Mechanisms of Microtubule Dynamics Regulation by the MICROTUBULE ORGANIZATION 1 Protein

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Abstract

The *Arabidopsis thaliana* MOR1 (MICROTUBULE ORGANIZATION1) protein belongs to the MAP215/Dis1 family of microtubule-associated proteins. The temperature-sensitive *mor1* mutants have N-terminal amino acid substitutions, which lead to cortical microtubule disorganization (Whittington et al., 2001). Here I demonstrate by use of live cell imaging and immunolabelling that MOR1 is important for function and organization of all microtubule arrays during cell division and keeps microtubules highly dynamic. Although disruption of mitotic and cytokinetic microtubule arrays is not detected in all dividing *mor1-1* cells, quantitative analysis identified distinct defects in preprophase bands, spindles and phragmoplasts. In nearly half of dividing *mor1-1* cells, preprophase bands are not detected, and those that do form are often disrupted. *mor1-1* spindles and phragmoplasts are short and abnormally organized and persist for longer times than in wild-type, leading to aberrant chromosome arrangements, misaligned cell plates and multinucleate cells. Immunofluorescence indicates that the mutant mor1-1L174F protein remains associated along the full length of all microtubule arrays, in spite of their disorganization. This suggests the N-terminal region altered by the *mor1-1* mutation does not regulate the binding of MOR1 to microtubules, but that it instead plays a role in microtubule dynamics. Microtubule dynamics were therefore measured in living leaf cells expressing three microtubule reporter proteins, GFP-TUA, *CMV35S::GFP-EB1* and *ProEBI::EB1-GFP*. Dynamics analysis indicates that MOR1 promotes constant and rapid growth and shrinkage and prevents pausing of microtubules. Integrating this new information with previous observations showing that MOR1 and its tobacco homologue MAP200 can bind tubulin oligomers (Twell et al., 2002; Hamada et al., 2004), and that XMAP215 speeds up microtubule growth and shrinkage in 40-60nm increments (Kerssemakers et al., 2006), I postulate that MOR1 might promote microtubule
growth and shrinkage by adding and removing tubulin oligomers. Consistent with this idea, the
N-terminal region of MOR1 consists of 5 TOG domains, which each span the approximate
length of one tubulin dimer within a protofilament chain. I define experiments and present
preliminary data to test the hypothesis that each MOR1 protein can add or remove up to 5
tubulin dimers at a time.
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Chapter 3

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Chapter 1: General Introduction
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1.1 MICROTUBULE STRUCTURE AND NATURE

Microtubules are essential for all eukaryotic cells to divide, change shape and are often involved in transporting organelles. Microtubules are dynamic and form various arrays to accomplish different tasks.

1.1.1 Microtubule structure

Microtubules are long hollow cylinders about 25nm in diameter (Figure 1.1a (Hashimoto, 2003)) (Alberts et al., 1994). Microtubules are typically formed by 13 linear protofilaments aligned in parallel, each of which is composed of α-tubulin and β-tubulin heterodimers. α-tubulin and β-tubulin are globular polypeptides that tightly link to each other to form the heterodimer subunits of microtubules. One protofilament has a repetitive order of α-tubulin and β-tubulin. The end of the protofilament where β-tubulin is exposed is more reactive to an incoming tubulin heterodimer than the other end where α-tubulin is exposed. This provides a protofilament/microtubule with an inherent polarity. The end where αβ-tubulin dimers are exchanged more rapidly is called the ‘plus-end’, and the other end at which α-tubulin is exposed and where microtubule subunits are exchanged more slowly, is called the ‘minus-end’ (Hashimoto, 2003; Howard and Hyman, 2003). The term minus refers the tendency for subunits to be lost from the minus end, while they are being added to the plus end during treadmilling, a process that can occur when tubulin concentrations are below the minus end’s critical concentration for assembly.
Figure 1.1 Microtubules are composed of α- and β-tubulin heterodimers and are highly dynamic.
(reproduced from Hashimoto (2003) with permission from Elsevier)

(a) α- and β-tubulin dimers are polymerized at the microtubule plus-end, and GTP in β-tubulin (orange) is hydrolyzed to GDP (dark green). Growing microtubules are believed to have a sheet-like structure with protofilament extensions. When the GTP-cap is lost, protofilaments curve and tend to splay outward, and the microtubule shrinks (catastrophe). The accidental gain of a GTP-cap and transition from shrinkage to growth are called rescue. GDP in β-tubulin of dissociated free dimers is exchanged with abundant GTP in the cytoplasm.

