Large-scale Identification of Tubulin-binding Proteins Provides Insight on Subcellular Trafficking, Metabolic Channeling, and Signaling in Plant Cells*[S]

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Microtubules play an essential role in the growth and development of plants and are known to be involved in regulating many cellular processes ranging from translation to signaling. In this article, we describe the proteomic characterization of *Arabidopsis* tubulin-binding proteins that were purified using tubulin affinity chromatography. Microtubule co-sedimentation assays indicated that most, if not all, of the proteins in the tubulin-binding protein fraction possessed microtubule-binding activity. Two-dimensional gel electrophoresis of the tubulin-binding protein fraction was performed, and 86 protein spots were excised and analyzed for protein identification. A total of 122 proteins were identified with high confidence using LC-MS/MS. These proteins were grouped into six categories based on their predicted functions: microtubule-associated proteins, translation factors, RNA-binding proteins, signaling proteins, metabolic enzymes, and proteins with other functions. Almost one-half of the proteins identified in this fraction were related to proteins that have previously been reported to interact with microtubules. This study represents the first large-scale proteomic identification of eukaryotic cytoskeleton-binding proteins, and provides insight on subcellular trafficking, metabolic channeling, and signaling in plant cells. *Molecular & Cellular Proteomics 3:970–983, 2004.*

The cytoskeleton is the single most important structure that contributes to the highly ordered organization of the eukaryotic cell. It provides a framework for cell division and the trafficking of organelles and macromolecules, and also serves to regulate important cellular processes such as signaling, translation, and metabolism. The cytoskeleton plays a key role in a number of plant-specific processes, such as assisting in the formation of the cell plate, regulating cell-to-cell movement, and influencing the direction of cell elongation (1). A role for the microtubule (MT)[1] component of the cytoskeleton in many of these processes has been demonstrated, and a number of MT-binding proteins that are responsible for regulating these events have been identified.

Plant MTs are assembled into four distinct arrays during the cell cycle (2). Three of these arrays—the interphase cortical array, the pre-prophase band, and the phragmoplast—have no counterpart in animal cells. The cortical MT array has been linked to the regulation of cellulose microfibril deposition and, hence, a role in cell expansion, while the pre-prophase band and the phragmoplast have important roles in the positioning and synthesis of the new cell plate in dividing cells. The fourth array, the spindle, has an evolutionarily conserved role in the segregation of chromosomes during cell division. The organization and dynamics of MTs in these arrays depend on the activity of various MT-associated proteins (MAPs). Several plant MAPs have been identified, including the 65-kDa MAPs, MAP 190, and MOR1 (3). These proteins are important in cross-bridging MTs, linking MTs with actin filaments, and stabilizing MTs. Many other proteins are known also to bind to MTs but do not function as MAPs. These proteins, often called MT-interacting proteins (MIPs) (2, 4), likely bind to MTs as a mechanism to regulate their own activity, to direct their subcellular localization, or as a concentrating mechanism at specific locations within the cell. The interaction of these proteins with MTs is often transient, making it difficult to visualize their interaction with MTs *in situ* (5). The large surface area provided by the MT network, and the cytoskeleton as a whole, likely serves as a matrix for the binding of hundreds of proteins to an extent that is dependent on cell type and environmental conditions (6).

Several approaches have been used to identify plant MT-binding proteins. These have included biochemical purification methods, mutant screens, and the identification of plant homologs to animal MT-binding proteins using antibody

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[1] The abbreviations used are: MT, microtubule; CLASP, cytoplasmic linker protein-associating protein 1; EF-1α, elongation factor 1-α; MAP, microtubule-associated protein; MFP, multifunctional protein; MIP, microtubule-interacting protein; PABP, polyadenylation-binding protein; RNP, ribonucleoprotein; TCP-1, T-complex protein-1.

cross-reactivity and sequence database mining (7–10). One of the first biochemical purification approaches involved the polymerization of endogenous plant tubulin in the presence of taxol, followed by pelleting of the resulting MTs and their associated proteins. This procedure was often followed by rounds of MT assembly and disassembly to enrich the MT-binding proteins (9, 11). The addition of taxol to assist in the stabilization of plant MTs was necessary due to the low concentration of endogenous tubulin in plant cells. The addition of high concentrations of neuronal MTs has been used as an alternative to facilitate the recovery of MT-binding proteins (7). Modifications to these techniques have been aimed at eliminating problems associated with the tough cell wall and lytic vacuoles in plant cells, and the development of "gentle" extraction buffers that maintain cytoskeleton integrity has made the plant cytoskeleton purification procedure more efficient (12).

MT-binding proteins have also been successfully purified from both animal and plant cells using tubulin affinity chromatography techniques. Two studies demonstrated that the SDS-PAGE profiles of plant proteins that eluted from tubulin and MT affinity chromatography columns were qualitatively similar (5, 13), and essentially all proteins that bound to the tubulin affinity columns subsequently co-sedimented with MTs in pelleting assays (14). This indicates that tubulin affinity chromatography can be used to successfully purify MT-binding proteins. Indeed, tubulin affinity chromatography has been used to identify three authentic plant MT-binding proteins—elongation factor-1α (EF-1α) (15), the peroxisomal multifunctional protein (5), and phospholipase D (14)—as well as additional authentic and putative MT-binding proteins from both plant and animal cells (16–18).

Although numerous reports on the identification of cytoskeleton-binding proteins have been published, only a few of these reports have described the identification of multiple proteins of this type. For instance, in neutrophil cells the purification of a detergent-resistant membrane cytoskeleton led to the identification of 19 cytoskeleton-associated proteins involved in signaling (19). Similarly, a study of the actin cytoskeleton in thrombin-stimulated human platelet cells identified 27 actin-binding proteins from a detergent-resistant cell extract (20). These types of studies have also been performed in plants. Using a detergent-resistant cytoskeleton/protein body fraction from rice and maize endosperm cells, 15 and 5 cytoskeleton-associated proteins were identified, respectively (21, 22). Another plant study identified 8 proteins from a detergent-resistant protein fraction from pea stems (12). Here we report the identification of 122 proteins that were purified from an *Arabidopsis* cell suspension culture extract using tubulin affinity chromatography. Most, if not all, of these proteins possessed MT-binding activity *in vitro*, and almost one-half of these proteins were related to proteins that were previously shown to interact with MTs. The identification of these tubulin-binding proteins provides insight on the function of specific MT/protein interactions in plant cells.

EXPERIMENTAL PROCEDURES

Growth of Arabidopsis Suspension Cells—*Arabidopsis* suspension cells were cultured in Murashige and Skoog medium (pH 5.8, M-6899; Sigma, St. Louis, MO) containing 3% (w/v) sucrose and supplemented with 1 mg/liter 1-naphthaleneacetic acid and 1 mg/liter kinetin (23). The cells were incubated at 23 °C in the dark and subcultured every 7 days by pipetting the suspension (one-tenth of the final volume) into fresh medium. Cells were harvested 3 days after subculturing, and the cell paste was frozen in liquid nitrogen and stored at −80 °C.

