The Putative RNA-Processing Protein, THO2, is a Microtubule-Associated Protein in Tobacco

Takahiro Hamada¹,²,⁴,*, Hisako Igarashi¹,³,⁴, Ryoichi Taguchi¹, Masayuki Fujiwara², Yoichiro Fukao², Teruo Shimmen¹, Etsuo Yokota¹ and Seiji Sonobe¹

¹Department of Life Science, Graduate School of Life Science, University of Hyogo, Harima Science Park City, Hyogo, 678-1297 Japan
²Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, 630-0101 Japan
³National Institute for Basic Biology, Myodaiji-cho, Okazaki, Aichi, 444-8585 Japan

THO2 is a component of the THO–TREX (transcription and export factor) complex that participates in mRNA metabolism and export from the nucleus in yeast and animal cells. Here we report that tobacco putative THO2-related protein (NtTHO2) is a microtubule-associated protein, which directly binds to microtubules in vitro and co-localizes with cortical microtubules in vivo. We purified endogenous NtTHO2 by cycles of microtubule polymerization–depolymerization from crude extracts of tobacco BY-2 miniprotoplasts. Purified NtTHO2 sedimented with microtubules in vitro. Immunofluorescence revealed that NtTHO2 was present in both the nucleus and cytoplasm. In interphase, cytoplasmic NtTHO2 was localized along cortical microtubules. In the mitotic phase, NtTHO2 was localized to the mitotic spindle but not to either the preprophase band or the phragmoplast. In mature cells of seedling roots, and in BY-2 cells in which proliferation was stopped by removing 2,4-D, NtTHO2 staining was confined mainly to the nucleolus. These results suggest that NtTHO2 is a multifunctional protein that participates in mRNA metabolism, and also functions within the cortical microtubules and mitotic spindle.

Keywords: BY-2 cells • Microtubule • Microtubule-associated protein • Nicotiana tabacum • Nucleolus • THO2.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; LC, liquid chromatography; MAP, microtubule-associated protein; MS, mass spectrometry; MTP, microtubule protein; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; RNP, ribonucleoprotein; TREX, transcription and export factor.

Introduction

In plant cells, structures made with microtubules underlie various cellular functions in morphogenesis and mitosis. To fulfill these functions, the cell requires a suite of microtubule-associated proteins (MAPs), which play essential roles in governing microtubule behavior (Hamada 2007, Buschmann and Lloyd 2008, Sedbrook and Kaloriti 2008, Wasteneys and Ambrose 2009). For example, MOR1 and SPR2/SRL2 are involved in overall microtubule organization via regulation of microtubule dynamics (Whittington et al. 2001, Buschmann et al. 2004, Hamada et al. 2004, Shuji et al. 2004, Yao et al. 2008). Likewise, MAP65 and WVD2/WDL are involved in microtubule organization via bundling activity (Jiang et al. 1993, Yuen et al. 2003, Muller et al. 2004, Perrin et al. 2007). These are examples where the activity of the protein has been studied both in vivo and in vitro. There is a larger group of examples where microtubule-related activity has been inferred based on studies in vivo only, based for the most part on mutant phenotypes. Even including both types of example, we estimate that only a small minority of plant MAPs have been discovered so far.

MAPs were discovered originally based on microtubule affinity. Because assembled microtubules are massive structures compared with individual proteins, they can be pelleted from a cell extract, taking binding proteins down with them. The pellet is then resuspended and the microtubules disassembled, releasing all bound proteins. By repeating this several times, a population enriched in MAPs is obtained. Indeed, decades ago, this procedure was used to define the major families of MAPs in animals (Cassimeris and Spittle 2001). However, the affinity approach was not particularly...
successful in plants, probably because the concentration of such proteins in plant cell extracts is low; even in favorable cases, the outcome was often nothing more than indicating a band on a silver-stained gel. Therefore, plant scientists interested in finding MAPs turned to developmental genetics, and experienced greater success (Hamada 2007, Buschmann and Lloyd 2008, Sedbrook and Kaloriti 2008, Wasteneys and Ambrose 2009).

