 5′-AGTCGGAGACGGAAGAGCG-3′	*AtSGP2* (AB025634)
5′-GGAAGCAACAAGAAGAAGGGAG-3′ 5′-AGGGATCAAAGCCACAGCG-3′	*AtCycB1;1*
5′-GGACTCTTTACGGATTCGGTGG-3′ 5′-CATAACAAGAACCTACCGCAAACTAC-3′	*AtH4* (M17132)

techniques for transformation and genetic manipulation of *S. pombe* were used as described previously (Okasaki et al., 1990). *S. pombe spg1-B8-* and *sid1-239*-defective strains were used for complementation tests. Transformed colonies were replicated on EMM2-agar plates containing thiamine (non-inducing medium) or without thiamine (inducing medium). Nuclei and septa were labelled using DAPI and calcofluor, respectively, according to Fankhauser and Simanis (Fankhauser and Simanis, 1994).

Yeast two-hybrid experiments

The coding sequences of plant cDNAs were fused in frame to the GAL4 activation domain (AD) or the GAL4 binding domain (BD) by cloning into the pGAD424 or pGBT9 vectors, respectively. The coding regions were amplified by PCR using specific oligonucleotides: AtSGP1 5′-CTCGAATTCTCTGTTTCATCAATGGCC-3′, 5′-CTAGAATTCAATTAGGAATCGTAGTC-3′; AtSGP2, 5′-GTACGAATTCATGGCTCAATC-3′, 5′-ATAGGATCCCTAGAAGTCGATG-3′; modified with restriction sites (underlined sequences). DNA fragments corresponding to the full length *BnMAP3Kε1*, *BnMAP3Kα1* and *BnMAP3Kβ1* coding regions were generated by digestion and cloned into pGAD424 or pGBT9.

BD-cdc7, BD-spg1, BD-byr4 and AD-cdc14, AD-cdc7, AD-spg1, AD-byr4 constructs were a gift from V. Simanis. The BD and AD constructs were used to transform the yeast strain Y190 (Harper et al., 1993), and protein interactions were assayed using *lacZ*-filter lifts and by monitoring growth on SD-LTH medium containing 20 mM 3-amino-1,2,4-triazole (3-AT).

Construction, transformation and microscopy techniques for GFP detection

The coding sequences of plant genes were fused in frame with the smGFP encoding gene in the CD3-326 vector (Davis and Vierstra, 1998). The coding regions were amplified by PCR using specific oligonucleotides: *BnMAP3Kε1* 5′-AGGAATTCGAGCTCGGGATCCATGACG-3′, 5′-TTGGATCCACAAGACGGTGTTGATGTG-3′; *AtSGP1* 5′-GAATTCTCTGGATCCTCAATGGC-3′, 5′-GAATGGATCCAGGAATCGTAGTC-3′; *AtSGP2* 5′-GTACGGATCCATGGCTCAATC-3′, 5′-CAGGATCCAGAAGTCGATG-3′; *AtMAP4Kα1* 5′-GTTGGATCCATGGATGATGTTGCTGG-3′, 5′-TCTGTGAGGGATCCAACTTTGGTTAAGATCACGC-3′; *AtMAP4Kα2* 5′-TAGTGGATCCAGCGGGAGAGAAGGTGG-3′, 5′-GGGGATCCAAAGATCACGTGATGATTG-3′; modified with restriction sites (underlined sequences).

The cell suspension of the tobacco Bright-Yellow 2 (BY2) mutant was maintained according to standard protocols (Murashige and Skoog, 1962) and transformed with the plasmids mentioned above. 50 ml of cell suspension were harvested before it reached the stationary growth phase and subjected to cell wall digestion using the following mixture: 12% sorbitol, 0.1% pectolyase, 1% cellulase pH 5.5 at 30°C under gentle constant swirling. After completion (60-90 minutes), the protoplasts were washed twice with protoplast medium (0.10 M glucose, 0.25 M mannitol pH 5.8 in Murashige and Skoog medium). Transformations were carried out with 10 μg of plasmid per 10^6 protoplasts in the protoplast medium supplemented with 12.5% polyethylene glycol 4000. After dilution to a 5 ml volume with protoplast medium, protoplasts were cultured for 24-36 hours in the dark at 25°C prior to examination.

Cells were observed under the epifluorescence microscope after staining the nuclei with 4′-6 diamidino-2-phenyl indole (DAPI, 10 μg/ml) and the cell walls with Calcofluor (Tinopal LPW™, 100 μg/ml). GFP fluorescence was imaged with an epifluorescence confocal Sanastro 2000 microscope (Moleculars Dynamics). Excitation of in vivo GFP fluorescence was achieved by the Ar laser line at 488 nm, while the fluorescence emission was collected by a broad band pass filter (500-550 nm). The resulting signal was amplified, digitalized and the consistent picture reconstituted by Leica software. As classically used for animal cells, nucleolar localisation was ascertained by analysing the plant cells by phase contrast microscopy, DAPI staining and GFP observation (Sirri et al., 2002).

Results

Identification of *Arabidopsis thaliana* homologues of fission yeast spg1 and sid1

To identify plant genes that encode putative SIN components, fission yeast *spg1* and *sid1* sequences were used to screen the GenBank database using the Basic Local Alignment Search Tool. The *Arabidopsis* genome contains two homologues of each of the fission yeast genes, namely *AtSGP1*, *AtSGP2*, *AtMAP4Kα1* and *AtMAP4Kα2*, which were amplified by PCR from an *A. thaliana* root cDNA library. The genes *AtSGP1* and *AtSGP2* (for *Arabidopsis thaliana* spg1-like G protein) are highly similar to the gene encoding the yeast GTPase spg1p (Schmidt et al., 1997). *AtSGP1* and *AtSGP2* encode polypeptides of 290 and 292 amino acids, respectively, sharing 52% overall sequence identity. Spg1p from *S. pombe* showed sequence identities of 52% and 46% with AtSGP1 and AtSGP2, respectively (Fig. 2A). However, the plant and fungal proteins differ in their organisation in that the two plant proteins possess an amino terminal extension, consisting of 92 residues in AtSGP1 and 94 residues in AtSGP2, sharing 31% identity (Fig. 2A). The neighbour-joining dendogram shown in Fig. 2B indicates that spg1p and Tem1p from *S. cerevisiae*, GiTem1p from *Giardia intestinalis* and AtSGP1 and AtSGP2 constitute a new monomeric G-protein subfamily. None of these proteins possess the carboxyl-terminal CAAX prenylation motif, required for the association with cell membranes by lipid anchoring.