Preparation of the Protein Extract—Eighty grams of *Arabidopsis* cell culture were ground to a fine powder in liquid nitrogen, and 40 ml of cold extraction buffer (200 mM HEPES, pH 7.6, 10 mM $MgSO_4$, 10 mM EGTA, 20 mM DTT, 2 mM PMSF, and 5 μg/ml each of leupeptin, pepstatin, and aprotinin) was then added, followed by further grinding. After filtering through two layers of Miracloth (Calbiochem, La Jolla, CA), the extract was centrifuged for 30 min at 48,000 $\times$ *g* at 4 °C in an ultracentrifuge (model L7; Beckman, Fullerton, CA), and the supernatant was collected and centrifuged for 90 min at 100,000 $\times$ *g* at 4 °C to remove cellular debris and insoluble proteins. The supernatant from the 100,000 $\times$ *g* centrifugation step was then filtered through a 0.45-μm nylon filter.

Tubulin Affinity Chromatography and MT Co-sedimentation Assays—A bovine brain tubulin column with a 5-ml bed volume was prepared as described previously (5). A BSA control column was also prepared with an equivalent amount of protein as the tubulin column and using the same coupling procedure. Cell extract containing ~150 mg of protein was loaded onto the columns at a rate of 0.5 column volumes per hour at 4 °C. The columns were washed thoroughly with 20 column volumes of washing buffer (50 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 50 mM KCl, 0.5 mM DTT, and 2.5 μg/ml each of leupeptin, pepstatin, and aprotinin), and proteins that bound to the columns were eluted with washing buffer containing 500 mM KCl. The protein was acetone precipitated in preparation for gel electrophoresis, or desalted and concentrated using a microfiltration apparatus (Centricon, Millipore, Bedford, MA) for co-sedimentation experiments.

MT co-sedimentation assays were performed as described previously (5). Briefly, 5 μg of MTs and 2 μg of tubulin-binding proteins were incubated either alone or together in 20 mM HEPES, pH, 7.5, 50 mM KCl, 1 mM EGTA, 0.1 mM GTP, 1 mM DTT, and 2.5 μg/ml each of leupeptin, pepstatin, and aprotinin at 24 °C for 30 min and then centrifuged at 100,000 $\times$ *g* for 15 min in a benchtop ultracentrifuge (model TL-100; Beckman). The supernatant was carefully removed and the pellet was gently washed and then solubilized in SDS-PAGE gel-loading buffer, and the supernatants and pellets were then analyzed by SDS-PAGE.

Gel Electrophoresis—SDS-PAGE, two-dimensional NEPHGE, and immunoblot analysis were performed as described previously (5, 21). Protein gels were stained with either Coomassie Brilliant Blue stain (R-250) or by silver staining (Silver Stain Plus; Bio-Rad, Hercules, CA). For immunoblot experiments, rice multifunctional protein (MFP) and EF-1α antisera were both used at a dilution of 1:1,000, and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Sigma) were used at a dilution of 1:3,000.

Mass Spectrometry—Coomassie blue-stained protein spots were excised from the two-dimensional gel and analyzed individually by LC-MS/MS at the University of Victoria Genome BC Proteomics Centre. Protein samples were "in-gel" digested with trypsin in 50 mM ammonium bicarbonate overnight at 37 °C, followed by extraction of peptides with 10% formic acid. Samples were pumped onto a 300-μm inner diameter $\times$ 1-mm PepMap C18 5-μm, 100-A nano precolumn (LCPackings/Dionex, Sunnyvale, CA) to concentrate and desalt the peptide mixture before MS analysis. The peptides were

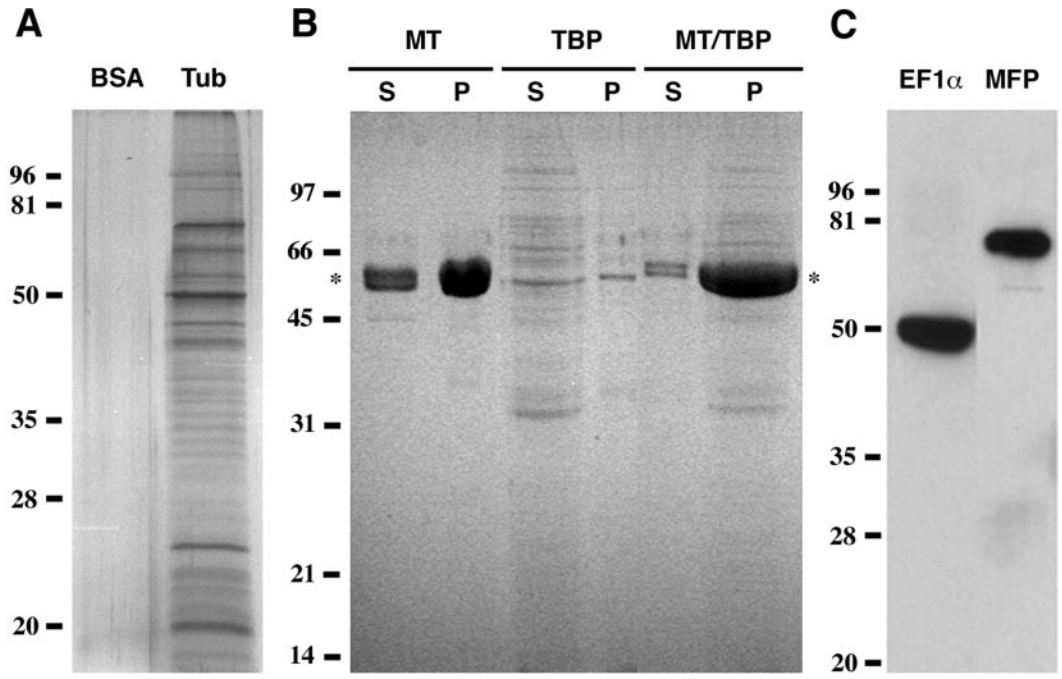

FIG. 1. **Affinity purification of *Arabidopsis* cell culture tubulin-binding proteins.** *A*, silver-stained SDS-PAGE gel of *Arabidopsis* cell culture proteins that bound to the tubulin affinity column (*Tub*). A BSA column served as a negative control for identifying any nonspecific protein binding (BSA). *B*, Coomassie blue-stained SDS-PAGE gel of the soluble (*S*) and pellet (*P*) fractions from MT co-sedimentation assays of the *Arabidopsis* tubulin-binding protein fraction. The α/β tubulin dimer is marked by an *asterisk*. *C*, an SDS-PAGE gel blot of the tubulin-binding proteins probed with a rice polyclonal EF-1α and MFP antisera. Molecular mass standards (in kDa) are indicated to the *left* of the panels.

then separated on a 75-μm inner diameter $\times$ 15-cm PepMap C18 3-μm, 100-A column (LCPackings/Dionex), followed by MS/MS on a PE Sciex QStar Pulsar I Q-TOF mass spectrometer (Sciex, Thornhill, Ontario, Canada). Proteins were identified using the MASCOT search program (version 1.9; Matrix Science, London, United Kingdom) with a confidence limit value set at <0.05, where *P* is the probability that the observed match is a random event. The comprehensive Mass Spectrometry Protein Sequence Database (MSDB) containing 1,165,316 protein entries was searched, and the oxidation of methionine was selected as a variable modification.