(b) If net depolymerization occurs at the lagging, or minus-end, and net polymerisation at the leading, or plus-end, then individual tubulin heterodimers flow from the plus end to the minus end of the polymer, in a process known as ‘treadmilling’.
1.1.2 Microtubules are highly dynamic.

Microtubules can change length by gaining or losing αβ-tubulin heterodimers at their exposed ends (Figure 1.1a). Polymerization of free tubulin dimers into microtubules relies on the presence of a GTP attached to the β-tubulin subunit. After a free tubulin dimer binds to the exposed β-tubulin subunit at the microtubule plus-end, the GTP bound to the β-tubulin can be hydrolyzed to GDP. Tubulin heterodimers can also bind to the minus-end of MTs but the polymerization rate is slow compared to the plus-end. When GTP hydrolysis in β-tubulins is slower than the addition of tubulin dimers, microtubules obtain a GTP-capped end, which makes microtubules stable and promotes their polymerization, called ‘growth’. When GDP-bound-β-tubulins are exposed at the end of microtubules, conformational changes in each β-tubulin subunit cause each protofilament to become curved, which destroys the structure of the microtubule and causes rapid depolymerization. The event of change from growth to the loss of the GTP-cap, from polymerization to rapid depolymerization, is called ‘catastrophe’, whereas the regain of a GTP-cap is called ‘rescue’ (Figure 1.1a). Depolymerization of tubulin dimers at the microtubule end cause microtubule ‘shortening’. The stochastic transition between ‘growth’ and ‘shortening’ is called dynamic instability. The state at which there is no net microtubule growth or shortening is termed ‘pause’. When polymerization at the plus-end and depolymerization at the minus-end take place at a same speed, the length of microtubule does not change but the position changes. This process is known as ‘treadmilling’ (Figure 1.1b).

1.2. MICROTUBULE ASSOCIATED AND REGULATORY PROTEINS

Microtubules have a variety of accessory proteins, called MAPs (Microtubule-Associated Proteins). Different MAPs provide a microtubule with distinct functions. In plants, there are structural MAPs such as the end-binding proteins (EB1), a class of cross-linking proteins called MAP65, and XMAP215/MOR1, regulatory MAPs, which include katanin, and motor MAPs,
which in plants belong to the kinesin family (Figure 1.2). There are some other MAPs whose identities are not so clear. These include such as SPIRAL1/SKU6 (Furutani et al., 2000; Nakajima et al., 2004; Sedbrook et al., 2004) and, SPIRAL2/TORTIFOLIA1 (Furutani et al., 2000; Shoji et al., 2004; Buschmann et al., 2004), which are involved in directional cell growth and probably plant-specific. A recently discovered MAP, AIR9, has been found to be active during cytokinesis. It is found in land plants and trypanosomatid parasites (Buschmann et al., 2006).
Plus-end-binding MAPs might stabilize microtubules by binding at the plus-end. Cross-bridge-forming MAPs connect microtubules in parallel alignment by forming dimers. The XMAP215 family protein is linear and stabilizes microtubules, possibly from depolymerization factor of Kin1 kinesin. Katanin, composed of 2 subunits, severs microtubules. (reproduced with permission from Hashimoto, 2003)
1.2.1 Microtubule-Associated Proteins in Plants