Nevertheless, over the years, techniques for biochemical analysis have greatly advanced (Jiang and Sonobe 1993, Igarashi et al. 2000, Yasuhara et al. 2002, Hamada et al. 2004). With the ability to identify peptides from extremely small samples with mass spectroscopy coupled to the availability of genomic databases, reviving an affinity-based approach to discovering MAPs is proving fruitful (Korolev et al. 2005, Buschmann et al. 2006, Hamada et al. 2006). Many such proteins are arguably beyond the reach of typical mutant screens, either because the proteins are essential or because their function is covered redundantly by another protein, at least under common laboratory conditions.

Starting with tobacco BY-2 cells, we used microtubule affinity to prepare a fraction enriched in MAPs. Validating our approach, one of the first proteins investigated turns out to be a homolog of THO2, known in yeast and animals to be active in RNA metabolism and export, but apparently never before detected in association with the microtubule cytoskeleton. Here, we report that plant THO2-related protein binds to microtubules directly in vitro and localizes to cortical microtubules in vivo.

Results

MAPs were purified from a crude extract of tobacco mini-protoplasts by two cycles of microtubule polymerization–depolymerization. We focused on a 195 kDa polypeptide in the MAP fraction and prepared a monoclonal antibody against it. In the crude extract, this antibody specifically recognized a 195 kDa polypeptide (Fig. 1A, Bb), but not other known high molecular weight MAPs, including MAP190 (Fig. 1Ba) and MAP200 (Fig. 1Bc) (Igarashi et al. 2000, Hamada et al. 2004).

Purification of the 195 kDa polypeptide

To begin characterizing the 195 kDa polypeptide, we purified it. The microtubule protein (MTP) fraction was applied to an anion-exchange column (DEAE-Sephacel) and eluted by a linear gradient of 50–600 mM NaCl. The 195 kDa polypeptide was eluted between 150 and 250 mM NaCl (Fig. 2A, lane a). These fractions were pooled and applied to a hydroxyapatite column. The 195 kDa polypeptide was eluted at around 100 mM potassium phosphate. These fractions contained polypeptides in addition the 195 kDa polypeptide (Fig. 2A, lane b); therefore, they were applied to a cation-exchange column (CM Sepharose CL-6B). The 195 kDa polypeptide was eluted between 150 and 250 mM NaCl (Fig. 2A, lane a). These fractions were pooled and applied to a hydroxyapatite column. The 195 kDa polypeptide was eluted at around 100 mM potassium phosphate. These fractions contained polypeptides in addition the 195 kDa polypeptide (Fig. 2A, lane b); therefore, they were applied to a cation-exchange column (CM Sepharose CL-6B). The 195 kDa polypeptide was eluted between 150 and 250 mM NaCl (Fig. 2A, lane a). These fractions were pooled and applied to a hydroxyapatite column. The 195 kDa polypeptide was eluted at around 100 mM potassium phosphate. These fractions contained polypeptides in addition the 195 kDa polypeptide (Fig. 2A, lane b); therefore, they were applied to a cation-exchange column (CM Sepharose CL-6B). The 195 kDa polypeptide was eluted between 150 and 250 mM NaCl (Fig. 2A, lane a). These fractions were pooled and applied to a hydroxyapatite column. The 195 kDa polypeptide was eluted at around 100 mM potassium phosphate. These fractions contained polypeptides in addition the 195 kDa polypeptide (Fig. 2A, lane b); therefore, they were applied to a cation-exchange column (CM Sepharose CL-6B). The 195 kDa polypeptide was eluted between 150 and 250 mM NaCl (Fig. 2A, lane a). These fractions were pooled and applied to a hydroxyapatite column. The 195 kDa polypeptide was eluted at around 100 mM potassium phosphate. These fractions contained polypeptides in addition the 195 kDa polypeptide (Fig. 2A, lane b); therefore, they were applied to a cation-exchange column (CM Sepharose CL-6B). The 195 kDa polypeptide was eluted between 150 and 250 mM NaCl (Fig. 2A, lane a). These fractions were pooled and applied to a hydroxyapatite column. The 195 kDa polypeptide was eluted at around 100 mM potassium phosphate. These fractions contained polypeptides in addition the 195 kDa polypeptide (Fig. 2A, lane b); therefore, they were applied to a cation-exchange column (CM Sepharose CL-6B).
polypeptide was eluted between 190 and 250 mM NaCl. In these fractions, on the basis of Coomassie blue staining, the 195 kDa polypeptide appeared free of contaminating polypeptides (Fig. 2A, lane c).