RESULTS

Purification of Tubulin-binding Proteins—Tubulin affinity chromatography was used to purify tubulin-binding proteins from an *Arabidopsis* cell suspension culture. Previously, we demonstrated that bovine brain tubulin affinity columns were as effective in purifying rice tubulin-binding proteins as were columns generated with rice tubulin as the ligand (5). In addition, tubulin and MT affinity chromatography of plant extracts resulted in the purification of proteins that had SDS-PAGE profiles that were qualitatively similar (5, 13). The ease of purifying large amounts of bovine brain tubulin and the enhanced stability of tubulin columns over MT columns prompted us to use bovine tubulin affinity columns in this study, as in our previous study (5).

The *Arabidopsis* protein fraction that bound to the bovine tubulin column was analyzed by SDS-PAGE (Fig. 1*A*). Numerous proteins were identified in this fraction, and the number and pattern of proteins observed was consistent with that of a similar fraction purified from rice and carrot cells (5, 13). In contrast to the tubulin-binding protein fraction, no proteins were evident in the eluate from a BSA control column (Fig. 1*A*). The BSA column served as an appropriate control column to identify any proteins that interacted solely by nonspecific ionic interactions, because the isoelectric points of BSA (pI = ~4.7) and tubulin (pI = ~4.6) are very similar. When the tubulin-binding protein fraction was used in MT co-sedimentation assays, most, if not all, of the proteins in this fraction co-sedimented with MTs (Fig. 1*B*). This was a similar observation to what has been reported previously (14) and is consistent with reports demonstrating that tubulin and MT affinity column eluates had similar SDS-PAGE protein profiles (5, 13). Additional evidence for the presence of MT-binding proteins in the tubulin-binding protein fraction came from the immunological detection of the known MT-binding proteins EF-1α (15) and the peroxisomal MFP (Ref. 5 and S. D. X. Chuong, R. T. Mullen, and D. G. Muench, unpublished observations), as shown in Fig. 1*C*.

Two-dimensional NEPHGE analysis of the tubulin-binding protein fraction followed by Coomassie blue staining identified ~100 prominent proteins spots (Fig. 2). Close examination of these gels indicated that there were many additional, less abundant proteins in this fraction. The two-dimensional gel profiles were consistent between independently purified

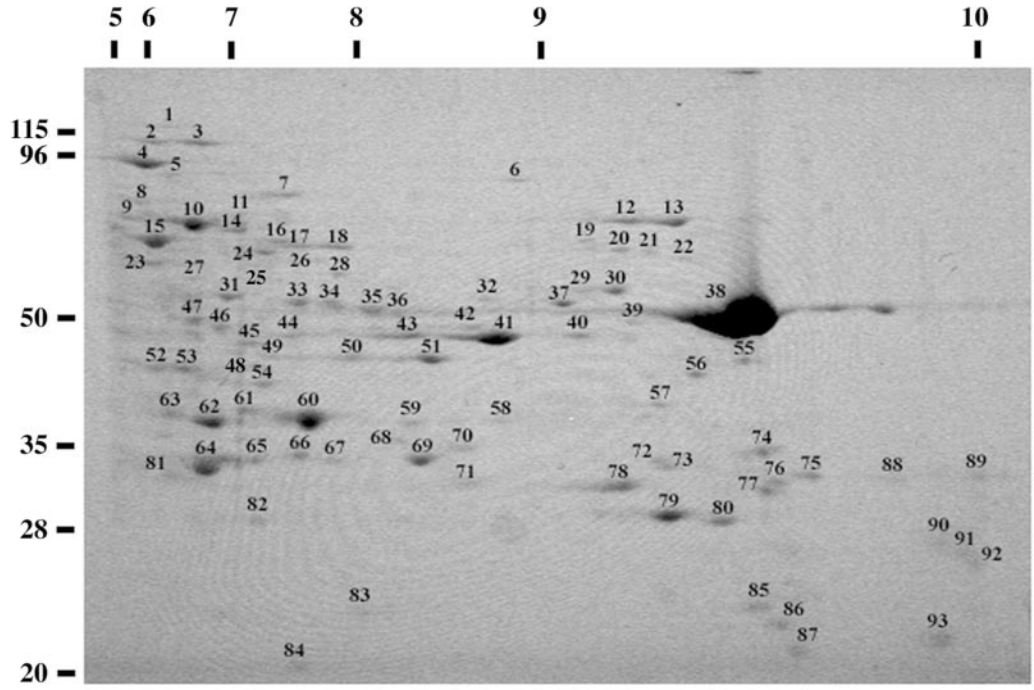

FIG. 2. **Two-dimensional NEPHGE of the tubulin-binding protein fraction.** Two hundred fifty micrograms of the tubulin-binding protein fraction was separated by two-dimensional NEPHGE and stained with Coomassie blue. Protein spots with relatively high staining intensity were candidates for MS analysis and were given a reference number as shown. The approximate pH value at specific positions of the first-dimension gel is indicated at the *top* of the gel. Molecular mass standards (in kDa) are indicated to the *left* of the gel.

tubulin column eluate fractions, showing only differences in spot intensity rather than in spot number or position (data not shown). These quantitative differences were likely the result of variation in the amount of protein loaded onto the columns. When a high concentration of protein was used, proteins with a higher affinity for tubulin could effectively outcompete other proteins for tubulin-binding sites. For instance, the relative abundance of EF-1α was variable between column purifications, as observed by comparing the relative intensities of the abundant protein in spot 38 in Fig. 2 with the less obvious corresponding band at the 50-kDa molecular mass marker position in the tubulin-binding protein fraction lane in Fig. 1*A*. We observed previously that the tubulin-binding protein fraction was composed mostly of basic proteins, as determined by NEPHGE (5). This was also the case here; therefore, our first dimension, nonequilibrium tube gels were run so as to observe the proteins within the pH 6–10 range of the gel to allow for better separation of proteins (Fig. 2). Due to the basic properties of these proteins, the NEPHGE system was optimal as it preserved the solubility characteristics of the protein fraction, whereas these proteins formed a precipitate in standard, fixed pH IEF gels strips. One disadvantage of using the NEPHGE system was that some smearing occurred at the acidic end of the first-dimension gels, often causing protein spots to overlap in that region of the gel (Fig. 2).

