SB401, a pollen-specific protein from Solanum berthaultii, binds to and bundles microtubules and F-actin

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Summary

We characterize a novel, pollen-specific, microtubule-associated protein, SB401, found in Solanum berthaultii. This protein binds to and bundles taxol-stabilized microtubules and enhances tubulin polymerization in a concentration-dependent manner, particularly at lower temperatures. Electron microscopy revealed that the protein decorates the entire length of microtubules. Cross-linking and electrophoresis studies showed that SB401 protein forms dimers, and suggest that dimerization could account for bundling. Double immunofluorescent staining of pollen tubes of S. berthaultii showed that SB401 protein co-localized with cortical microtubule bundles. SB401 protein also binds to and bundles actin filaments, and could connect actin filaments to microtubules. SB401 protein had a much higher affinity for microtubules than for actin filaments. In the presence of both cytoskeletal elements, the protein preferentially bound microtubules to form bundles. These results demonstrate that SB401 protein may have important roles in organizing the cytoskeleton in pollen tubes.

Keywords: microtubule-associated protein, microtubules, actin, pollen tubes, Solanum berthaultii.

Introduction

Microtubule-associated proteins (MAPs) regulate the dynamics and organization of microtubules (MTs). Recently, an increasing number of MAPs or MT-related proteins have been identified in plant cells. Some of these proteins types have homologues in animal cells, whereas others are unique to plants (Hussey et al., 2002; Lloyd and Chan, 2004; Lloyd and Hussey, 2001; Sedbrook, 2004; Wasteneys, 2000). For example, the plant MAP, MOR1 (microtubule organization 1) is a homologue of Xenopus MAP215 and has an important role in cortical MT organization (Whittington et al., 2001). The cross-linking MAP65 has been identified in carrot, tobacco and Arabidopsis (Chan et al., 1999; Jiang and Sonobe, 1993; Smertenko et al., 2000, 2004), and is homologous to the spindle mid-zone proteins Ase1p, found in yeast (Schuyler et al., 2003), and PRC1, found in human cells (Mollinari et al., 2002). BY-2 cells contain a 190 kDa protein that binds to both MTs and actin filaments, which suggests that it might play a role in the interaction between these two components of the cytoskeleton (Igarashi et al., 2000). A katanin-like protein, which has the ability to sever MTs, has been identified in Arabidopsis and alters the oriented deposition of cellulose microfibrils (Burk and Ye, 2002). A 90 kDa phospholipase D from tobacco BY-2 binds to MTs and the plasma membrane (Gardiner et al., 2001), and triggers the re-orientation of cortical MTs when activated (Dhonukshe et al., 2003). In addition to these investigations on the identity and function of MAPs, studies on the identity and function of kinesin-related microtubule motor proteins (KRP)s have also been performed in plant cells (reviewed by Lee and Liu, 2004). Collectively, these studies on MAPs have increased our understanding of the cellular functions of MAPs and MT-related proteins in plants. Pollen tube growth is essential for reproduction in higher plants. Through tip growth, the pollen tube grows towards the ovules and delivers the male germ unit to the embryo sac for fertilization. Accordingly, pollen tube growth is a good...
model system for investigating the control and regulation of cell growth in plant. The cytoskeleton is crucial for the tip growth of pollen tubes. A large body of evidence has established that actin filaments are fundamental, not only for delivering substances to the pollen tube tip by cytoplasmic streaming, but also because they are directly involved in tip growth in response to environmental cues (reviewed by Geitmann and Emons, 2000; Vidali and Hepler, 2001). Although the precise functions of MTs in pollen tube growth have yet to be elucidated, research has established that MTs do participate in pollen tip growth. For example, treatment with MT inhibitors partially blocks gymnosperm pollen germination and growth of pollen tubes, which results in an abnormal morphology and cytoplasmic architecture (Anderhag et al., 2000). Treating pollen tubes of the tobacco plant (Nicotiana sylvestris) with MT inhibitors results in attenuated movement of the vegetative nucleus and the generative cell from the pollen grain into the pollen tube, and disruption of the cellular polarity that is normally maintained by numerous cytoplasmic components (Äström et al., 1995; Joos et al., 1994). In addition, MTs form unique arrays and have special functions, such as mediating the migration of the nucleus to the generative pole during pollen development (Zonia et al., 1999). Although it is widely recognized that the movement of organelles in pollen tube cells involves actin, there is evidence that KRPs also participate in this process (Romagnoli et al., 2003). Moreover, MTs may have an important role in the guidance of tip growth, as seen in the tip growth of root hair cells of Arabidopsis (Bibikova et al., 1999; Ketelaar et al., 2003). Therefore, microtubules are probably involved in the tip growth of pollen tubes, but little is known about the dynamics and organization of MTs in this process. Furthermore, a pollen-specific MAP has not yet been identified.