Analysis of the *AtMAP4Kα1* and *AtMAP4Kα2* genes and their deduced protein sequences revealed that they are closely related to the *sid1* gene (Guertin et al., 2000) (Fig. 2C). *AtMAP4Kα1* and *AtMAP4Kα2* encode protein kinases of 708 and 711 residues, respectively, belonging to the SOK group of MAP4Ks as illustrated in Fig. 2D. They show an overall identity of 78% and are highly similar to *Brassica napus* BnMAP4Kα1 and BnMAP4Kα2, previously reported by Leprince et al. (Leprince et al., 1999). In vitro kinase assays demonstrated that the *BnMAP4Kα2* cDNA encodes a functional serine/threonine protein kinase with auto-phosphorylation activity, which is also able to phosphorylate myelin basic protein (data not shown). Fig. 2C shows that sid1p

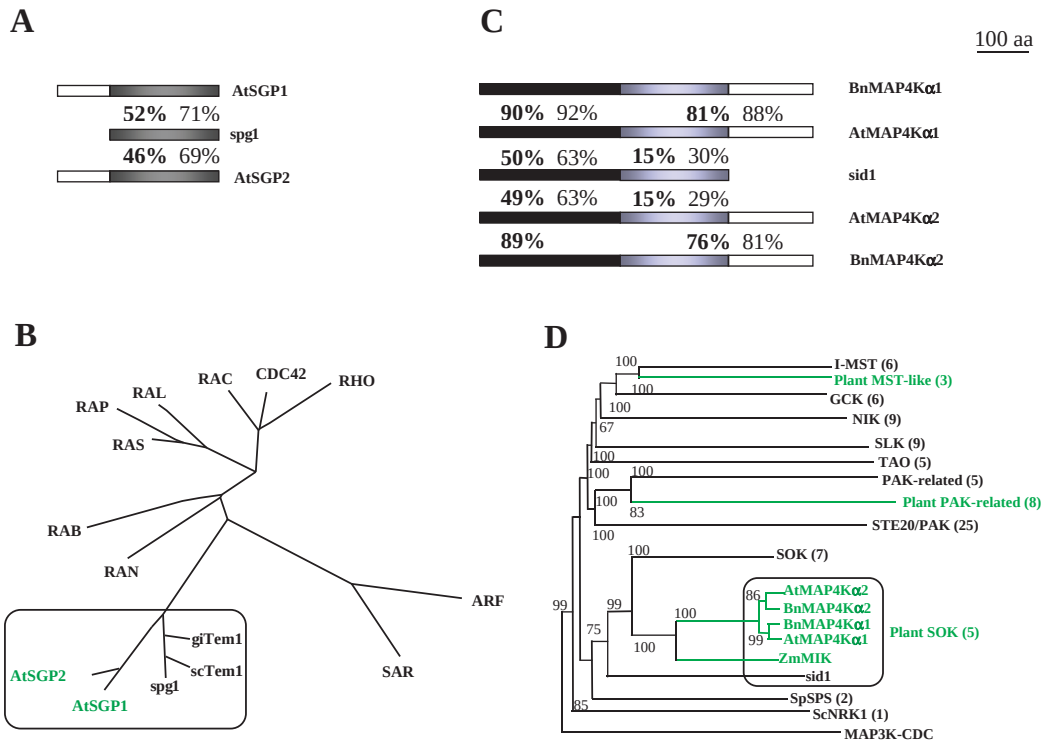

Fig. 2. Protein structure and relationship trees of monomeric G-proteins and MAP4Ks. (A) Comparison of the primary structure of AtSGP proteins with the homologous *S. pombe* protein. The plant-specific extensions are shown in white. Both identity (bold) and similarity percentages are shown. (B) Phenogram was generated using the Neighbour-Joining algorithm (Saitou and Nei, 1987) and illustrates the evolutionary relatedness of AtSGP1 and AtSGP2 to a representative subset of monomeric GTPases. The subfamily including AtSGP1, AtSGP2, spg1p from *S. pombe*, Tem1 from *S. cerevisiae* and GiTem1 from *G. intestinalis* is boxed. The sequences have been submitted to the EMBL database under the accession numbers AJ238846 (*AtSGP1*), AJ505743 (*AtSGP2*), AJ505744 (*AtMAP4Kα1*), AJ505745 (*AtMAP4Kα2*). (C) Comparison of the primary structures of MAP4Kα proteins with the homologous *S. pombe* proteins. The plant-specific extensions are shown in white, the catalytic domains in black. Both identity (bold) and similarity percentages are shown. (D) Schematic representation of the MAP4K groups based on Neighbour-Joining analyses of the catalytic domains. Number of proteins used to build the tree is indicated in brackets after each group of MAP4K.

and the two AtMAP4Kα proteins from *Arabidopsis* share 50% identity over the catalytic domain, whereas they share 30% similarity over the short carboxyl-terminal domain. Moreover, the organisation of the plant proteins differs from that of sid1p by a non-catalytic carboxyl-terminal extension, consisting of 178 and 179 amino acids in AtMAP4Kα1 and AtMAP4Kα2, respectively (Fig. 2C).

No significant similarities were observed between AtSGP1/2 or AtMAP4Kα1/2 and proteins from the fully sequenced genomes of *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. Hence, the genes and their corresponding proteins described in this study are designated as 'plant-specific SIN-related components'.