LC-MS/MS Protein Identification—Ninety-three protein spots containing the highest abundance of protein were identified on the two-dimensional gel (Fig. 2). Several of these proteins appeared to migrate as probable isoform pairs (for example, spots 2 & 3 and 12 & 13), and in these instances only a single member of each pair was selected for LC-MS/MS analysis. The large cluster of heavily stained proteins that migrated at the pH 9.3 position in the first-dimension gel and had a molecular mass of 50 kDa was assigned a single number (spot 38). A total of 86 of these spots were chosen for LC-MS/MS analysis. Sufficient protein was present in each spot to allow for the use of a single gel for protein spot excision and subsequent LC-MS/MS analysis. Protein spots were carefully excised from the gel, and tryptic peptides that were liberated from each spot were analyzed by LC-MS/MS. High-confidence peptide identity data ($p < 0.05$) was obtained from 74 of the 86 spots analyzed. An average of 5.0 peptides were identified for each protein, with a range of 1–22 peptides identified per protein. The high confidence limit settings that were used in the analysis of the peptide data coupled with the identification of multiple peptides for most of the proteins allowed for the unambiguous identification of individual proteins using this technique.

The MS analysis of single gel spots often identified peptides that corresponded to more than one protein, indicating that proteins within this fraction often migrated to the same location in the gel, or there was occasionally some overlap of spots due to protein streaking in the gel. In total, 122 proteins were identified, and these were grouped according to their predicted functions (Tables I–VI, Fig. 3). The experimentally determined molecular mass of each protein was generally

TABLE I
Proteins similar to known MT-associated proteins that were identified in the Arabidopsis cell culture tubulin-binding protein fraction

Spot no.	M_r theoretical (experimental)	Accession no.	Identity (no. of peptides matched)	References supporting a MT interaction
23	59.8 (62)	Q9SF16	Putative TCP-1 η subunit (4)	(28, 102)
40	159 (48)	Q8RWY6	Hypothetical protein with homology to CLASP1/ORBIT (3)	(24)
47	62.9 (50)	G96703	Similar to myosin heavy chain related protein/kinectin/ centriolin (4)	(103–105)
57	40.0 (39)	T48161	Heat shock protein 40 (2)	(28)
58	36.4 (38)	Q9SA50	Hypothetical protein with domain similar to myosin heavy chain/dynein/kinesin (4)	(105)
60	36.4 (37)	Q9SA50	Hypothetical protein with domain similar to myosin heavy chain/dynein/kinesin (4)	(105)
82	70.0 (29)	Q9LHA8	70-kDa heat shock protein (5)	(28)

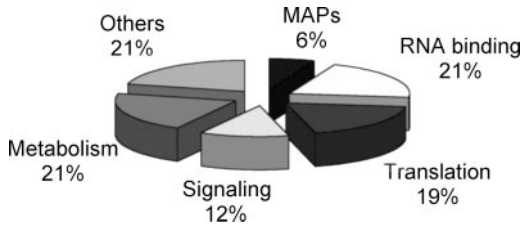

FIG. 3. **Distribution of tubulin-binding proteins according to their proposed function.**

consistent with the predicted value of the corresponding protein from the database (Tables I–VI). The discrepancy between the experimental and theoretical values observed for some proteins could be explained by proteolytic degradation of polypeptides during sample preparation, or variability arising from alternate splicing of mRNAs. Individual peptide sequences and their probability scores are listed in Supplementary Tables I–VI. Each of these peptides was confirmed as a tryptic digest product based on the presence of flanking arginine or lysine residues.

Microtubule-associated Proteins—Table I lists the tubulin-binding proteins that were identified as MAP-like proteins. One hypothetical protein (spot 40) possessed 42 and 44% amino acid similarity to the human cytoplasmic linker protein-associating protein 1 (CLASP1) and its *Drosophila* homolog ORBIT, respectively. This protein functions in spindle development as well as other roles in MT organization in animal cells (24, 25). HSP70, a well-characterized MAP (26–28), was also identified in this study (spot 82), as was its co-factor, HSP40 (spot 57). This chaperone complex is believed to regulate MT dynamics (28). In addition, the η subunit of the T-complex protein-1 (TCP-1) chaperone was identified (spot 23) and may assist the TCP-1 complex in the folding of α- and β-tubulin and in regulating MT nucleation (28, 29).

The remaining proteins listed in Table I contained domains that showed moderate to high amino acid similarity to motor proteins. Spot 47 contained a protein with 76% amino acid similarity to a myosin heavy chain-like protein from *Arabidopsis*, as well as 59% similarity to an 80-aa region from a human centriole-binding protein (centriolin), and 45% similarity in a 200-aa region with a chicken kinesin-binding protein (kinectin). A protein that was present in spots 58 and 60 contained an amino-terminal domain of ~100 aa that has 52–57% similarity to mouse myosin heavy chain, human kinesin, and fungal dynein.

RNA-binding Proteins—A large number of proteins identified in the tubulin-binding protein fraction are well-characterized RNA-binding proteins or are hypothetical proteins containing RNA-binding domains (Table II). Among these were a SNc/Tudor domain protein (spot 3), a polyadenylation-binding protein (PABP; spot 10), and several RNA helicases (spots 10, 15, 22, 23, 30, 32, 37). Homologs of these proteins have been shown to associate with MTs, either directly or indirectly. For example, PABP co-purified with MTs from sea urchin embryos and HeLa cells (30, 31), and a human RNA helicase (Vasa) interacts with a MT-binding protein that regulates MT nucleation (32). A protein similar to the SNc/Tudor domain-containing protein has also been identified in cytoskeleton fractions from rice and pea (33, 34). The SNc domain is involved in single-stranded nucleic acid binding, while the Tudor domain has been suggested to function in RNA trafficking/processing (34, 35). This protein is involved in seed storage protein mRNA localization to subdomains of the endoplasmic reticulum in rice endosperm cells (T. W. Okita, personal communication).