XMAP215/MOR1

The XMAP215 family proteins, which are believed to form linear structures, have a general function in microtubule assembly and are well conserved throughout eukaryotes (Figure 1.2). All XMAP215 orthologues except those found in plants have been demonstrated to regulate spindle formation (Gard et al., 2004). These proteins are localized at centrosomes and spindle pole bodies, and are distributed along the lengths of microtubules, or concentrated at microtubule plus-ends (Gard et al., 2004). Plant homologues include MOR1/GEM1 (MICROTUBULE ORGANIZATION 1/GEMINI POLLEN 1) in *Arabidopsis thaliana* – hereafter referred to as arabidopsis (Whittington et al., 2001; Twell et al., 2002) and MAP200 (Hamada et al., 2004) in tobacco. Like most homologues identified so far, MOR1 occurs as a single copy gene. The arabidopsis temperature-sensitive mutant *mor1-1* undergoes rapid cortical microtubule disorganization when shifted to restrictive temperature (Whittington et al., 2001). The *gem1* mutants of arabidopsis show aberrant cytokinesis at pollen mitosis I, and can only be maintained as heterozygotes (Park et al., 1998; Park and Twell, 2001; Twell et al., 2002). The *root initiation defective5* (rid5) mutant of arabidopsis is another temperature-sensitive mutant allelic to *mor1*. *rid5* shows a similar phenotype as the *mor1* mutants (Konishi and Sugiyama, 2003). MAP200 has been isolated from tobacco BY-2 culture cells and been shown to bind to tubulin oligomers *in vitro* (Hamada et al., 2004).

End-binding proteins

EB1 (end binding protein 1) and CLIP-170 (cytoplasmic linker) preferentially localize to the plus ends of microtubules (Galjart and Perez, 2003) (Figure 1.2). The arabidopsis genome encodes three EB1 homologues (Mathur et al., 2003; Bisgrove et al., 2004). In arabidopsis
transgenic culture cells, EB1 tagged with GFP and expressed at high levels was found at microtubule plus ends with strong fluorescence but also at minus ends with weak fluorescence (Chan et al., 2003). EB1-GFP with its native promoter localizes to only growing microtubules (Dixit et al., 2006), indicating that the expression level of a protein can alter localization patterns. In animal cells, CLIP-170 has been demonstrated to localize to microtubule plus-ends. Based on sequence analysis there are no obvious CLIP-170 homologues in plants. YFP-CLIP-170, however, incorporates at microtubule ends when expressed in tobacco culture cells, indicating that microtubule plus ends in plant cells retain capacity for CLIP-170 binding (Dhonukshe and Gadella, 2003). In addition to modifying the microtubule plus-end so that growth can continue, end-binding proteins can interact with cell membranes by recruiting other cytoplasmic proteins. In arabidopsis leaf cells, EB1 has also been localized to endomembranes, suggesting EB1's role in endomembrane organization (Mathur et al., 2003). In animal cells, CLIP-170 associates with and pulls along membrane tubules (Pierre et al., 1992; Waterman-Storer et al., 1995). EB1 has been suggested to interact with the APC (adenomatous polyposis coli) tumor suppressor protein (Barth et al., 2002) and CLIP-170 interacts with CLASP1 (CLIP-ASSOCIATED PROTEIN 1)/MAST (MULTIPLE ASTERS) (Maiato et al., 2003). The tubulin-binding domain of CLASP1 has significant homology to APC region of TAN1 (TANGLED1) in arabidopsis (Smith et al., 2001). TAN1 is important for microtubule organization during cell division in maize (Cleary and Smith, 1998; Smith et al., 2001).

MAP65

The MAP65 family proteins are plant-specific and bundle microtubules. MAP65 proteins form dimers and are shown to make 25-30nm cross-bridges between microtubules in vitro (Chan et al., 1999; Smertenko et al., 2004) (Figure 1.2). In arabidopsis, there are nine MAP65 genes, AtMAP65-1 to AtMAP65-9. In vitro experiments show that AtMAP65-1 bundles microtubules
but does not promote polymerization or stabilize microtubules against cold-induced microtubule depolymerization (Smertenko et al., 2004). MAP65s are associated with microtubules throughout the cell cycle, especially where microtubules overlap. One of the MAP65 proteins, MAP65-3 is known to regulate phragmoplast integrity during cytokinesis (Muller et al., 2004).