The SB401 protein was first identified in 1997 by the Thompson group from a cDNA library of in vitro-germinated pollen of the diploid potato species, Solanum berthaultii (Liu et al., 1997). SB401 belongs to the ‘late’ gene group of pollen-expressed genes. It is exclusively expressed in anthers: SB401 mRNA was not detected before the mid-binucleate stage or in other vegetative tissues (Liu et al., 1997). It is expressed in the late stage of pollen maturation, throughout pollen germination and is enriched in extracts of mature pollen grains and in vitro-germinated pollen. Most interestingly, this protein contains six imperfectly repeated motifs of the sequence V-V-E-K-K-N/E-E, which resembles a repetitive domain responsible for MT binding of the microtubule-associated protein, MAP1B, found in murine cells (Noble et al., 1989). However, preliminary experiments showed that SB401 was not associated with MTs (Liu et al., 1997), and so further investigation was necessary to establish whether SB401 is a MAP.

Here, we report our investigations into the properties of SB401. These studies demonstrate that SB401 can indeed bind to MTs and causes their bundling in vitro. Double staining of MTs and SB401 showed that SB401 is associated with cortical MTs in the cortex of pollen tubes. In addition, SB401 also binds to and bundles actin filaments. Hence, SB401 may play a role in the regulation of MT organization, and function as a link between the actin and MT cytoskeleton.

Results

Purification of the recombinant SB401 protein

A construct of the cDNA responsible for encoding SB401 protein was created using pET-30a(+) vectors, and the recombinant SB401 protein was purified (see Experimental procedures). Figure 1 shows the mass of recombinant SB401 protein determined using polyacrylamide gels with various concentrations of SDS. The predicted molecular mass of SB401 protein is 30.137 kDa. On 10% SDS–polyacrylamide gels, the molecular mass of SB401 is 50 kDa, whereas it is 60 kDa on 8% SDS–polyacrylamide gels. Liu et al. (1997) have described and discussed the mobility of SB401 protein on SDS–polyacrylamide gels.

SB401 protein binds to MTs and enhances tubulin polymerization

Co-sedimentation experiments were performed to determine whether the SB401 protein binds to MTs. After incubation with taxol-stabilized MTs, SB401 protein was co-sedimented with MTs by centrifugation. SB401 protein

Figure 1. Coomassie blue-stained gel of expressed and purified recombinant SB401 protein.

Lane 1, total extract from bacterial cells (10 μg); lane 2, total extract from bacterial cells for SB401 protein without IPTG induction (10 μg); lane 3, total extract from IPTG-induced bacterial cells for SB401 protein expression (20 μg); lane 4, purified SB401 after Ni-NTA agarose purification (3 μg). The purity of SB401 protein after purification was estimated to be about 95%, according to gel scanning.
was not detected in the pellets in the absence of MTs (Figure 2a). The quantity of SB401 protein in the pellets increased when a higher concentration of MT SB401 protein was added. Saturation was reached when the concentration of added SB401 reached 6 μM (Figure 2b). The binding ratio between MTs and SB401 protein was 1:0.4 after measuring the concentration of tubulin dimers and SB401 in the pellets at saturation level (Figure 2b). This result indicates that recombinant SB401 protein binds to taxol-stabilized MTs in vitro.

To test whether SB401 protein has any effect on tubulin polymerization, we performed turbidimetric assays. Figure 3 shows typical processes of tubulin polymerization in the presence of various concentrations of SB401 protein at various temperatures. At equilibrium, both the rate of tubulin polymerization and the quantity of MTs were increased significantly by adding SB401 protein. These effects occurred in a concentration-dependent manner. When tubulin polymerization was performed at 35, 30 and 20°C, the enhancement effect on tubulin polymerization after adding SB401 protein was more pronounced at low temperatures (Figure 3). Tubulin usually did not polymerize at 20°C, but some polymerization did occur after adding SB401 protein (Figure 3).