A number of the RNA-binding proteins identified here are predicted to have a nuclear localization. For instance, nucleolin (spots 8, 14) functions in the synthesis, assembly, and maturation of ribosome components and appears to shuttle between the nucleus and cytosol (36). Also identified here was a protein similar to the La protein (spot 20) that functions in the nucleus to stabilize small RNAs, such as tRNA, and also shuttles between the nucleus and the cytosol (37, 38). Spots 54, 64, 79, and 80 contained proteins identified as components of small nuclear ribonucleoproteins (snRNPs), a protein class that functions in splicing. Although there are no reports of snRNPs having MT binding activity, two of these proteins (spots 54, 64) have sequence conservation (50% similarity) with fungal β-transducins, a WD-40-containing protein that binds to MTs in sea urchin cells (39). A transcriptional co-

TABLE II
Proteins with RNA binding activity that were identified in the Arabidopsis cell culture tubulin-binding protein fraction

Spot no.	M_r theoretical (experimental)	Accession no.	Identity (no. of peptides matched)	References supporting a MT interaction
3	108 (107)	AAL57629	SNc/Tudor domain protein (19)	(33, 34)
8	58.8 (78)	A96527	Probable nucleolin protein (4)	(106)
10	67.6 (71)	Q8LA13	ATP-dependent RNA helicase (7)	(32)
10	72.7 (71)	C96534	Poly(A) binding protein (8)	(30, 31)
14	58.8 (68)	A96527	Probable nucleolin protein (4)	(106)
15	65.8 (66)	Q9LU46	DEAD-box RNA helicase (4)	(32)
20	55.8 (65)	Q94K80	Hypothetical RNA binding protein with homology to La protein (6)	
22	53.1 (63)	Q8W4R3	DEAD box RNA helicase (6)	(32)
23	66 (62)	Q8LA13	ATP-dependent RNA helicase (3)	(32)
30	65.4 (56)	AAM13255	DEAD box RNA helicase (16)	(32)
32	55.6 (54)	B96593	Ethylene response RNA helicase (5)	(32)
37	55.6 (52)	Q9C718	Ethylene-response RNA helicase (22)	(32)
41	38.9 (47)	AAL09735	Nuclear RNA-binding protein A-like protein (16)	
42	38.9 (49)	Q8LB56	Nuclear RNA-binding protein A-like protein (6)	
43	38.9 (46)	G714444	Nuclear RNA-binding protein (3)	
44	41.7 (48)	Q94AC3	Hypothetical protein with RRM domain (1)	
51	38 (44)	Q8LDQ7	Nuclear RNA-binding protein A-like protein (10)	
53	45 (43)	B96600	Protein with PPR domain involved in RNA stability (3)	
54	37.9 (42)	C84870	Probable small nuclear RNP (2)	
64	28.1 (32)	S30580	U2 small nuclear RNP A (3)	
72	33.5 (33)	Q8RWN5	RNA-binding protein-like (1)	
79	26.2 (29)	Q8LB63	Putative small nuclear RNP U2B (2)	
80	26.2 (28)	C84706	Small nuclear RNP U2B (3)	
90	25.9 (27)	Q9C7C3	Hypothetical protein with KH domain (4)	(107)
91	25.7 (26)	Q8L773	Transcription coactivator-like protein with RRM domain (2)	(108)
93	25.3 (21)	Q8LDY1	Hypothetical protein with RRM domain (3)	(107)

activator protein with an RNA-recognition motif was also identified (spot 91) and has high sequence similarity to the mammalian ALY protein that is involved in RNA export from the nucleus (40, 41).

Proteins Involved in Translation—It is now well established that the cytoskeleton has an important role in regulating translational efficiency (1, 42). Several proteins that are known to function in this process were identified in the tubulin-binding protein fraction (Table III). As demonstrated previously using rice and carrot cell extracts (5, 13) and here by protein gel blot analysis (Fig. 1*C*) and LC-MS/MS (Table III), EF-1α (spot 38) was an abundant protein in the tubulin-binding protein fraction. Several other elongation and initiation factors were also identified, some of which have been shown to bind to MTs and other cytoskeletal structures in previous studies (Table III) (12, 43–46). These included EF-2 (spot 4) and subunits of the eukaryotic translation initiation factors eIF3 and eIF4 (spots 7, 18, 24, 27, 50, 54, 70). Spot 30 contained a hypothetical protein with 56% amino acid similarity throughout its sequence to the PINHEAD/ZWILLE AND ARGONAUTE1 proteins that are involved in vegetative development and are members of a family of proteins that includes eIF2C (47). Additional evidence for the role of cytoskeletal elements in translation comes from reports that aminoacyl-tRNA synthetases and ribosomes can associate with the cytoskeleton (21, 31, 48–51). Several aminoacyl-tRNA synthetases were identified here (spots 5, 8, 9, 23, 85), as were various isoforms of large and small ribosomal subunits (spots 75, 86 to 91, 93). These ribosomal proteins may function to anchor ribosomes to MTs (51) or may have functions that extend beyond translation (52).

Proteins Involved in Signaling—Table IV lists a number of potential cell-signaling proteins that were identified in the tubulin-binding protein fraction. These included several protein kinases, including casein kinase I and II (CK; spots 68, 73), a cyclin-dependent protein kinase (CDK; spot 39), and several other serine/threonine kinases (spots 5, 15, 70, 84). In addition to the protein kinases, a hypothetical protein that contains a protein phosphatase 2A (PP2A) catalytic domain (spot 32) and a PP2A regulatory subunit (spot 33) were also identified. Other proteins in the tubulin-binding protein fraction with probable signaling roles included a leucine-rich repeat (LRR) containing protein (spot 22) involved in disease-resistance signaling (53). This protein shows 55% amino acid similarity to a *Drosophila* protein, FLIGHTLESS, which is thought to link the cytoskeleton to signal transduction pathways, and which binds to MTs and actin filaments (54). A hypothetical protein was identified (spot 44) that contains a SWI/SNF domain first identified in yeast (55). This domain is important in regulating transcriptional activity by remodeling chromatin, and it also localizes to the kinetechores of mitotic chromosomes where the SWI/SNF complex may interact with

TABLE III
Proteins involved in translation that were identified in the Arabidopsis cell culture tubulin-binding protein fraction

Spot no.	M_r theoretical (experimental)	Accession no.	Identity (no. of peptides matched)	References supporting a MT interaction
4	94 (94)	AAK96653	EF-2 (9)	(109)
5	89.8 (86)	T05247	Methionyl-tRNA synthetase (4)	(48)*
7	82.9 (80)	Q8H179	eIF4-like protein (17)	(44)
8	77.2 (78)	Q8LBY0	Glycyl tRNA synthetase (4)	(48)*
9	77.3 (72)	O04630	Threonyl tRNA synthetase (6)	
18	59.3 (65)	Q9M7E8	eIF4B (8)	(44)
23	60.8 (62)	T48004	Multifunctional aminoacyl-tRNA synthetase-like protein (4)	(48)*
24	57.7 (64)	Q9LIN5	eIF4B (8)	(44)
27	57.6 (58)	Q9SQK7	eIF4B (5)	(44)
30	129.1 (56)	AAF24586	Hypothetical protein similar to eIF2C/ARGONAUTE1/PINHEAD/ZWILLE (1)	(109)
38	49.8 (50)	AY048275	EF-1α (11)	(15)
39	49.8 (50)	Q8GTY0	EF-1α (3)	(15)
50	39.2 (45)	Q9C8J1	eIF4A (5)	(110)*
54	46.7 (42)	Q8LBZ6	eIF4A (6)	(110)*
70	32.6 (34)	Q9C5Y9	eIF3G (5)	(45)
75	27.5 (32)	Q9FJA6	Ribosomal protein S3 (4)	(21)*
85	20 (23)	AAM15519	Tyrosyl tRNA synthetase (4)	(48)
86	22.9 (22)	P51427	Ribosomal protein S5–2 (4)	
87	22 (21)	Q8LCC2	Ribosomal protein L9 (5)	
88	27 (32)	T45927	Ribosomal protein S3a (7)	(21)*
89	30.9 (33)	AY085726	Ribosomal protein S2 (3)	
90	24.5 (27)	Q8VZB9	Ribosomal protein L10a-1 (3)	
91	27.9 (26)	Q940T2	Ribosomal protein L7 (4)	
93	22 (21)	Q8LD03	Ribosomal protein L7 (2)	

* Proteins described in these references were identified from a detergent-treated fraction containing total cytoskeleton proteins.