AtSGP1, *AtSGP2* and *BnMAP4Kα2* are functional homologues of spg1p and sid1p

In fission yeast, the SIN pathway couples the end of anaphase with the initiation of cytokinesis (McCollum and Gould, 2001). The bioinformatical studies described here show that the plant SIN-related proteins are highly similar. Therefore, it was tempting to speculate that these plant proteins can complement the spg1 or sid1 yeast mutants by reconstituting a functional SIN pathway. To test this hypothesis, yeast complementation experiments, overexpression and two-hybrid assays were performed. The *AtSGP1*, *AtSGP2* or *BnMAP4Kα2* coding regions were linked to the thiamine-repressible *nmt1* promoter in the vector pREP3ΔATG, and introduced into the *spg1-B8* and *sid1-239* mutant *S. pombe* strains. A comparison of the mutant phenotypes under permissive (Fig. 3A1,B1) and restrictive conditions (i.e. 36°C) showed that cells at 36°C failed to form septa (Fig. 3A2,B2). These cells go through additional nuclear division cycles without septation, leading to the production of elongated and multinucleated cells (Schmidt et al., 1997; Guertin et al., 2000). By contrast, 4 hours after the shift to 36°C, the yeast cells expressing *AtSGP1*, *AtSGP2* or *BnMAP4Kα2* produced normal septa (Fig. 3A3,B3). In addition, about 10% of the yeast cells expressing *AtSGP1* produced additional septum material at the cell tips (Fig. 3A3). A very similar phenotype was previously observed after complementation of the yeast *cdc7-24* mutant by the plant BnMAP3Kε1 protein kinase (Jouannic et al., 2001), which suggests that the two proteins transduce the same signal in *S. pombe*. No noticeable abnormalities were observed in cells containing either the constructs *AtSGP1*, *AtSGP2* or *BnMAP4Kα2* under non-inducing conditions (data not shown), or the empty pREP3ΔATG vector under inducing conditions (Fig. 3A2,B2).

In fission yeast, overexpression of *spg1* and *cdc7*, using the

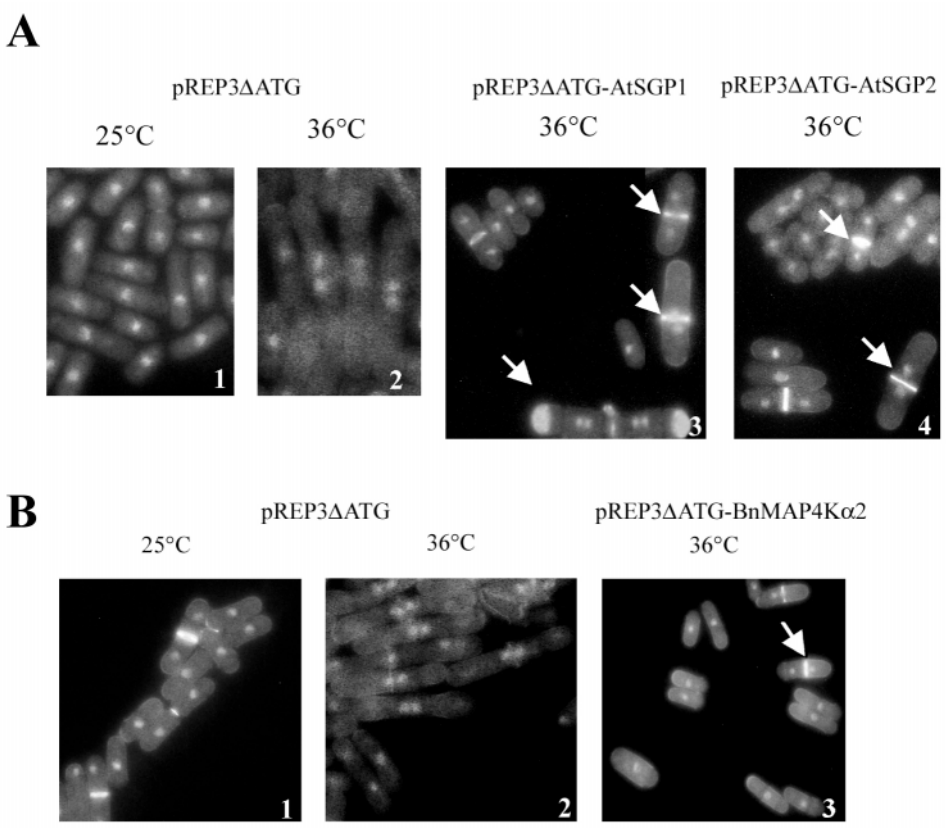

Fig. 3. Complementation of the *S. pombe* *spg1-B8* and *sid1-239* mutants. (A) *spg1-B8* mutant cells in inducing conditions transformed with: (1) pREP3ΔATG, the thiamine-repressible promoter p_{nmt1} vector at 25°C (permissive temperature), and (2) at 36°C (restrictive temperature); (3) pREP3ΔATG-AtSGP1 at 36°C; and (4) pREP3ΔATG-AtSGP2 at 36°C. (B) *sid1-239* mutant cells in inducing conditions transformed with: (1) pREP3ΔATG at 25°C and (2) at 36°C; (3) pREP3ΔATG-BnMAP4Kα2 at 36°C. Nuclei and septation material were labelled with DAPI and calcofluor, respectively. Representative examples of the septation material are indicated by arrows.

thiamine-repressible expression vector pREP3ΔATG, induces multiple rounds of septum formation without cell cleavage (Fankhauser and Simanis, 1994; Schmidt et al., 1997). In contrast, overexpression of *sid1* does not compromise the signalling of cytokinesis (Guertin et al., 2000). In order to investigate further the role of the plant homologues of *cdc7* (Jouannic et al., 2001), *spg1* and *sid1* genes, we overexpressed the *A. thaliana* and *B. napus* proteins in the wild-type *S. pombe* background. Upon withdrawal of thiamine, growth of the yeast cells carrying the empty pREP3ΔATG plasmid was not affected (Fig. 4A1). In contrast overexpression of either *AtSGP1* or *BnMAP3Kε1* (i.e. homologues of cdc7) disturbed the formation of the septum in part of the cells, resulting in multiseptate cells (Fig. 4A2,A4). Furthermore, 50% of the septate cells overexpressing *AtSGP2* showed abnormal cytokinesis, forming cells that were either multiseptate or uninucleate with one or more septa (Fig. 4A3). No abnormal phenotype was observed among the cells overexpressing *BnMAP4Kα2* (Fig. 4A5). Cell populations overexpressing either *AtSGP1* or *BnMAP3Kε1* formed twice as many septa as wild-type cell populations (Fig. 4B). Conversely, cells carrying the *BnMAP4Kα2* gene did not differ from the wild type with regard to the percentage of septum-forming cells. These results show that overexpression of either the plant BnMAP4Kα2 (this study) or sid1p (Guertin and McCollum, 2001) does not affect the cytokinesis process, and that overexpression of the plant homologues of spg1p or cdc7p is able to control, at least partially, cytokinesis in fission yeast.