**SB401 protein bundles MTs in vitro**

To further investigate the effect of SB401 on MTs, we used confocal microscopy to examine the effect of adding SB401 protein to rhodamine-conjugated MTs. In the presence of

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**Figure 2.** Recombinant SB401 protein binds to taxol-stabilized MTs. Pre-formed taxol-stabilized MTs polymerized from 2 μM tubulin were incubated with various concentrations of SB401, as shown.

(a) After centrifugation, the supernatants (S) and pellets (P) were analysed by 8% SDS-PAGE.

(b) Binding to MTs was saturated at a stoichiometry of 0.4 mol SB401 per mol of tubulin dimer, estimated by gel scanning.

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**Figure 3.** SB401 protein enhances tubulin polymerization.

Tubulin (50 μM) was polymerized at 20, 30 or 35°C in the presence of varying concentrations of SB401 protein, and changes in the turbidity of the tubulin suspension were monitored over 30 min. The polymerization rate and quantity of MTs at equilibrium both increased significantly in the presence of SB401 protein, especially at lower temperatures.

SB401 protein, MTs became organized into densely packed bundles in a concentration-dependent manner (Figure 4). When SB401 protein was absent, MTs were scattered individually throughout the solution (Figure 4a). After adding 1 μM SB401 protein, short and thin MT bundles appeared (Figure 4b). When 2 μM SB401 protein were added, the MT bundles became densely packed and formed disconnected aggregates that ultimately meshed into a large meshwork composed of long and wavy MT bundles (Figure 4c). The addition of denatured SB401 protein, prepared by boiling the native protein for 1 min, had no effect on MT bundling (Figure 4d).

Although the formation of MT bundles proceeded rapidly at high temperatures, the MT-bundling effect of SB401 protein was quite conspicuous even at low temperatures. At
SB401 binds to and bundles microtubules and F-actin

0 or 4°C, the taxol-stabilized MTs formed bundles about 10 min after the addition of SB401 protein. At 37°C, bundles formed almost immediately after addition of SB401 protein. Once formed, the MT bundles persisted for as long as 24 h at room temperature.

To investigate further the process of MT bundling, we added increasing concentrations of NaCl to detach SB401 protein from the MTs (Figure 4). Before adding NaCl, MT bundles were pre-formed using 2 μM SB401 protein (Figure 4e). Addition of 50 mM NaCl reduced the mass of MT bundles. The large aggregated MT bundles lost their wavy shape and were transformed into bundles in which the MTs tended to be straight (Figure 4f). Further increases in the NaCl concentration resulted in loosening of MT bundles. Approximately half of the MTs remained in bundles after addition of 100 mM NaCl (Figure 4g). When the NaCl concentration was increased to 200 mM, the bundles broke down, and apparently single, scattered MTs were observed (Figure 4h).

Next, we examined the structure of SB401 protein–MT bundles using electron microscopy. In the presence of SB401 protein, the MTs were tightly bunched along their whole length (Figure 4i,j). On the electron micrographs, negatively stained dots were observed between the individual MTs in the bundles (Figure 4k), and the distance between them was about 6 nm (Figure 4l).

SB401 protein forms polymers

In order to answer the question of whether SB401 protein formed dimers, we conducted cross-linking experiments and gel analysis using native protein treated with EDC [1-ethyl-3-(3-dimethylamino-propyl) carbodiimide]. After incubating SB401 protein with EDC, the product was run on an SDS-PAGE gel. Three bands appeared (Figure 5a, lane 2), whereas only one band was present if the protein was not exposed to EDC (Figure 5a, lane 1). To confirm this observation, we also performed acrylamide gel analysis using native SB401
protein, and an identical result to that obtained using SDS–PAGE was obtained. Three protein bands appeared on the gel at 30, 60 and 120 kDa (Figure 5b). We concluded from these experiments that SB401 protein can form 60 kDa dimers in solution. The detection of a 120 kDa band suggests that the protein may be also capable of forming tetramers.