The 195 kDa polypeptide binds directly to microtubules in vitro

To analyze the microtubule-binding property of the 195 kDa polypeptide, we performed a microtubule co-sedimentation assay (Fig. 2B). We prepared samples containing a constant quantity of the 195 kDa polypeptide (1.06 µg) and different quantities of taxol-stabilized microtubules (0–4 µg). The samples were centrifuged and the amount of 195 kDa polypeptide recovered in the pellet was quantified by densitometry. The assay revealed that the 195 kDa polypeptide could bind to microtubules directly and, further, that the molar ratio between the 195 kDa polypeptide and tubulin dimer was 1:2.1. We subsequently analyzed microtubule bundling with electron microscopy and microtubule dynamics with dark-field microscopy, but we did not find any notable effect of the 195 kDa polypeptide.

Identification of the 195 kDa polypeptide as a tobacco homolog of THO2 (NtTHO2)

We identified amino acid sequences from the 195 kDa polypeptide by liquid chromatography/tandem mass spectrometry (LC/MS). The analyzed sample was prepared from the purified fraction from the hydroxyapatite column followed by immunoprecipitation using the 195 kDa polypeptide monoclonal antibody. Mass spectrometry revealed that amino acid sequences of the 195 kDa polypeptide are identical to those from an Arabidopsis thaliana protein (At1g24706.1). A BLAST search indicated that the A. thaliana protein is similar to a component of the putative THO–TREX (transcription and export factor) complex, THO2, which has been studied previously in animals and fungi in connection with RNA metabolism. Comparison of the deduced amino acid sequences among THO2 genes shows that the identity/similarity of the A. thaliana protein (At1g24706.1) is 25/58% to Homo sapiens THO2, 24/56% to Drosophila melanogaster THO2 and 21/54% to Schizosaccharomyces pombe THO2 (Supplementary Fig. S1). In addition, the arabidopsis protein sequence has 51% identity and 78% similarity to a translated sequence present in the rice genome. THO2 is a large protein and is conserved throughout, although conservation is lower in the C-terminal quarter of the polypeptide. Based on these results, we define the 195 kDa polypeptide as the tobacco putative THO2-related protein (NtTHO2).

In animals and yeast, THO2 is a component of the THO–TREX complex, involved in transcription (yeast), splicing (animals) and mRNA export (both). Several components of the THO–TREX complex are different between animals and yeast (Read and Cheng 2005). We found that the A. thaliana genome contains putative homologs for all eight of the proteins so far identified as components of the animal THO–TREX complex, implying that the entire THO–TREX complex is conserved in plants. Furthermore, these homologs were present in a fraction containing arabidopsis MAPs (our unpublished data). The results suggest that NtTHO2 might interact with microtubules as part of the THO–TREX complex.

NtTHO2 localizes not only to the nucleus but also to the spindle and cortical microtubules

To characterize NtTHO2 further, we examined its intracellular localization by immunofluorescence microscopy. In interphase BY-2 cells, NtTHO2 staining was bright in the nucleus (Fig. 3A, B). Confocal microscopy revealed that...
NtTHO2 staining was punctate (Fig. 3C). The nuclear localization is consistent with the canonical THO2 function in mRNA export and splicing.