**Katanin**

Katanin functions as a microtubule-severing factor (Figure 1.2). Katanin forms heterodimers of a catalytic subunit and a regulatory subunit: the 60 kDa catalytic subunit has ATP-dependent microtubule severing activity, while the 80 kDa subunit targets the complex to the reaction sites on the microtubule surface and thereby regulates the activity of a 60 kDa subunit (Quarmby, 2000). The arabidopsis genome encodes one 60kDa subunit and four 80kDa subunit-like proteins (Bouquin et al., 2003). There are some mutant alleles of the 60kDa katanin subunit characterized in arabidopsis, *boterol* (Bichet et al., 2001), *fragile fiber 2* (Burk et al., 2001; Burk and Ye, 2002), *ectopic root hair 3* (Webb et al., 2002) and *LUC-super-expressor 1* (Bouquin et al., 2003). *boterol*, *fragile fiber 2* and *LUC-super-expressor 1* show aberrant cortical microtubule organization.

**Kinesin**

Kinesins have microtubule-binding domains and move along microtubules using the energy of ATP hydrolysis. Kinesins transports various vesicles and organelles along microtubules. The arabidopsis genome encodes at least 61 kinesins (Reddy and Day, 2001). In addition to their transport functions, some kinesins are important for microtubule assembly (Lee and Liu, 2004). For example, POK1 and POK2, members of Kin12 family, are involved in orienting cytokinetic arrays in arabidopsis (Muller et al., 2006). A recent study suggests that arabidopsis ATK5 of the Kin14 family coaligns mitotic spindle microtubules (Ambrose and Cyr, 2007). KinI or MCAK is known to depolymerise microtubules by binding to the plus ends of microtubules in animal cells.
(Figure 1.2) (Walczak, 2003). There is no clear evidence, however, that arabidopsis KinI homologues can function in a similar manner (Lee and Liu, 2004; Lu et al., 2004).

1.2.2 MOR1 is well conserved throughout eukaryotes

MOR1 orthologues are probably found in all eukaryotic cells. They include XMAP215 of Xenopus, Msps of Drosophila, ch-TOG of human, Zyg9 of C. elegans, MAP200 of N. tabacum, STU2 of Saccharomyces cerevisiae and Dis1 and Alp14 of Schizosaccharomyces pombe and DdCP224 of Dictyostelium (Gard et al., 2004). An extensive search of the public database revealed about 40 new members of the MAP215 family (Gard et al., 2004). The most characteristic feature of the XMAP215 family is the conserved TOG domains (from Tumor Overexpressed Gene of human) of ~200 amino acid residues, which range in number from 2 to 4 or possibly 5 and make up the protein’s N-terminal region. Each of these domains, in turn, contains up to 5 HEAT repeats (Ohkura et al., 2001). HEAT repeats consist of 35-50 amino acid domains of tandemly arranged α-helices constructed with hydrophobic or charged residues (Andrade and Bork, 1995; Andrade et al., 2001) and are believed to mediate protein-protein interactions (Neuwald and Hirano, 2000).

1.2.3 MOR1’s functional properties

Stabilizing microtubules is one of the functions of the XMAP215 family proteins. How does the MOR1 protein stabilize microtubules? In Xenopus, the C-terminal region binds to microtubules in vitro and the well-conserved N-terminal region is suggested to suppress catastrophe by antagonising XKCM1, a member of the microtubule destabilising kinesin-13/KinI family (Popov et al., 2001), yet there is no clear evidence that any XMAP215 orthologue directly interacts with members of the KinI family. In arabidopsis, the C-terminal region, including the putative fifth TOG domain, has been reported to bind to microtubules in vitro (Twell et al., 2002). The activity
of XMAP215 may be regulated by phosphorylation of Cdc2 kinase in a cell cycle-dependent manner (Gard and Kirschner, 1987; Vasquez et al., 1999). There is a fairly conserved putative phosphorylation site, the KIGX motif (Gard et al., 2004), and XMAP215 (Gard and Kirschner, 1987; Vasquez et al., 1999) and Disl (Nabeshima et al., 1995) were shown to be phosphorylated. Serine at the X residue or KIGX is the target for MAP kinases. In MOR1, serine is substituted with aspartate, which is expected to mimic phosphorylation (Gard et al., 2004). This suggests that in plants, and some other organisms, this putative mechanism for regulation is not conserved.