SB401 protein co-localizes with MTs in pollen tubes

To examine the association of SB401 protein with MTs, we raised an SB401 protein antibody and observed the localization of SB401 protein in the pollen tubes of *S. berthaultii* by double staining SB401 protein and MTs. Western blotting established that the antibody was specific for SB401 protein isolated from the protein extracts of *S. berthaultii* pollen (Figure 6a). Under the confocal microscope, we observed large bundles of cortical MTs all along the shank of the pollen tube, but they were fragmented at the apical region (Figure 6b). SB401 protein was distributed as dot-like structures throughout the cytoplasm and cortex (Figure 6c). SB401 protein was found in the cell cortex and co-localized mostly with large MT bundles (Figure 6d). Following treatment of the pollen tubes with 1.5 μM propyzamide and disassembly of the MTs, the SB401-labelled structures dispersed (Figure 6e,f). Staining with pre-immune serum showed no detectable signals in pollen tube cells of *S. berthaultii* (Figure 6h–j), which confirmed the specificity of the SB401 protein antibody.

SB401 protein binds to and bundles F-actin

The co-sedimentation experiments established that SB401 protein binds not only to MTs but also to actin filaments. When increasing concentrations of SB401 protein were

Figure 5. SB401 protein forms dimers.

(a) SB401 protein dimers were analysed by EDC cross-linking experiments and native acrylamide gels. Three major bands appeared on the gel (lane 2), while only one band was present if the protein was not cross-linked by EDC (lane 1).

(b) Native SB401 protein was run on a native acrylamide gel and three major bands were detected.

Figure 6. SB401 co-localizes with MTs in *Solanum berthaultii* pollen tubes.

(a) Western analysis showed that the antibody specifically recognized SB401 from the protein extracts of *S. berthaultii* pollen tubes.

(b–g) Double staining of MTs and SB401 in *S. berthaultii* pollen tubes by confocal immunofluorescence microscopy was performed. (b) Cortical MTs formed large bundles all along the shank of the pollen tube. (c) Dot-like structures, presumed to be SB401 protein, are visible in the cytoplasm and cortex. (d) Overlay image of (b) and (c) showing that the SB401 protein (dots) at the cell cortex of the pollen tube shank were mostly co-localized with large MT bundles. (e) MTs after depolymerisation using propyzamide. (f) SB401 protein-labelled elements became more dispersed when MT aggregates were disassembled. (g) Overlay image of (e) and (f).

(h–j) Staining with pre-immune serum showed no detectable signals in *S. berthaultii* pollen tubes. (h) Cortical MTs stained with tubulin antibody. (i) No signal was detected with Pre-immune serum to stain SB401. (j) Overlay image of (h) and (i).

Bar in (j) = 5 μm, and applies to (b–j).
incubated with F-actin, the pellets following centrifugation became enriched with SB401 protein in a concentration-dependent manner. The binding ratio of monomeric actin:SB401 protein was approximately 1:0.22 at the saturation concentration (Figure 7). The presence and absence of SB401 protein did not affect the extent of actin polymerization. When observed under a confocal microscope, the actin filaments bundled together in a fashion similar to the formation of MT bundles when SB401 protein was present (Figure 8). Pre-formed single actin filaments remained scattered throughout the suspension if SB401 protein was not added (Figure 8a). However, actin bundles appeared when 1 μM SB401 protein was added (Figure 8b). In the presence of 3 μM SB401 protein, more actin bundles were formed and these bundles aggregated (Figure 8c). It took much longer for the actin filaments than the MTs to form bundles (30 min versus 5 min). The addition of 200 mM NaCl to detach SB401 protein from the actin filaments also resulted in disassembly of the F-actin bundles (Figure 8d). When observed using electron microscopy, actin bundles were formed in the presence of SB401 protein (Figure 8e,f). The distance between actin filaments in the bundles was approximately 6 nm, the same distance as between MTs in the SB401 protein–MT bundles.

**SB401 protein preferentially bundles single-filament MT compared to single-filament actin**

In view of the results demonstrating that SB401 protein can bind to both MTs and actin filaments, we considered whether it could function as a connector between these two cytoskeletal components. To address this issue, we performed several experiments involving 0.5 μM taxol-stabilized rhodamine-conjugated MTs and 0.5 μM F-actin polymerized with 100 nM Alexa-488 phalloidin. In the absence of SB401 protein, F-actin remained as single filaments (Figure 9a). When 0.25 μM SB401 protein was added to the suspension, actin filaments were induced to form bundles (Figure 9b). After the actin bundles formed, pre-formed taxol–MTs were then added to the suspension. The MTs became bundled within 5 min after their addition to the suspension, with the bundles being mostly separate from...