In addition, we found punctate staining in the cytoplasm (Fig. 3A, C), consistent with microtubule binding in vitro. Many NtTHO2-positive puncta were observed in the cortical region of interphase cells, some of them seeming to co-localize with microtubules (Fig. 4). To examine the localization more clearly, we used BY-2 membrane ghosts (Fig. 5). On ghosts, membrane-binding structures (including cortical microtubules and associated proteins) remain, but most of the cytoplasm is lost during preparation. On the ghosts, NtTHO2 puncta were fewer in number and smaller in size compared with intact cells, and appeared to be closely associated with microtubules. These results substantiate the microtubule association of THO2.

We also localized NtTHO2 at mitosis (Fig. 6). During the transition from interphase to the mitotic phase, the pre-prophase band is formed in the cortical region around the nucleus. NtTHO2 puncta remained in the cytoplasm and were not concentrated in the pre-prophase band (Fig. 6A–C). At metaphase, NtTHO2 puncta associated with the mitotic spindle (Fig. 6D–F), and were also observed at or near the plasma membrane (Fig. 6D), even though cortical microtubules are no longer present. At telophase, when the nuclear membrane is reconstituted and the phragmoplast expands outward to the cell periphery, most of the NtTHO2 staining was localized to the nucleus and there were a few NtTHO2 puncta dispersed throughout the cytoplasm (Fig. 6G–I). Therefore, NtTHO2 appeared to associate with the spindle but with neither the pre-prophase band nor the phragmoplast.

Nucleolar localization of NtTHO2 in non-proliferating cells

We also localized NtTHO2 in root cells of tobacco seedlings. Unexpectedly, NtTHO2 staining was prominent in the nucleolus (Fig. 7A, B). Prominent nucleolar localization of NtTHO2 staining was also observed in log-phase BY-2 cells (Fig. 7E), but only in approximately one-fifth of the culture (Fig. 8). Because the root cells in which we observed nucleolar localization were not dividing, we removed 2,4-D from the BY-2 cells' growth medium, a treatment known to curtail division and enhance elongation (Hasezawa and Syono 1983). With increasing time after removal of 2,4-D, cell division decreased and the percentage of cells showing prominent nucleolar localization increased (Fig. 8). Nuclear localization of NtTHO2 was not altered at 12 h after removing 2,4-D (data not shown). At 1 d after removal, nucleolar staining became evident (Fig. 7D, and Fig. 8: 1d), and 3–4 d after removal it became predominant, with little NtTHO2 staining elsewhere in the nucleus (Fig. 8: 3d, 4d). These results suggest that localization of NtTHO2 is distinct in proliferating and elongating cells.

Discussion

In yeast and animals, THO2 is part of the THO–TREX complex that participates in mRNA export from the nucleus to the cytosol (Reed and Cheng 2005, Kohler and Hurt 2007). Yeast THO2 is also involved in transcription; on the other hand, animal THO2 is involved in mRNA splicing. To accomplish mRNA maturation and export, eukaryotic mRNAs form messenger ribonucleoprotein (mRNP) complexes with various proteins that mediate 5' end capping, splicing, 3' end...
cleavage, polyadenylation, and mRNA surveillance and export (Aguilera 2005). Finally, mature mRNP is exported to the cytosol. Our finding that tobacco THO2 is present in the nucleus and nucleolus, particularly in elongating cells, suggests that the plant THO–TREX complex likewise plays a role in RNA processing.