TABLE IV
Proteins involved in signaling that were identified in the Arabidopsis cell culture tubulin-binding protein fraction

Spot no.	M_r theoretical (experimental)	Accession no.	Identity (no. of peptides matched)	References supporting a MT interaction
5	65.8 (86)	B84833	Hypothetical protein with serine-threonine kinase domain and HEAT repeat (2)	
15	79.0 (66)	T05148	Serine/threonine protein kinase homolog (1)	
22	64.0 (63)	Q9FHL8	Putative disease resistance protein family with LRR domain (5)	(54)
32	27.7 (54)	C96606	Hypothetical protein with PP2A domain (2)	(85, 86)
33	45.0 (53)	Q94A11	PP2A regulatory chain (1)	(85, 86)
39	72.4 (50)	Q8W2N0	Cyclin-dependent kinase CDC2C (1)	(80)
44	42.7 (48)	Q8LFA6	Hypothetical protein with SWI/SNF subunit domain (2)	
45	46.5 (47)	T46063	Hypothetical protein with SEC14 lipid-binding domain (4)	
64	35.7 (32)	AAF78369	G-protein β-subunit/contains WD-40 repeat (4)	(57)
65	35.8 (33)	Q8LAF2	G-protein β-subunit/contains WD-40 repeat (2)	(57)
68	47.1 (35)	Q8H120	Casein kinase II α chain 2 (5)	(84)
70	36.2 (34)	Q9FNL4	Serine/threonine protein kinase (1)	(87)
73	33.5 (33)	E85088	Casein kinase I-like protein (1)	(82)
75	64.8 (32)	Q8W4K6	Hypothetical protein with GTPase-activating domain(2)	(111)
84	87.0 (21)	Q9C833	Serine/threonine protein kinase (2)	(87)

MTs (56). Two β-subunits of G-proteins were identified in this fraction (spots 64, 65), and were previously shown to bind to MTs in mammalian cells (57). Proteins with probable roles in G-protein activation were also identified and included a hypothetical protein with a SEC14 lipid-binding domain known to associate with G-protein subunits (spot 45) and a hypothetical protein with a GTPase-activating domain (spot 75).

Proteins Involved in Metabolism—A large number of proteins in the tubulin-binding protein fraction were identified as metabolic enzymes (Table V). Five of these enzymes (spots 15, 23, 31, 35, 67/69) are involved in catalyzing reactions in folate-dependent pathways of one-carbon metabolism (58), and five others (spots 13, 50, 51, 64/81, 82) have roles in fatty acid metabolism in the peroxisome (59). Evidence for perox-

TABLE V
Proteins involved in metabolism that were identified in the Arabidopsis cell culture tubulin-binding protein fraction

Spot no.	M_r theoretical (experimental)	Accession no.	Identity (no. of peptides matched)	References supporting a MT interaction
8	73.4 (78)	T06034	Phosphoenolpyruvate carboxykinase (8)	
13	77.8 (72)	T08956	AIM protein (peroxisomal MFP) (8)	(5)
15	67.8 (66)	C96541	10-Formyltetrahydrofolate synthetase (7)	
23	64.9 (62)	Q8RWT5	Phosphoribosylaminoimidazole carboxamide formyltransferase (2)	
28	59.2 (59)	T52611	Glucose-6-phosphate 1- dehydrogenase (7)	
31	51.7 (55)	O23254	Serine hydroxymethyl- transferase (14)	
33	58.0 (53)	T06104	2-Dehydro-3-deoxyphospho- heptonate aldolase (2)	
35	50.7 (52)	T05362	Glycine hydroxymethyl- transferase (3)	
47	52.9 (50)	Q9FWA3	6-Phosphogluconate dehydrogenase (5)	
50	47.6 (45)	T46895	Acyl-CoA dehydrogenase (2)	
51	48.6 (44)	Q9S7M3	3-Ketoacyl-CoA thiolase (11)	
53	47.3 (43)	T04668	Phosphoserine transaminase (7)	
53	43.3 (43)	Q8H1Y0	Pyruvate dehydrogenase E1a-like subunit IAR4 (3)	
57	34.4 (39)	T10203	Protein from the alcohol dehydrogenase family (6)	
59	36.7 (38)	H84898	3-Methyl-2-oxobutanoate hydroxy-methyl-transferase (4)	
60	36.7 (37)	O82357	3-Methyl-2-oxobutanoate hydroxy-methyl-transferase (9)	
61	37.4 (39)	T47945	3-Methyl-2-oxobutanoate hydroxy-methyl-transferase (2)	
62	36.9 (38)	Q8LAS0	Glyceraldehyde-3-phosphate dehydrogenase C subunit (8)	(62)
64	31.7 (32)	Q9LDF5	3-Hydroxyacyl-CoA dehydrogenase-like protein (5)	
66	37.5 (34)	G84616	Glyoxosomal malate dehydrogenase (6)	
67	31.6 (33)	Q8LEA3	5,10-Methylenetetrahydrofolate dehydrogenase (4)	
69	31.6 (33)	Q8LEA3	5,10-Methylenetetrahydrofolate dehydrogenase (13)	
81	31.7 (32)	Q9LDF5	3-Hydroxyacyl-CoA dehydrogenase-like protein (3)	
81	36.5 (32)	T46192	Acetylglutamate kinase-like protein (2)	
82	29.9 (29)	Q9FHR8	Enoyl CoA hydratase (3)	

isomal enzyme interactions with MTs has been reported previously (5, 16, 60, 61). The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also identified in this fraction (spot 62) and has been previously shown to have MT-binding activity (22, 62, 63). Additional proteins were identified that function in other metabolic pathways, including the pentose phosphate pathway (spots 31, 47), phosphate metabolism (spot 33), amino acid biosynthesis (spots 53, 81), the tricarboxylic acid cycle (spot 53), anaerobic glycolysis (spot 57), and pantothenate biosynthesis (spots 59–61).