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**Figure 7.** Recombinant SB401 protein binds to F-actin. F-actin was pre-formed in a 2 μM actin solution, and incubated with 0–8 μM SB401. (a) After centrifugation, the supernatants (S) and pellets (P) were analysed by SDS–PAGE. (b) Binding to F-actin was saturated at a stoichiometry of 0.22 mol SB401 per mol of F-actin, estimated by gel scanning.

**Figure 8.** SB401 protein bundles F-actin. Pre-formed F-actin (10 μM) was labelled with Alexa-488 phalloidin. Various concentrations of SB401 to 0.5 μM actin were then added, and the mixture was incubated for 60 min before observation. Confocal microscopic images of F-actin (a) in the absence of SB401 protein, (b) after adding 1 μM SB401 protein, (c) after adding 2 μM SB401 protein and (d) after adding 200 mM NaCl to the suspension containing 2 μM SB401 protein. The F-actin bundles were dispersed into single actin filaments. Bar in (d) = 10 μm, and applies to (a–d). (e and f) Electron micrographs of negatively stained samples of the experiments depicted in (a) and (c). (e) Actin filaments in the absence of SB401 protein. (f) Actin bundles with SB401. The distance between actin filaments in the bundles is approximately 6 nm. Bar in (f) = 50 nm, and applies to (e) and (f).
the actin bundles (Figure 9c). After 60 min, more MT bundles were formed, and the actin bundles separated into single actin filaments (Figure 9d). When pre-formed single-filament taxol–MTs (Figure 9e) were bundled by the addition of 0.25 μM SB401 protein (Figure 9f), and then F-actin was added to the suspension (Figure 9g), the MT bundles remained intact and actin bundles were not observed after 60 min (Figure 9h). This observation indicates that SB401 protein preferentially binds to MTs when it is not present in sufficient quantities to cause bundling of single-filament MTs and actin.

In a series of additional experiments, we first mixed 0.5 μM pre-formed MTs and 0.5 μM actin filaments in the absence of SB401 protein. Both MTs and actin filaments remained as distinct single filaments, scattered randomly throughout the suspension. Co-localization of MTs and actin filaments was not observed in the double-stained preparation (Figure 9i). After the addition of 1 μM SB401 protein, a concentration four times greater than that used in the previously described co-localization experiments involving MT and actin, MT bundles were formed before the formation of actin bundles (Figure 9j). This result confirms our previous finding that the time taken for actin bundles to form in the presence of SB401 protein is longer than that needed for MT bundles to form. With time, actin bundles began to co-localize with MT bundles (Figure 9k), which suggests that actin bundles and MT bundles might be connected by SB401 protein. When SB401 antibody was added to the connected MT and actin bundles in the presence of SB401 protein, the bundles broke down (Figure 9l). For this breakdown to have occurred, it is likely that a dynamic exchange took place between the free SB401 protein in the suspension and the bound SB401 protein on the MTs and actin filaments. Accordingly, we suggest that SB401 protein plays an active role in binding MTs to actin filaments.

Discussion

A previous report showed that the glutamic acid-rich protein, SB401, contains repeated motifs of the sequence V-V-E-K-K-N/E/E (Liu et al., 1997), with the K-K-N/E-E core motif resembling the MT-binding domain of murine MAP1B.
(Noble et al., 1989). This basic MT-binding region has no structural relationship with the MT-binding domains of other classes of MAP, such as kinesin, MAP2 or tau (Noble et al., 1989).

A BLAST search showed that proteins containing this imperfect repeated motif of sequence V-V-E-K-K-N/E-E are present in a variety of plant species, including ST901 from Solanum tuberosum cv. Desiree (accession number AAS17876), a pollen-specific lysine-rich protein SBgLR from S. tuberosum (accession number AAR29265, Lang et al., 2004) and TSB from Lycopersicon esculentum (accession number AAM53961, Zhao et al., 2004). A BLAST search for sequences in the Arabidopsis genome identified also a gene (At5g44610), located on chromosome 5, that encodes a protein with unknown function and that contains repeated V-E-E-K-K motifs. It would be of interest to research the interaction of these proteins with MTs and actin in the future. In addition, a recent report demonstrated that significant homology exists between the autophagic protein AtAtg8 and the microtubule binding, light chain 3 of MAP1A and B (Ketelaar et al., 2004). Comparison of these proteins with SB401 shows that ST901 from S. tuberosum cv. Desiree has 73% homology, SBgLR from S. tuberosum has 71% homology, and TSB from L. esculentum has 66% homology. However, the two MT-binding proteins described in Arabidopsis do not exhibit any homology with the proteins found in Solanum species. Therefore, although the repeated motif is found in proteins of several plant species, the SB401 protein appears to be a genus-specific protein.