The involvement of THO2 in RNA export is apparently highly regulated. In D. melanogaster, the THO2 complex is involved in the export of specific mRNAs stimulated by heat shock, but is not involved in the constitutive export of mRNAs (Rehwinkel et al. 2004). We found that, in tobacco, the abundance and localization of THO2 within the nucleus were a function of cellular development. Elongated root cells and non-dividing BY-2 cells had modest THO2 staining in the nucleus, whereas dividing BY-2 cells had predominant nuclear localization. Comparing dividing vs. elongating cells, the expression pattern of mRNAs is dramatically different, as is auxin-mediated transcriptional activation and repression (Okushima et al. 2005). The changes observed in NtTHO2 localization are consistent with differentiation to an elongating or mature cell state being accompanied by an increasing demand for THO2 in one or more steps of mRNP formation.

Surprisingly, we identified NtTHO2 as a novel MAP that binds microtubules directly in vitro. We also confirmed that NtTHO2 localizes to cortical microtubules and to the mitotic spindle in vivo. These results suggest that NtTHO2 functions in the cytoplasm. Although roles for the THO–TREX complex in the nucleus are well known, this is apparently the first report suggesting a role for a member of the THO–TREX complex in the cytoplasm.

Fig. 5 Localization of NtTHO2 on a BY-2 membrane ghost. (A) NtTHO2. (B) Tubulin. (C) Merge. (D) A higher magnification of a region from (C). Many NtTHO2 puncta abut cortical microtubules. Bar = 5 µm (A–C) and 2.5 µm (D).
complex in the cytoplasm. Although we did not detect NtTHO2 influencing microtubule behavior in vitro, it is possible that such functionality requires a complete THO–TREX complex.

Interestingly, the organization of the mitotic spindle has been reported to require nuclear- and nucleolar-localized RNPs (Zatsepina 1999, Rabitsch et al. 2003, Raemaekers et al. 2003, Blower et al. 2005, Ma et al. 2007, Montembault et al. 2007, Eliscovich et al. 2008). Genome-wide RNAi (RNA interference) screening of spindle organization in Drosophila S2 cells revealed that spindle morphology is defective in about 100 cases where transcriptional or translational proteins were silenced, including splicing factors, RNA polymerase, poly(A)-binding proteins, ribosomal proteins, elongation factors and undefined nucleolar proteins (Goshima et al. 2007). These RNPs localize to the nucleus in interphase and around the spindle at mitosis, in a manner reminiscent of what is reported here for NtTHO2. However, to our knowledge, the reason for spindle localization remains to be elucidated for any RNP.

NtTHO2 also localizes to cortical microtubules in interphase cells. Punctate staining along cortical microtubules has been reported for several plant MAPs that are implicated in microtubule organization, including γ-tubulin, MAP200

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**Fig. 6** Localization of NtTHO2 during the mitotic phase. (A, D, G) NtTHO2. (B, E, H) Tubulin. (C, F, I) Merge. A BY-2 cell in (A–C) prophase, (D–F) metaphase and (G–I) telophase. NtTHO2 puncta accumulate in the spindle (F) but not in the pre-prophase band (A) or in the phragmoplast (G). Bar = 20 µm.
and SPR2 (Hamada et al. 2004, Murata et al. 2005, Yao et al. 2008), suggesting that NtTHO2 may be involved in organization of cortical microtubules. On the other hand, several organelles also localize to the cytoskeleton in plants. For example, the prolamine body–endoplasmic reticulum, mitochondria and Golgi vesicles localize along both microtubules and actin microfilaments (Muench et al. 2000, Romagnoli et al. 2007). In animal cells, both actin and microtubules host RNA granules, which play a role in the distribution of translation-restrictive mRNA (Ross et al. 2008). If similar granules occur in plants, then NtTHO2 might act to anchor the particle on cortical microtubules by virtue of its microtubule-binding activity.