Cdc7p and spg1p interact both genetically and physically (Schmidt et al., 1997). Byr4p binds to cdc16p and spg1p (Furge et al., 1998). In order to investigate a possible interaction between the plant SIN-related homologues and *S. pombe* SIN proteins, we performed yeast two-hybrid experiments. The assays based both on growth on medium lacking His and on β-galactosidase activities (Table 2), indicate an interaction between AtSGP1 or AtSGP2, and byr4p. Furthermore, we also detected positive interactions of cdc7p with either AtSGP1 or AtSGP2. Conversely, no interaction could be detected between BnMAP3Kε1 and spg1p. To test the ability of BnMAP3Kε1, AtSGP1 and AtSGP2 to interact, two-hybrid experiments were conducted. These experiments showed that BnMAP3Kε1, but not BnMAP3Kα1 or BnMAP3Kβ1, could interact with AtSGP1 in yeast (Table 2). No interaction was detected between AtSGP2 and BnMAP3Kε1 (Table 2), suggesting a functional difference between the two AtSGP proteins.

Thus the complementation of the yeast *sin* mutants by AtSGP1, AtSGP2 or BnMAP4Kα2 and the effects of the overexpression of these proteins and BnMAP3Kε1 on the division of yeast as well as their interactions in the yeast two-hybrid system with one another and with the yeast SIN proteins, provide complementary evidence that the plant SIN-related proteins are, in fission yeast cells, functional homologues of spg1p, cdc7p and sid1p.

AtSPG1, *AtMAP3Kε1*, *AtMAP3Kε2* and *AtMAP4Kα2* transcripts are up-regulated during cell division

We measured the relative transcript level of *AtSGP1/2*, *AtMAP3Kε1/2* and *AtMAP4Kα1/2* genes in various *Arabidopsis* organs, using real-time RT-PCR (Bustin, 2000). The actin genes *ACT2* and *ACT8*, amplified simultaneously,