**SB401 protein binds to both MTs and actin filaments**

Preliminary experiments by Liu et al. (1997) indicated that SB401 protein does not bind to MTs. Nevertheless, our present study demonstrated that SB401 protein binds to MTs. Several reasons may explain these different experimental results. Liu et al. (1997) reported that recombinant SB401 protein formed inclusion bodies and therefore may be inactive. In addition, the SB401 protein used in their experiments was a truncated protein, starting at amino acid 23 and finishing at the C-terminal end. Whether this molecule possesses MT-binding activity has yet to be determined.

Our experiments using cross-linked proteins and native gels suggest that SB401 protein forms dimers to bundle MTs. The protein AtMAP65-1 from Arabidopsis can also form dimers to link MTs, with the MT-binding site at its C-terminal end and bundling activity at its N-terminus (Smertenko et al., 2004).

Various lengths for the inter-MT bridges induced by MAPs have been reported. The proteins of the MAP65 family are capable of forming inter-MT cross bridges of 25–30 nm in length (Chan et al., 1999; Smertenko et al., 2004). This distance contrasts with the smaller cross-bridge distance of 6 nm that we observed when SB401 protein was used. The 65 kDa MAP isolated from tobacco BY-2 cells induces cross bridges of 10–12 nm length between adjacent MTs (Jiang and Sonobe, 1993). The cross bridges in the MT bundles induced by purified tobacco 65 kDa MAP and in the cycled cortical MTs are 2–3 nm long, which is much shorter than the cross bridges of 30–35 nm length observed in isolated plasma membrane vesicles and isolated cortical MTs (Sonobe et al., 2001). The results of our experiments also showed that the addition of SB401 protein induces the formation of wavy MTs. This formation of wavy MT bundles by SB401 protein contrasts with the action of other plant MAPs. For example, proteins of the MAP65 family usually characteristically induce the formation of large and straight MT bundles (Chan et al., 1999; Mao et al., 2005; Smertenko et al., 2004; Wicker-Planquart et al., 2004). We conjecture that the variation of inter-MT spacing in MT bundles might be related to the organization of MTs.

Our studies also indicate that SB401 protein, similar to MAP1B, binds to and bundles actin filaments, although the protein binds preferentially to MTs. The KKEE motif, which appears in both proteins, is also present in the F-actin binding domain of villin (Friederich et al., 1992). It would be of interest to establish whether these repeated motifs account for the binding of SB401 protein to filaments of MTs and actin. If so, this may well explain the nature of the competitive binding of MTs and F-actin for SB401 protein. In the presence of MTs and actin filaments, the SB401 protein binds preferentially to MTs and binding to actin does not occur if free or excess SB401 protein is not available.

**SB401 protein may play a role in mediating interaction between microtubules and membrane organelles in pollen tubes**

We have shown here that the SB401 protein may be associated with organelles in pollen tube cells. The decoration of large MT bundles in the shank, and shorter, possibly fragmenting, MTs at the tip, are consistent with the guidance or movement of materials to the tip. The globular pattern of their decoration has also been observed with other plant MAPs. For example, the AtMAP65-6 isoform is associated with mitochondria and is seen as a dot-like structure in a suspension of Arabidopsis cells (Mao et al., 2005). Two proteins, with apparent M of 161 and 90 kDa and found in tobacco pollen tubes, bind to MTs and are associated with the plasma membrane compartment (Cai et al., 2005). In the present study, we observed dot-like structures localized with cortical MTs. We propose that these dots represent the punctate attachment of organelles to the microtubular cytoskeleton. It is likely that SB401 protein targets specific organelles to MTs, and functions as
a transport conduit or a platform for a subsequent reaction.