In conclusion, our results illustrate the usefulness of a biochemical approach for discovering MAPs. Further, our results provide insight into the surprising diversity of THO2 function in eukaryotes. The next key steps are to determine

Fig. 7 Nucleolar localization of NtTHO2 in tobacco seedlings and BY-2 cells. (A, B) Tobacco roots double-stained with anti-NtTHO2 (green) and anti-tubulin (red). B is a higher magnification of A. (C–E) Examples of NtTHO2 staining seen in BY-2 cells. C shows even staining throughout the nucleus. D shows a pattern with both the nucleus and nucleolus stained. E shows predominant nucleolar staining of NtTHO2 (left panel) and a DIC image of the same cell (right panel). Bars = 20 µm.

Fig. 8 Kinetics of the appearance of NtTHO2-stained nucleoli following 2,4-D removal from BY-2 cells. Categories counted are as shown in Fig. 7 (C, D, E). The number of observed cells was 215, 155, 95 and 48 for 0, 1, 3 and 4 d, respectively.
the role of THO2 in mRNA metabolism in plants, whether THO2 binds microtubules in animals and what service THO2 binding to microtubules performs for the plant cell.

Materials and methods

Cell culture and plant material

Tobacco BY-2 cells (Nicotiana tabacum ‘Bright Yellow 2’) were cultured as described by Nagata et al. (1981). For 2,4-D removal, culture medium was prepared without 2,4-D. Tobacco seedlings were grown on 1/2 strength Murashige and Skoog basal medium (Sigma Aldrich, St Louis, MO, USA), containing both 1% sucrose and 1% agar, under long days (16 h light, 8 h dark). For immunostaining, 7-day-old BY-2 cells and 10-day-old tobacco seedlings were used.

Preparation of MTPs

Preparation of MTP containing tubulin and MAPs was performed according to Hamada et al. (2004) with slight modifications. An 800 g aliquot of 5-day-old BY-2 cells was used to prepare tubulin and MAPs. Mini-protoplasts were prepared from BY-2 protoplasts by density gradient centrifugation on a Percoll solution [37% Percoll (Amersham Bioscience, Uppsala, Sweden), 6.5 mM HEPES-KOH pH 7.3, 0.49 M sucrose, 0.62 M sorbitol, 0.04 M MgCl₂] at 25,000×g for 30 min. Mini-protoplasts were suspended in 10 vols. of ice-cold extraction buffer [50 mM PIPES-KOH pH 7.0, 10 mM EGTA, 15% sucrose, 2 mM MgCl₂, 1% casein, 20 µg ml⁻¹ leupeptin, 20 µg ml⁻¹ pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol (DTT)] and homogenized with a Teflon homogenizer. The homogenate was centrifuged at 170,000×g at 2°C for 30 min. The supernatant, supplemented with 0.5 mM GTP and 20 µM taxol (LC Laboratories, Woburn, MA, USA), was incubated at 30°C for 10 min to polymerize tubulin. Microtubules were collected by centrifugation at 23,000×g for 10 min, suspended in a cold depolymerization buffer (20 mM PIPES-KOH pH 7.0, 0.4 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 µg ml⁻¹ leupeptin, 20 µg ml⁻¹ pepstatin A, 1 mM PMSF and 1 mM DTT) and left standing for 20 min on ice. MTP was recovered in the supernatant after centrifugation at 250,000×g for 5 min at 2°C. MTP was diluted with 4 vols. of the extraction buffer and further purified by a second cycle of microtubule polymerization–depolymerization using the procedure described above.

Isolation of 195 kDa polypeptides from MTPs

All procedures below were performed at 4°C and polypeptides were resolved by SDS–PAGE on 5% acrylamide gels. The MTP fraction was diluted 8-fold with MEP solution (1 mM MgCl₂, 2 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 50 µg ml⁻¹ leupeptin and 25 mM PIPES-KOH pH 7.0), and applied to a DEAE-Sephadex (Amersham Pharmacia Biotech AB) column pre-equilibrated with MEP solution supplemented with 50 mM NaCl. The adsorbed materials were eluted with a linear concentration gradient of 50–600 mM NaCl in the MEP solution. Fractions containing the 195 kDa polypeptides were pooled and diluted 2-fold with a solution containing 3 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF, 50 µg ml⁻¹ leupeptin and 25 mM PIPES-KOH (pH 7.0). The diluted sample was then chromatographed on a hydroxylapatite (Bio-Rad) column pre-equilibrated with a solution containing 150 mM NaCl, 1 mM EGTA, 2 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF, 50 µg ml⁻¹ leupeptin and 25 mM PIPES-KOH (pH 7.0). The adsorbed materials were eluted with a linear concentration gradient of 80–200 mM potassium phosphate buffer (pH 7.0) in the pre-equilibrated solution.