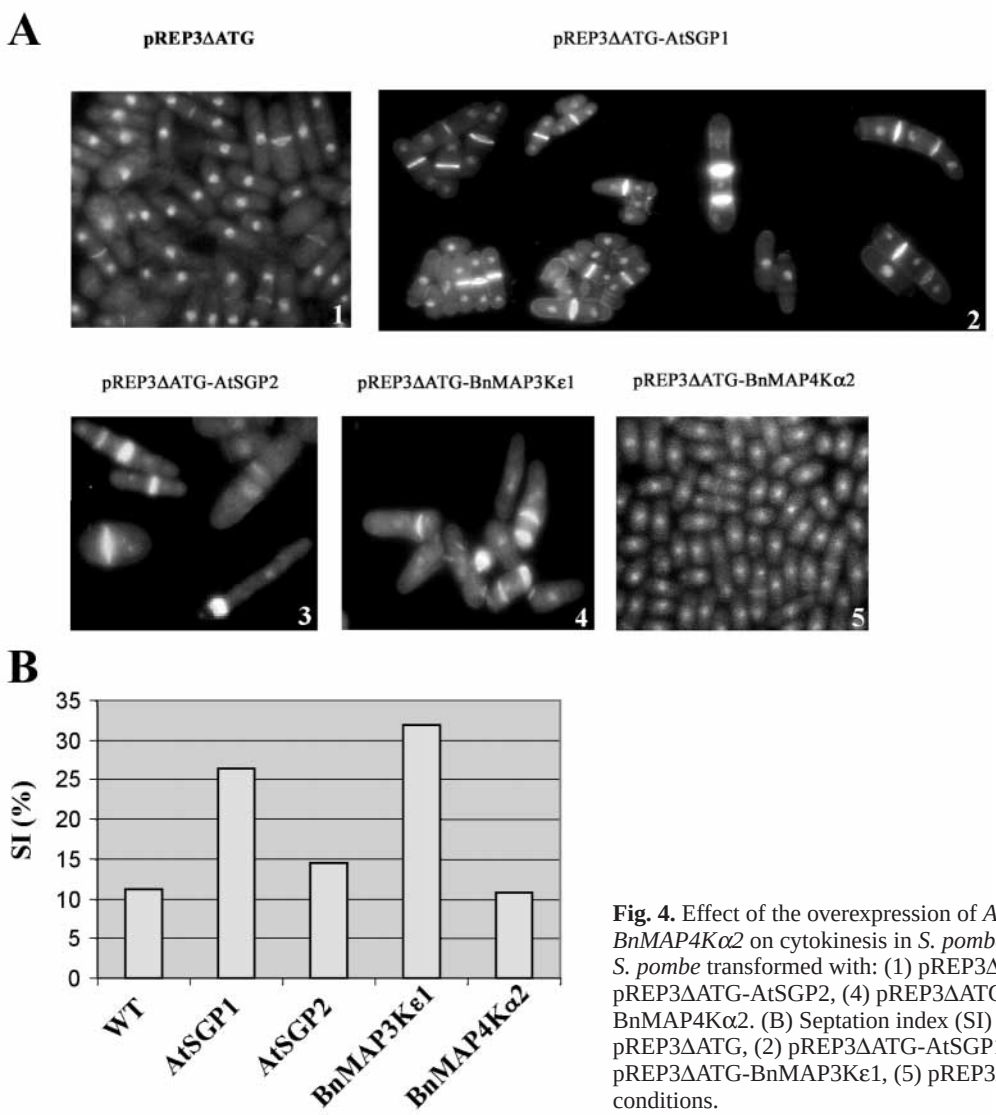

Fig. 4. Effect of the overexpression of *AtSGP1*, *AtSGP2*, *BnMAP3Kε1* and *BnMAP4Kα2* on cytokinesis in *S. pombe*. (A) DAPI calcofluor-stained cells of *S. pombe* transformed with: (1) pREP3ΔATG, (2) pREP3ΔATG-AtSGP1, (3) pREP3ΔATG-AtSGP2, (4) pREP3ΔATG-BnMAP3Kε1 and (5) pREP3ΔATG-BnMAP4Kα2. (B) Septation index (SI) in cells transformed with: (1) pREP3ΔATG, (2) pREP3ΔATG-AtSGP1, (3) pREP3ΔATG-AtSGP2, (4) pREP3ΔATG-BnMAP3Kε1, (5) pREP3ΔATG-BnMAP4Kα2 under inducing conditions.

were used as a standard because of their 'housekeeping' functions, and demonstrated stable expression profiles (An et al., 1996). The *AtSGP1*, *AtMAP3Kε1*, *AtMAP3Kε2*, *AtMAP4Kα1* and *AtMAP4Kα2* genes were ubiquitously expressed, with slightly higher transcript levels in flower buds and mature flowers (Fig. 5A). In contrast, the *AtSGP2* gene displayed a significant differential expression, with transcript levels 20 times higher in flower buds and 15 times higher in mature flowers than in other organs (Fig. 5A).

Jouannic et al. previously showed that the *AtMAP3Kε1* transcript levels are regulated during the cell cycle, showing a peak of expression at the G_2-M phase transition (Jouannic et al., 2001). In order to get an overview of the expression of each *A. thaliana* SIN-homologue during the cell cycle, we performed expression analyses of the *AtMAP3Kε1*, *AtMAP3Kε2*, *AtMAP4Kα1*, *AtMAP4Kα2*, *AtSGP1* and *AtSGP2* genes in aphidicoline-synchronised cell suspension cultures of *Arabidopsis* using real-time RT-PCR (Fig. 5B). As reference genes we monitored the expression of the cell cycle phase-specific markers histone *AtH4* (Reichheld et al., 1995) and cyclin *CycB1;1At* (Shaul et al., 1996), marking the S phase and the G_2-M transition, respectively. Examination of the transcript levels of these eight genes during the cell cycle revealed two types of expression patterns. The first expression profile is similar to the profile of *CycB1;1At*, and comprises transcripts profiles of *AtSGP1*, *AtMAP3Kε1*, *AtMAP3Kε2* and *AtMAP4Kα2* genes, which are all co-expressed at a higher level at the G_2-M transition. The second type consists of the transcript expression patterns of *AtMAP4Kα1* and *AtSGP2*. *AtSGP2* transcripts were markedly induced during S phase and the steady state level remained stable during G_2. In contrast to the other genes, *AtMAP4Kα1* displayed a quasi-constitutive expression profile during the course of the cell cycle. Hence, expression of several of the plant SIN-related genes is tightly regulated during the cell cycle with respect to their transcription and/or transcript stability, with the highest mRNA accumulation at the G_2-M phases.

AtSGP1, *AtSGP2*, *BnMAP3Kε1*, *AtMAP4Kα1* and *AtMAP4Kα2* co-localise to the nucleolus

In *S. pombe* a major part of the regulation of the SIN pathway proteins resides in a strict control of their subcellular localisation

Table 2. Interactions between plant SIN-related homologues and *S. pombe* SIN proteins in the yeast two-hybrid system

Plasmids		His auxotrophy	β-galactosidase activity
pGAD424	pGBT9	–	–
pGAD424-BnMAP3Kε1	pGBT9	–	–
pGAD424	pGBT9-BnMAP3Kε1	–	–
pGAD424-AtSGP1	pGBT9	–	–
pGAD424-AtSGP2	pGBT9	–	–
pGAD10-spg1	pGBT8-cdc7	+	++
pGAD10-spg1	pGBT8-byr4	+	++
pGAD424-BnMAP3Kε1	pGBT8-spg1	–	–
pGAD424-AtSGP1	pGBT8-byr4	+	+
pGAD424-AtSGP2	pGBT8-byr4	+	+
pGAD424-AtSGP1	pGBT8-cdc7	+	+
pGAD424-AtSGP2	pGBT8-cdc7	+	+
pGAD424-AtSGP1	pGBT9-BnMAP3Kε1	+	+
pGAD424-AtSGP2	pGBT9-BnMAP3Kε1	–	–
pGAD424-AtSGP1	pGBT9-BnMAP3Kα1	–	–
pGAD424-AtSGP1	pGBT9-BnMAP3Kβ1	–	–

β-galactosidase activity was determined by a filter assay as described in the Materials and Methods, for the yeast strains containing the indicated plasmids.

++, a strong positive blue colour; +, a positive pale blue colour and –, no β-galactosidase activity in filter assays. Identical results were obtained in at least three independent experiments.

during the cell cycle (Simanis, 2003). To investigate whether a similar regulation occurs in plant cells, the localisation of the plant SIN-related proteins was determined using transiently expressed GFP fusions. Because of their amenability to microscopic analysis, protoplasts of tobacco BY2 cells were used for these studies. Twenty-four hours after protoplast transformation, all GFP fusions with the different plant SIN-related homologues were localised to the nucleus of interphase cells (Fig. 6). The data shown in Fig. 6 illustrate that each of the GFP-tagged proteins appeared concentrated in the non-DAPI staining region of the nucleus, i.e. the nucleolus. These interphase cells were characterised by a large nucleolus. A faint, diffuse signal was also detectable in the nucleoplasm, all around the nucleus and in the cytoplasm (Fig. 6B2,B4-B6). In control cells transformed with GFP alone, fluorescence was localised both to the nucleus and to the cytoplasm, including cytoplasmic strands (Fig. 6B1). GFP alone is smaller than the accepted exclusion size limit of nuclear pores, in agreement with its distribution both in the cytoplasm and the nucleus (Chytilova et al., 2000). However, the size of the plant SIN-like component GFP fusion proteins, are above the exclusion size limit of nuclear pores indicating that the plant GFP-tagged proteins are translocated to the nucleus by an active process. We were unable to identify potential nuclear localisation signals similar to the ones identified by Kalderon et al. and Robbins et al. from database searches (Kalderon et al., 1984; Robbins et al., 1991). In conclusion, these results show that, in plant cells, all of the plant SIN-related components AtSGP1, AtSGP2, BnMAP3Kε1, AtMAP4Kα1 and AtMAP4Kα2 localise to the nucleolus.