Other Proteins with Known or Unknown Functions—The tubulin-binding proteins listed in Table VI likely participate in cellular activities other than those described above. These proteins have a wide range of functions, and at least one of these proteins has previously been reported to bind to MTs. Spot 81 contained a regulatory subunit of the 26S proteosome, a complex that was shown previously to bind to multiple MT arrays in plants (64). Other interesting proteins were identified that have not been shown previously to bind to MTs; however, their predicted functions implicate a possible MT association. For instance, the sorting nexin-like protein (spots 45, 46) is involved in vesicle trafficking in both plants and animals and has a role in axonal guidance, a form of cell movement requiring cytoskeletal rearrangements (65). Actin-11 (spot 4), a unique and ancient actin subclass in the *Arabidopsis* actin family that has a divergent sequence from other actins, was also identified in this study. This protein likely participates in specific developmental roles (66) and may function to link actin filaments and MTs together. Several proteins were identified that possessed DNA-binding domains (spots 8, 46/47, 56, 84), including the transcription factor Scarecrow. Transcription factor activity is known to be regulated in some instances by its shuttling into and out of the nucleus in a MT-dependent manner, as was reported for the mammalian tumor suppressor protein p53 (67). A group of proteins that were unexpectedly represented in the tubulin-binding protein fraction were those involved cell wall modification and included β-galactosidase (spot 6), xylosidase (spot 19), pectate lyase (spot 58), xyloglucan endo-1,4-beta-D-glucanase (spot 71), and endoxyloglucan glycosyl-transferase (spot 78).

DISCUSSION

Here we report the identification of 122 tubulin-binding proteins that were purified from *Arabidopsis* suspension cells using tubulin affinity chromatography, a procedure that has been used previously to purify *bona fide* MT-binding proteins (5, 13–15, 17). The following observations indicated that tubulin affinity chromatography also served as an effective method for the purification of MT-binding proteins in the present study. First, antibodies raised against two known MT-binding proteins, EF-1α (15) and the peroxisomal MFP (5),

TABLE VI
Proteins with other known or unknown functions that were identified in the Arabidopsis cell culture tubulin-binding protein fraction

Spot no.	M_r theoretical (experimental)	Accession no.	Identity (no. of peptides matched)	References supporting a MT interaction
4	41.9 (92)	P54396	Actin 11 (3)	
6	93.6 (86)	CAB64737	β-Galactosidase (11)	
8	79.0 (78)	Q9LDX1	Hypothetical protein with XS zinc finger nucleic acid binding domain (4)	
19	83.5 (68)	Q9FGY1	Xylosidase (4)	
20	63.1 (65)	Q9FK40	Histone acetyltransferase (5)	
23	56.9 (62)	G86349	Hypothetical protein with homology to auxilin, trichohalin, and has a DnaJ domain (3)	
45	46.5 (47)	Q9FG38	Sorting nexin-like protein (7)	
46	46.5 (48)	Q9FG38	Sorting nexin-like protein (9)	
46	44.0 (48)	T51151	Nuclear DNA-binding protein G2p (7)	
47	44.0 (50)	T51151	Nuclear DNA-binding protein G2p (7)	
50	42.5 (45)	T05870	Cell division protein pelota (3)	
56	169.4 (42)	Q9LNX6	Scarecrow-like transcription factor (2)	
56	49.0 (42)	Q9FF81	Vacuolar protein sorting 36 (2)	
58	46.0 (38)	Q9LTZ0	Putative pectate lyase 11 precursor (4)	
59	35.7 (38)	Q8W457	Hypothetical protein with cysteine	
65	36.5 (33)	T52133	Potassium channel protein, b subunit (5)	
67	34.3 (33)	B96602	Hypothetical protein with homology to myosin heavy chain (3)	
70	51.8 (34)	Q93Y09	Serine carboxypeptidase II (4)	
71	32.8 (31)	C49539	Xyloglucan endo-1,4-β-D-glucanase (4)	
76	33.6 (31)	Q8LB34	Phosphate-induced protein 1 (2)	
77	172 (30)	T06677	Protein with Bromo adjacent homology domain (1)	
78	32.8 (30)	Q39099	Endoxyloglucan glycosyl- transferase (12)	
81	33.5 (32)	Q8LD11	26S proteasome non-ATPase regulatory subunit (6)	(64)
84	22.3 (21)	BT004166	Protein with single-stranded DNA-binding domain (2)	
84	20.6 (21)	Q8RXZ8	Hypothetical protein (2)	

cross-reacted with proteins of the expected size on protein gel blots containing the tubulin-binding proteins (Fig. 1*C*). The presence of these two proteins was confirmed by LC-MS/MS analysis (results from spots 38 and 13 in Tables III and V, respectively). Second, it appears that most, if not all, of the proteins that bound to the tubulin affinity column, pelleted with MTs in co-sedimentation assays (Fig. 1*B*). A similar observation was made previously (14) and is consistent with observations that SDS-PAGE profiles of proteins that bound to tubulin and MT affinity columns were qualitatively similar (5, 13). Third, 43% of the proteins identified in this study (52/122) are related to proteins previously reported to have an association with MTs (see references in Tables I–VI). These observations gave us confidence that the proteins identified here are authentic MT-binding proteins.

The tubulin-binding proteins that were identified in this study were grouped according to their predicted primary function as either MAPs, RNA-binding proteins, translation factors, metabolic enzymes, signaling proteins, or proteins with other functions (Tables I–VI, Fig. 3). Although we expected to identify a broad range of protein types based on the identities of previously characterized plant MT-binding proteins (1, 2, 10), certain groups of these proteins would not be expected to bind to MTs because of their known subcellular location. In these cases, the association of these proteins with MTs may play a role in their trafficking within the cell. Alternatively, these proteins may possess multiple functions and carry out the additional function(s) in the cytosol in association with MTs. A good example of a group of tubulin-binding proteins identified here that is known to be localized to a particular membrane-bound organelle were the peroxisomal matrix enzymes. Five of these enzymes catalyze reactions in fatty acid β-oxidation (Table V, spots 13, 50, 51, 64/81, 82), and one functions in the glyoxylate cycle (malate dehydrogenase; spot 66). Each of these enzymes possesses a putative amino- or carboxyl-terminal signal sufficient to target the protein to the peroxisome matrix (68). Peroxisomal matrix proteins are imported into the peroxisome in their fully folded conformation and, therefore, have the potential to bind to MTs prior to their import. We have performed extensive studies on the MT binding activity of the rice peroxisomal MFP (5) and have demonstrated that it does indeed bind to MTs *in vivo* (S. D. X. Chuong, R. T. Mullen, and D. G. Muench, unpublished observations). We proposed previously that the binding of MFP to MTs may be important in facilitating its import into the peroxisome (69). The fact that at least six peroxisomal matrix enzymes were present in the tubulin-binding protein fraction in this study (Table V), and one reported in another study (16), supports a general role for MTs in peroxisomal matrix protein trafficking in plant cells.