Fractions containing 195 kDa polypeptides were pooled and then dialyzed against a solution of 15 mM NaCl, 2 mM EGTA, 1 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF, 50 µg ml⁻¹ leupeptin and 25 mM PIPES-KOH (pH 7.5) on ice. The dialysate was then applied to a CM Sepharose CL-6B column pre-equilibrated with dialyzed solution. The adsorbed materials were eluted with a linear concentration gradient of 90–300 mM NaCl in the pre-equilibrated solution. The fractions containing the 195 kDa polypeptide were pooled and dialyzed against a solution of 20% sucrose, 60 mM NaCl, 2 mM EGTA, 1 mM MgCl₂, 0.5 mM DTT, 1 mM PMSF, 50 µg ml⁻¹ leupeptin and 25 mM PIPES-KOH (pH 7.0) on ice. After centrifugation at 40,000×g for 20 min, the supernatant was used as the 195 kDa polypeptide fraction for the cosedimentation assay described below.

Co-sedimentation analysis of the 195 kDa polypeptide with microtubules

Tubulin was purified from BY-2 cells according to a method described previously (Hamada et al. 2004) and polymerized as described above. Microtubules at various concentrations were mixed with the 195 kDa polypeptide fraction, and left standing for 10 min at room temperature. To prevent depolymerization, taxol at a final concentration of 10 µM was added to the mixture. As a control, the 195 kDa polypeptide fraction alone was treated in the same manner. The final concentration of protein in the 195 kDa polypeptide fraction was 13.3 µg ml⁻¹. The samples were centrifuged at 40,000×g for 5 min. Pellets were resuspended in the dialysis solution. Both the supernatants and pellets were analyzed by SDS–PAGE. The amount of 195 kDa polypeptide recovered in the pellets was measured by densitometry (Densitograph pattern analyzer model no. EPA-3000, Cosmo Bio. Co., Ltd., Tokyo, Japan) of Coomassie blue-stained gels.

Antibody production

The band at 195 kDa was excised from an SDS–polyacrylamide gel, sonicated with TiterMax Gold (CytRx Corporation, Norcross, GA, USA) and injected into female BALB/c mice. Spleen cells were fused with P3×63Ag.8.653 mouse
myeloma cells. Screening of monoclonal antibodies was performed by Western blotting. Production of antibodies against MAP190 and MAP200 was described in Igarashi et al. (2000) and Hamada et al. (2004), respectively.

**Immunoblotting**

Crude extract of tobacco BY-2 cells was obtained according to Igarashi et al. (2000). After SDS–PAGE, polypeptides were transferred to a PVDF membrane. Antibodies against MAP190, MAP200 and 195 kDa polypeptides were used as the primary antibodies. Alkaline phosphatase-conjugated antibody against mouse IgG (EY Laboratories Inc., San Mateo, CA, USA) was used as the secondary antibody.