**Does SB401 protein play a role in coordination of the microtubule and actin cytoskeletons in pollen growth?**

SB401 protein is specifically expressed in the late stage of pollen maturation and throughout pollen germination (Liu et al., 1997). Therefore, we wished to examine the protein's role in this process. The actin cytoskeleton is of fundamental importance for pollen tube growth. Most of the actin filaments form bundles orientated longitudinally in cortical and endoplasmic regions of the pollen tube cells, and are organized into a reverse fountain pattern for cytoplasmic streaming. In addition to the actin cytoskeleton, many MTs are oriented longitudinally and sometimes adopt a slight helical distribution in these cells (Geitmann and Emons, 2000; Hepler et al., 2001). Because treatment of pollen tubes cells with an MT depolymerization agent has little effect on germination or elongation of angiosperm pollen, MTs have attracted relatively little attention regarding their role in pollen tube growth. However, recent studies have shed new light on their function in tip growth (Sieberer et al., 2005). Apart from maintaining polarity in pollen tubes, movement of the male germ unit and vesicle transport in pollen tubes, MTs may also play a role in determining the direction of tip growth. Depolymerization or stabilization of MTs resulted in broadening of the tip expansion area, whereas results show that it binds preferentially to MTs rather than to actin filaments when both of these cytoskeletal components are present. Two putative casein kinase phosphorylation sites within two units of the repeat motifs suggest that phosphorylation may be crucial in the regulation of SB401 protein activity (Liu et al., 1997). Pedrotti and Islam (1996) reported that dephosphorylated, but not phosphorylated, MAP1B binds to microfilaments. Therefore, it is reasonable to assume that phosphorylation could modulate the binding of SB401 protein to MTs and actin filaments. In addition, a recent study showed that the MT-bundling activity of a MAP, NtMAP65-1, may be regulated by a mitogen-activated protein kinase in tobacco cells (Sasabe et al., 2006). The interaction between AtMAP65-1 and MTs is also regulated by phosphorylation and dephosphorylation of the protein (Smertenko et al., 2006). Therefore, future experiments could be aimed at exploring the role of phosphorylation of SB401 protein in its MT-bundling activity.

**Experimental procedures**

**Preparation of recombinant SB401 protein and its antibody**

The cDNA sequence is available from EMBL Nucleotide Sequence Database GenBank (accession number X95984). The GSB plasmid containing the SB401 gene was kindly provided by Dr Junqi Liu at China Agricultural University. The cDNA sequence encoding SB401 protein was amplified by PCR using two primers: forward 5'-GGAGGATCATGCTGGGTTTTGATG-3' (BamHI site underlined) and reverse 5'-GGGAAGCTTAAAAGCCAGGATTTA-3' (HindIII site underlined). The full-length cDNA for the SB401 gene was reconstructed into pET-30a(+) vector.

The recombinant protein with a six-His tag on the N-terminus was expressed in Escherichia coli strain BL21 (DE3). Bacterial cultures were grown in LB (Luria–Bertani) broth medium supplemented with 50 mg l⁻¹ kanamycin at 37°C until the culture reached an optical density of 0.4–0.6 measured spectrophotometrically at 600 nm. The induction of protein expression was performed by adding 1 mM
isopropylthio-galactoside, and allowed to progress for 4 h. The
to a thin section, MTs were collected after centrifugation at 50 000

g. The amount of SB401 protein bound to MTs was deter-
mined by gel scanning with an Alpha™ 2200 (Alpha; http://
www.alphamettas.com).

For the F-actin and SB401 protein co-sedimentation assay, rabbit
muscle actin and SB401 protein were centrifuged at 100 000 g
for 60 min at 4°C before use. Then, 2 μM F-actin and 0–8 μM SB401
were incubated in a 100 μl volume of PEBM buffer. After 30 min incubation
at room temperature, samples were centrifuged at 100 000 g
for 60 min at room temperature. The protein concentration was
measured three times using the method described above.

Microtubule polymerization assay
To monitor the time course of tubulin polymerization, varying
concentrations (0, 0.2, 0.4 or 0.8 μM) of SB401 protein were added to
50 μM tubulin in PEBM buffer containing 1 mM GTP. Microtubule
polymerization was monitored spectrophotometrically at OD350 in a
0.4 cm-wide quartz cuvette at 20, 30 and 35°C for 30 min using a
beckmancoulter.com) in a temperature-controlled compartment.
The polymerization experiments were repeated at least three times
at each temperature and with the various SB401 protein concen-
trations.

Microtubule and F-actin bundling assays
Taxol-stabilized MTs (0.5 μM) were incubated with 0, 0.5, 1, 2, 3, 4 or
5 μM SB401 protein in PEBM buffer at room temperature in a 100 μl
volume, and fixed with 1% glutaraldehyde after various incubation
periods (5, 30 or 60 min). Aliquots (1 μl) of the samples were placed
onto a slide and observed using a confocal microscope (Meta 510,
Zeiss; http://www.zeiss.com/) with a Zeiss 100× magnification oil
objective (NA 1.3).