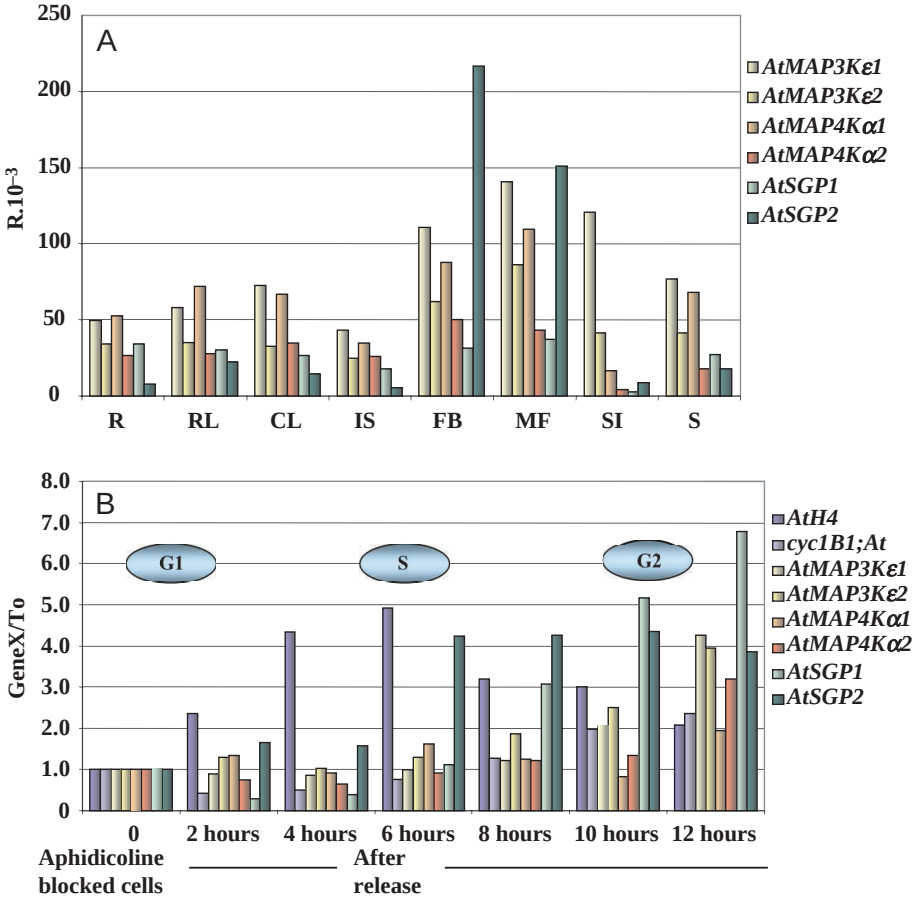

Fig. 5. *A. thaliana* SIN-related genes expression in various organs (A) and during the cell cycle (B). (A) The abundance of the *AtSGP1*, *AtSGP2*, *AtMAP3Kε1*, *AtMAP3Kε2*, *AtMAP4Kα1* and *AtMAP4Kα2* transcripts was examined in various organs using real-time quantitative PCR. The transcript levels were measured in RNAs extracted from roots (R), rosette leaves (RL), cauline leaves (CL), inflorescence stems (IS), flower buds (FB), mature flowers (MF), siliques (SI) and seedlings (S). Transcript levels are represented as a ratios (R) of the absolute value of the studied gene to the absolute value of the *ACT2/ACT8* genes. (B) Analyses of the relative expression levels of *AtSGP1*, *AtSGP2*, *AtMAP3Kε1*, *AtMAP3Kε2*, *AtMAP4Kα1* and *AtMAP4Kα2* during the cell cycle. Cultured *A. thaliana* cells were arrested at the G_1-S phase transition using aphidicolin. After removal of the drug (i.e. time 0), RNA extracts were prepared at the indicated time points and analysed. Accumulation of the G_2-M phase-specific genes *Cyc1B1;1* and *AtMAP3Kε1*, and the S phase-specific gene *AtH4* were simultaneously monitored. Transcript levels are represented as ratios (R) of the absolute value of the studied gene to the absolute value of each gene at T0.