**Immunofluorescence microscopy**

BY-2 cells were fixed by a cryo-fixation protocol. Cells were attached to a polylysine-coated coverslip and were frozen in liquid propane for 30 s. Cells were then substituted in 100% methanol at −80°C for 1 week, and sequentially warmed at −20, 4 and 25°C for 12, 2 and 1 h, respectively. The cells were rehydrated by progressive replacement of methanol with phosphate-buffered saline (PBS) during 4 h. The preparation of BY-2 membrane ghosts was described in Hamada et al. (2004). Fixed BY-2 cells or membrane ghosts were incubated in PBS supplemented with both a mouse monocular antibody against the 195 kDa polypeptide and a rabbit polyclonal antibody against BY-2 tubulin for 1 h at room temperature. After washing twice with PBS, the cells were incubated in PBS supplemented with both a goat Alexa-488-conjugated anti-mouse IgG and a goat Alexa-594-conjugated anti-rabbit IgG (1/1,000 dilution; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. DNA was stained with Hoechst (No. 33258) in PBS.

Tobacco seedlings were stained with a protocol slightly modified from Friml et al. (2003). Seedlings were fixed in 2% paraformaldehyde in PEM buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO₄) containing 0.05% NP-40 for 1 h. Fixation was performed under vacuum for the first 10 min. After washing in PEM buffer (three times for 5 min), cell walls were partially digested with an aqueous solution containing 0.5% Macerozyme R-10 (Yakuruto Co. Ltd., Tokyo, Japan) and 0.05% Pectriase Y-23 (Yakuruto Co. Ltd.) for 15 min at room temperature. After washing in PEM buffer (three times for 5 min), the seedlings were treated with PEM buffer containing 10% dimethylsulfoxide (DMSO) and 3% NP-40 for 1 h. After extensive washing in PEM buffer (five times for 10 min), blocking was performed with 2% bovine serum albumin (BSA) in PEM for 1 h. Antibody treatment was carried out in PEM containing 1% BSA supplemented with both the mouse monocular antibody against the 195 kDa polypeptide and rabbit polyclonal antibody against BY-2 tubulin for 1 h at 4°C overnight. After washing with PEM buffer (three times for 5 min), seedlings were incubated in PEM supplemented with both a goat Alexa-594-conjugated anti-mouse IgG and a goat Alexa-488-conjugated anti-rabbit IgG (1/500 dilution; Invitrogen) for 2 h at room temperature.

A fluorescence microscope (BX-50; Olympus, Japan) and confocal laser scanning microscope (LSM 510; Carl Zeiss Co., Ltd. or FV1000; Olympus, Japan) were used for observation.

**Mass spectrometry**

Mass spectrometry was performed according to Fujiwara et al. (2006). For samples run on SDS–PAGE and Coomassie blue stained, protein bands were de-stained with 50% (v/v) acetonitrile and 25 mM ammonium bicarbonate for 10 min, twice. Gels were then treated with DTT and iodoacetamide for 30 min successively, and then dried in a vacuum concentrator. Dried gels were digested with 0.25 µg of trypsin (Promega, Tokyo, Japan) in 50 µl of 25 mM ammonium bicarbonate, for 16 h at 37°C. Peptides were extracted from gels by 30 µl of 5% (v/v) formic acid and 20% (v/v) acetonitrile for 30 min, twice, and the solution of extracted peptides was dried using an evaporator. The dried sample was solubilized with 12 µl of 5% formic acid and 5% acetonitrile. Trypsin-digested peptides were loaded on the column (PEPMAPC18, 5 µm, 75 µm internal diameter, 15 cm; Dionex) using the CapLC system (Waters). Buffers were 0.1% formic acid in 5% acetonitrile (A) and 0.1% formic acid in 95% acetonitrile (B). A linear gradient from 5 to 45% of B for 25 min was applied, and peptides eluted from the column were introduced directly into a Q-TOF mass spectrometer (Waters) with a flow rate of 100 nl min⁻¹. Ionization was performed with a PicoTip nanospray source (New Objective, Woburn, MA, USA). Tandem MS spectra were subjected to the PEAKS program (Bioinformatics Solutions Inc., Waterloo, Canada) against a protein database (Viridiplantae, green plants) from the National Center for Biotechnology Information.

**Supplementary data**

Supplementary data are available at PCP online.

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