The identification of numerous cytosolically localized metabolic enzymes in the tubulin-binding protein fraction (Table V) is consistent with reports suggesting that sequential enzymes from individual metabolic pathways can cluster on the cytoskeleton to assist in the efficient and regulated control of flux through those pathways in a process called metabolic channeling (70). For instance, five enzymes that catalyze coupled reactions in the folate-dependent pathways of one-carbon metabolism (58) were identified as components of the tubulin-binding protein fraction (Table V, spots 15, 23, 31, 35, 67/69). This suggests that these cytosolic enzymes bind to MTs individually or as a complex to facilitate channeling of intermediates through these pathways. An interaction between homologs of some of the one-carbon metabolism enzymes that were identified here has been implicated in facilitating metabolic channeling in mammalian cells (58, 71, 72). Our data suggests that any metabolic channeling that results from such an interaction would also require the binding of this complex to the MT matrix in order to stabilize these interactions. The identification of other classes of metabolic enzymes in this study (Table V) indicates that MTs may have an important role in regulating the activity of many metabolic processes.

Interestingly, several enzymes that were identified in the tubulin-binding protein fraction have cell wall modification activities (spots 6, 19, 58, 71, 78), including endo-1,4-β-glucanase and endoxyloglucan glycosyltransferase (Table VI). These enzymes are known to have a cooperative role in the modification of cell wall architecture that is required for cell elongation and fruit ripening (73). The complex organization of the cell wall requires a highly coordinated activation of wall-loosening proteins to achieve precise control of wall disassembly. Perhaps an interaction occurs between some of these proteins to form a multicomponent system of enzymes that functions to channel substrates in the modification and degradation of xyloglucans. This would be analogous to the plant cell wall-degrading "cellulosome" of certain bacteria (74). An endo-1,4-β-glucanase that contains a transmembrane domain is localized to the plasma membrane and Golgi in expanding cells of tomato (75) and could potentially interact with MTs through its cytosolic domain. An interaction between endo-1,4-β-glucanase and MTs across the plasma membrane, perhaps as a component of an enzyme complex in the cell wall, could function to precisely regulate the activity of these wall-loosening enzymes. Several examples of linker proteins that are components of the cytoskeleton-plasma membrane-cell wall continuum in plants have been identified and appear to mediate communication across the plasma membrane (76).

In plant cells, the cortical MT network lines the plasma membrane and responds to a diverse array of extracellular stimuli (77–79). Several putative signaling proteins that were identified in the tubulin-binding protein fraction (Table IV) have been shown previously to interact with MTs directly or indirectly through their association with MAPs and play an important role in regulating the cell cycle, cytoskeletal dynamics, and stress responses. For instance, a cyclin-dependent kinase binds to MTs in the spindle and phragmoplast in plant cells (80), casein kinases regulate MT organization by interacting with MAPs (81–84), and PP2A assists in regulating MT organization (85). MAPs are a target of kinases and phosphatases, and their modification by phosphorylation/dephosphorylation can result in changes in MT organization and dynamics, as well as the activation of signaling cascades (86–90). In plants, rearrangement of cortical MTs is known to occur in response to various signals, including cold and aluminum stress, wounding, and pathogen attack (91–93). The putative signaling proteins identified in the tubulin binding protein fraction may be involved in the transduction of signals generated from abiotic and biotic stresses such as these, or in response to light or developmentally regulated signals (95). As very little is known about cytoskeleton-associated proteins and their role in plant-specific signaling pathways, these will serve as useful target proteins in future signaling studies.

Many proteins identified in this study have predicted roles in RNA binding and translation (Tables II and III, Fig. 3). Proteins that function in translation have been reported to bind to the cytoskeleton (21, 42), and several of these proteins were identified here, including translation initiation and elongation factors, aminoacyl-tRNA synthetases, and ribosomal proteins. This concentration of the translational machinery on the cytoskeleton may function to increase the efficiency of translation, or serve as a mechanism to regulate translation rate (42). Some of the RNA-binding proteins identified in this fraction may function to anchor RNAs to MTs in a specific or general manner in order to facilitate translation, as has been described previously (95, 96). Alternatively, these proteins may be involved in trafficking mRNAs from the nucleus to regions of the cell where they are to be translated. RNA localization is well characterized in many species and functions to contribute to the localized synthesis of protein at distinct subcellular regions (97). The initial step in RNA trafficking involves nuclear export with the assistance of nuclear/cytosolic shuttling proteins. These shuttling proteins can remain as a component of RNP particles that move specific RNAs throughout the cell along MTs (98). The transcriptional co-activator protein identified here (spot 91) is homologous to the animal nuclear RNA export factor ALY, which has a general role in RNA export from the nucleus and in the initial stage of localization of specific RNAs (41, 99). The identification of several types of RNA-binding proteins and translation factors in the tubulin-binding protein fraction is consistent with reports describing the existence of highly organized and efficient RNA localization and translation machineries that operate in association with the cytoskeleton (42).

A group of proteins that represented a relatively small percentage of the total proteins in the tubulin-binding protein fraction were the MAPs (Table I). Proteins such as EF-1α and

GAPDH also have MAP activities (13, 100) and could have been included in this group; however, these were grouped according to their originally documented functions (Tables III and V). The absence in this fraction of well-characterized plant MAPs, such as the 65-kDa MAPs, MAP190, or MOR1 (3), may have been due to the fact that the tubulin column does not bind these proteins with high affinity. The 65-kDa MAPs and MAP 190 were originally purified by pelleting endogenous taxol-stabilized plant MTs after rounds of polymerization and depolymerization (11, 102). Perhaps these proteins were in low abundance in our tubulin-binding protein fraction and were not visible on the Coomassie blue-stained gel, and therefore were not selected for MS analysis. The presence in this fraction of MAPs that are common in eukaryotes, such as molecular chaperones, CLASP, and putative motor proteins, indicates that at least some MAPs are enriched using this procedure.

The identity of the proteins reported in this study supports previous evidence suggesting that MTs serve as a matrix where proteins can bind and carry out numerous functions within the cell, and provides insight on the mechanisms responsible for macromolecule trafficking, metabolic channeling, and signaling in plant cells. Additional, more direct methods will be required to confirm that a *bona fide* interaction occurs between these proteins and MTs, as it is possible for some proteins that their interaction with tubulin *in vitro* occurs as a result of nonspecific binding during the chromatography procedure. Also, some of the proteins identified here may associate indirectly with MTs by interacting with other MT-binding proteins as a component of a protein complex.

There were many additional, less abundant proteins in the tubulin-binding protein fraction that were not identified in this study. Future cytoskeleton studies from plants and other eukaryotic species using similar and different methods of purification will be important in identifying additional MT-binding proteins, as well as proteins that bind to the actin component of the cytoskeleton. Technological advances that eliminate the need for gel electrophoresis and allow for the identification of low-abundance proteins will also accelerate the characterization of the cytoskeleton-binding proteome.

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