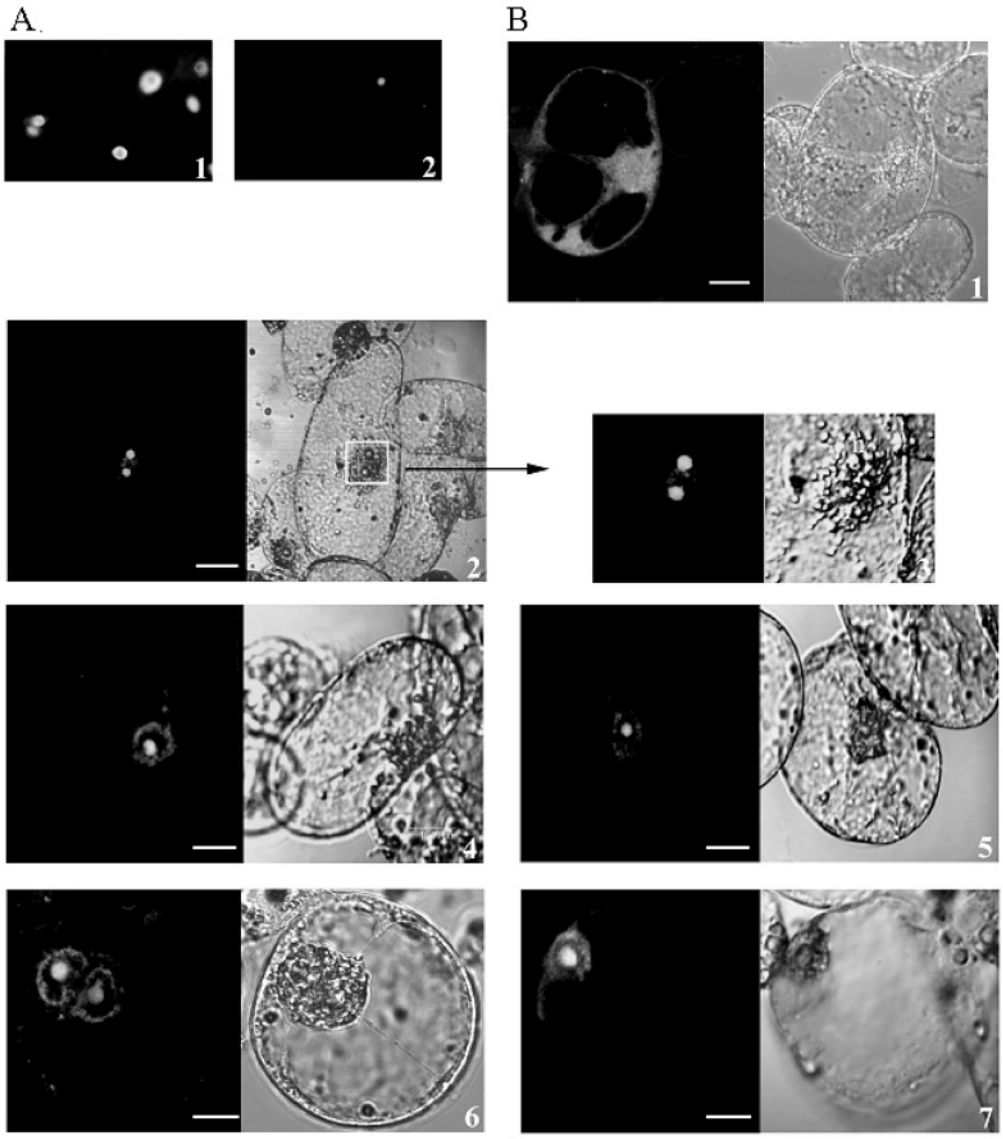

Fig. 6. Localisation of plant SIN-related proteins. (A) Interphase cell nuclei stained with DAPI (1) and epifluorescence microscopic image of BnMAP3Kε1-GFP (2). (B) Confocal fluorescent microscopic images (left) and the corresponding DIC images (right) of: (1) smGFP, (2) BnMAP3Kε1-GFP, (3) BnMAP3Kε1-GFP at a higher magnification showing a single nucleus with two nucleoli, (4) AtMAP4Kα1-GFP, (5) AtMAP4Kα2-GFP, (6) AtSGP1-GFP, (7) AtSGP2-GFP. Scale bars, 15 mm.

Discussion

The plant SIN-related components are functional homologues of spg1p, cdc7p and sid1p in fission yeast

A recent fundamental advance in understanding the mechanisms that coordinate mitosis and cytokinesis is the discovery of the SIN pathway. We present evidence that plants have SIN-related proteins that are related to the spg1p, cdc7p and sid1p proteins of fission yeast. Specifically, we established that (i) the *Arabidopsis* genome encodes homologues of the yeast spg1p and sid1p proteins; (ii) *Arabidopsis* AtSGP1, AtSGP2 and the *Brassica napus* BnMAP4Kα2 can complement the fission yeast septation initiation mutants *spg1-B8* and *sid1-239*; (iii) overexpression of the plant homologues of spg1p and cdc7p interferes with cytokinesis in fission yeast, and (iv) the *Arabidopsis* AtSGP1 and AtSGP2 proteins interact with relevant partners of the yeast SIN pathway (i.e. cdc7p and byr4p) in the yeast two-hybrid system. These results suggest that AtSGP1, AtSGP2 and BnMAP4Kα2 function in yeast cells as orthologues of *S. pombe* SIN pathways proteins. The molecular similarities observed between the plant genes and their yeast counterparts, known to be involved in cytokinesis, indicate that the plant and the *S. pombe* sequences are probably derived from common ancestral genes. However, rescue of the mutant phenotype and dominant-negative effects, show a partial functional divergence between the plant and the yeast proteins. The amino acid sequences of the plant SIN-related components show some differences with the *S. pombe* proteins spg1p, cdc7p and sid1p, such as plant-specific amino- or carboxyl-terminal domains. This suggests that the plant SIN-related proteins perform novel functions compared to the *S.*

pombe couterparts, by using these additional domains for distinct regulations or interactions.

Do the plant SGP, MAP3Kε and MAP4Kα proteins act in the same signalling pathway?

We provided independent sets of data, which are compatible with the hypothesis that plant SIN-related elements act in a same signalling pathway. We have shown that the plant proteins SPG, MAP3Kε and MAP4Kα can functionally replace spg1p, cdc7p and sid1p, which are involved in the SIN signal transduction network in fission yeast. AtSGP1 and BnMAP3Kε1 interact as observed for spg1p and cdc7p in the yeast two-hybrid system. Schmidt et al. showed that one possible role for spg1p in fission yeast is to direct cdc7p to its correct subcellular location in order to activate substrates such as sid1p (Schmidt et al., 1997). It is noteworthy that in the same system, however, the protein BnMAP3Kε1 failed to interact with the plant paralogous sgp1p-like protein AtSGP2. One interpretation of this finding is that there is a functional difference between the two AtSGP proteins. Alternatively, the lack of interaction could be a yeast two-hybrid artefact or a difference between *Brassica napus* and *Arabidopsis* proteins. Additional experiments are required to resolve these issues.