F-actin (0.5 μM) was incubated with 0, 0.5, 1, 2, 3, 4 or 5 μM SB401
protein at room temperature for 5, 30 or 60 min, then fixed with 1%
glutaraldehyde. Aliquots (1 μl) of the samples were placed onto a
slide and observed using a confocal microscope. The results were
compared to those obtained using SB401 protein that had been
denatured by boiling for 1 min before adding to the incubating
solution.

Bundles of taxol-stabilized MTs were formed by adding 2 μM
SB401. NaCl was then added so that the final NaCl concentrations
were 50, 100, 150, 200 or 250 mM. The samples were incubated for
10 min and then observed under the confocal microscope.

For the electron microscopy studies, MTs or actin filaments were
observed either by negative staining or thin sectioning. Negative
staining was performed using saturated uranyl acetate. To prepare
the thin section, MTs were collected after centrifugation at 50 000 g
for 30 min at 25°C. The pellets were fixed in 1% v/v glutaraldehyde in
PEBM buffer followed by 1% w/v osmium tetroxide, and then
embedded in LR White acrylic resin. Samples were then sectioned,
and stained with uranyl acetate and lead citrate. The sections were
observed under a Hitachi 7500 electron microscope.

To observe the linkage of MTs and F-actin by SB401, 0.5 μM taxol-
stabilized MTs were mixed with 0.5 μM pre-formed actin filaments
polymerized with 100 μM Alexa-488 phalloidin, 1 μM SB401 protein
was added, and the reaction was fixed in 1% glutaraldehyde after 5
or 60 min incubation. Aliquots (1 μl) of the suspension were placed
SB401 dimer assays

To assess the extent of dimer formation of SB401 protein, cross-linking experiments were performed using EDC [1-ethyl-3(3-dimethylaminopropyl)carbodiimide; Sigma] (Doi et al., 1987). EDC was added to PEM buffer containing 16 μM SB401 protein to give a final concentration of 4 mM. The sample was then incubated at room temperature for 1 h. The cross-linking reaction was stopped by adding SDS–PAGE loading buffer and then boiling for 5 min. The proteins were separated on a 10% gel, running at 180 V for 120 min.

The acrylamide gel analysis was performed as described by Daniel et al. (1996). Native SB401 protein and proteins of known molecular weight (ovalbumin, 43 kDa; albumin, 67 kDa; lactate dehydrogenase, 140 kDa; catalase, 232 kDa; ferritin, 440 kDa; thyroglobulin, 669 kDa) were electrophoresed on 8–12% native acrylamide gel. Protein mobility (Rf) was calculated by dividing the distance of protein migration by the distance of migration of the dye front. Plotting the log (100 Æ Rf) against the acrylamide concentration creates a line whose slope defines the molecular weight of the protein. The slopes generated from the experiments show a linear relationship with the molecular weight. The molecular mass of SB401 protein and its oligomers were extrapolated from the molecular weight standards.

Localization of SB401 protein, microtubules and F-actin in pollen tubes

Pollen of S. berthaultii was collected from the potato fields in Zhangjiako, Hebei Province, China, and stored at −20°C. The pollen was germinated in germination medium (20-30 mg pollen grains/ml) (H2BO3, 0.01% KNO3, 0.02% MgSO4.7H2O, 0.07% Ca(NO3)2, 2% sucrose, 15% PEG-6000) at 25°C for 45 min. The fixed pollen tubes were fixed with 4% formaldehyde in 2% sucrose, 15% PEG-6000, 50 mM PEM buffer, pH 6.9, for 45 min. The fixed cells were treated with 1% w/v cellulase (Sigma, http://www.sigmaaldrich.com/Local/SA-Splash.html) and 1% w/v pectolyase (Fluka; http://www.sigmaaldrich.com/Brands/Fluka_Riedel_Home.html) in PBS for 15 min. SB401 protein was probed with rabbit SB401 antibody diluted 1:100 in PBS containing 3% w/v BSA (Sigma) and 0.1% w/v Tween-20 at 4°C overnight. The samples were washed three times with PBS before adding mouse anti-


acquired using a Zeiss confocal microscope with a Zeiss 100x oil objective (NA 1.4).

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