SIN-related gene expression is not regulated in an organ-specific fashion. Similarly, *BnMAP3Kε1* gene expression is not organ dependent, however, using in situ hybridisation signal quantification, *BnMAP3Kε1* gene expression is higher in dividing cells than non-dividing ones (Jouannic et al., 2001). The expression of *AtSGP1*, *AtMAP3Kε1*, *AtMAP3Kε2* and *AtMAP4Kα2* genes increased coordinately during late G_2 phase of the cell cycle in *Arabidopsis* suspension cells, in contrast to *AtSGP2*, which is induced during S phase. The timing of mRNA accumulations was almost identical to *CycB1;1At* cyclin mRNA accumulation marking the G_2-M transition (Shaul et al., 1996). Similarly, we observed an accumulation of *BnMAP4Kα2* transcripts in freshly isolated microspores (data not shown), whose nuclei are in late G_2 (Binarova et al., 1993). The expression profile of these elements in plant cells might be compatible with a role in control of cell division. *AtMAP4Kα1* gene expression is not regulated in a cell cycle-dependent manner, suggesting that the two *AtMAP4Kα* genes possess different functions. In fact, in a previous study we showed that *AtMAP4Kα1* positively responded at the transcriptional level to both saline and PEG treatments by contrast to *AtMAP4Kα2*, suggesting that *AtMAP4Kα1* is involved in stress response (Charrier et al., 2002).

Huh et al. suggest that in yeast, the combination of transcriptional co-expression and protein co-localisation can be used to corroborate or predict the function of proteins (Huh et al., 2003). Our results indicated that all the proteins AtSGP1, AtSGP2, BnMAP3Kε1, AtMAP4Kα1 and AtMAP4Kα2 localised to the nucleolus during interphase. Such a compartmentalization of the plant elements should be compared to the co-localisation of various *S. pombe* SIN pathway proteins to the spindle pole body. One of the most striking examples is provided by the yeast protein phosphatase clp1p. This protein, acting downstream of the SIN pathway, appears in the nucleolus during interphase, but later during mitosis it exits from the nucleolus and interacts with the mitotic spindle and the actomyosin ring in a SIN-dependent signalling mechanism (Trautmann et al., 2001). Recent analysis of the human nucleolar proteome (Andersen et al., 2002) reinforces the hypothesis of a multi-protein complex of clustered network components. Andersen and collegues report a proteomic analysis using a combination of mass spectrometry and sequence database searches and highlight the dynamic nature of the nucleolar proteome (Andersen et al., 2002). Newly proposed roles for the nucleolus may include its function in the sequestration and the release of proteins involved in cell division, gene silencing and senescence.

In order to identify other components of the putative plant SIN-related pathway, such as regulators and targets of the cascade, an extensive search in the *A. thaliana* genomic sequence database was carried out. Although we have shown that AtSGP1 and 2 interact with the *S. pombe* cdc16p-associated protein byr4p, no putative homologues of byr4p have been identified in the available plant genome sequence databases. Furthermore, the *Arabidopsis* genome appears to lack genes homologous to the polo-like kinase plo1p and the protein phosphatase clp1p, acting upstream and downstream, respectively, of the SIN pathway. It has been shown (Sparks et al., 1999) that the sid2p and mob1p proteins from *S. pombe* form a complex, which seems to act downstream sid1p. Interestingly, mob1p-like protein-encoding genes are present in the *A. thaliana* genome (i.e. *At5g45550* and *At4g19050*), but at present it is unclear whether plant genomes encode homologues of sid2p and cdc14p (i.e. sid1p-interacting proteins). Our yeast two-hybrid experiments did not show interaction between the sid1p-like protein BnMAP4Kα2 and cdc14p (unpublished data). These results indicate that some of the plant SIN pathway regulators and possible targets may have diverged during evolution to such an extent that interaction between the yeast and plant proteins is no longer possible. Moreover, sequence database analyses reveal that the SIN pathway components are differentially conserved between the three eukaryotic kingdoms as suggested by the fully sequenced genomes from *S. cerevisiae*, *A. thaliana*, *Drosophila melanogaster*, *Anopheles gambiae*, *Caenorhabditis elegans* and *Homo sapiens*. Some of them seem to be conserved in the three kingdoms such as mob1p- and sid2p-related proteins (Xu et al., 1995; Tao et al., 1999). Some elements are conserved in fungi and metazoan but not in plants, such as the polo kinases and the Cdc14p-clp1p protein phosphatases (Donaldson et al., 2001; Gruneberg et al., 2002). A few elements seem to be specific to fungi such as the cdc16-byr4/Bub2p-Bfa1p two-component GTPase-activating protein complexes and the Lte1p guanine exchange factor. Conversely, the core elements spg1p-, cdc7p- and sid1p-related proteins are conserved only in the fungal and plant kingdoms.

These observations raise the possibility that functional counterparts of several SIN regulators exist in higher eukaryotes.

The role of plant SIN-related proteins in plant cytokinesis remains to be functionally demonstrated. Nevertheless, based upon the homology with the SIN pathway from *S. pombe* and our data, we can propose the following model. AtMAP3Kε1, recruited by AtSGP1, is able to activate AtMAP4Kα2 resulting in the activation of downstream processes (Fig. 1). Several questions still have to be addressed, in particular the effect on plant development of either the overexpression or knock-down

of the SIN-related gene and the localisation of these plant proteins during mitosis, in order both to clarify their roles in plant cell division and to understand the molecular network including AtSGP, AtMAP3Kε and AtMAP4Kα proteins.

We are grateful to C. Bergounioux and colleagues at the IBP for providing help with the cell cycle analysis. We would like to thank S. Brown from the ISV for many fruitful discussions and his expertise with confocal microscopy. We thank A. Mingam and A. Lecharny for their helpful discussions concerning the real-time RT-PCR technology. We gratefully acknowledge the assistance of I. Gy, R. Boyer and J.-P. Bares for DNA sequencing, for technical help with photographic art work and for access to plant growth facilities, respectively.

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