1.2.4 The morl mutant alleles in arabidopsis

MOR1 was initially discovered through the isolation of two mutants that both undergo temperature-dependent cortical microtubule disorganization, which leads to the left-handed twisting, and eventual radial swelling of organs. Both mutations substitute single amino acids (morl-1 - L174F, and morl-2 - E195K) in an N-terminal HEAT repeat, one of many such motifs found extensively along the length of MOR1 and other MAP215/Disl family proteins (Whittington et al., 2001). Another morl allele, rid5, has a similar morphological phenotype to the morl mutants. The rid5 mutation substitutes a single amino acid near the N-terminus and was identified in a screen for a temperature-sensitive impairment of auxin-dependent adventitious root formation (Konishi and Sugiyama, 2003). MOR1 occurs as a single copy gene in arabidopsis (Whittington et al., 2001) and severe alleles are considered to be homozygous-lethal (Twell et al., 2002). Understanding the function of MOR1 therefore relies on the identification of weak alleles or ones that generate severe defects only under specific conditions. The temperature-sensitive morl-1, morl-2 and rid5 alleles are clearly valuable tools for understanding the function of wild-type protein.
1.3. MICROTUBULE ORGANIZATION THROUGH THE CELL CYCLE IN PLANTS

The organization of microtubules dramatically changes during cell division. Microtubules form three typical structures during cell division including preprophase bands, spindles and phragmoplasts, and during interphase form cortical microtubule arrays (Figure 1.3).
Figure 1.3 Microtubule organization during cell cycle in plants.
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(A) The prophase band is a girdle of microtubules formed around nucleus, is linked to the nuclear envelope and marks the future site of cytokinesis.

(B) The metaphase spindle aligns chromosomes at the metaphase plane. Spindle poles are less focused compared to animal spindles.

(C) Early stage of the phragmoplast formation comprises a thick bundle of microtubules formed between two daughter nuclei.

(D) The phragmoplast expands centrifugally towards the periphery of the cell cortex at the site previously occupied by the prophase band. The cell plate is constructed within the ring structure of the phragmoplast.

(E) After completion of cytokinesis, microtubules extend from the nuclear envelope toward the cell cortex. Microtubules associated with the plasma membrane start to appear.

(F) Interphase microtubules in elongating plant cells are aligned parallel to each other and perpendicular to the cell’s growth axis.
1.3.1 Preprophase bands

The preprophase band (PPB) is a girdle of microtubules that appears prior to prophase and predicts the position of the new cell plate. Before PPB formation, the cortical microtubules disappear and then the PPB is formed at the cortex in the vicinity of the nucleus. PPBs mark the site of attachment of the future cell plate (Mineyuki and Gunning, 1990; Mineyuki, 1999). TONNEAU is required for the formation of PPBs and transverse arrays of cortical microtubules (Traas et al., 1995). The ton mutants lack the PPBs, resulting in misaligned cell division plane, but still form spindles and phragmoplasts (Traas et al., 1995). A recent study identified a novel MAP, AIR9 (auxin-induced in root cultures), that decorates cortical microtubules and PPBs. AIR9 disappears during mitosis and reappears precisely at the former PPB sites when phragmoplasts contact the PPB sites (Buschmann et al., 2006). In addition, AIR9 promotes maturation of the cell plate after the cell plate contacts the PPB sites (Buschmann et al., 2006). Despite accumulating findings, it is still unclear how the PPB leaves cues for cell division and how the cytokinetic apparatus recognizes and follows the cues.

1.3.2 Spindles

The spindle has a bi-polar anti-parallel structure and moves duplicated chromosomes apart to form the two daughter nuclei during anaphase. Live cell imaging using microtubule markers, GFP-MAP4, GFP-TUA and GFPEB1, have provided detailed information on spindle formation in tobacco BY-2 cells (Dhonukshe et al., 2006). The spindle starts to form during prometaphase. Microtubules surrounding the nucleus penetrates into nuclear region and form a few bi-polar microtubule bundles. During metaphase, spindles grow mainly from spindle poles until or past the metaphase plate (Dhonukshe et al., 2006). For chromosome separation, two different forces generated by the spindle are required. One is generated by kinetochore microtubule disassembly at the site where the kinetochore is attached to microtubules (Anaphase A) (Alberts et al., 1994).
This leads to chromosome movement towards the spindle poles. The other is generated by sliding of anti-parallel spindle microtubules (Anaphase B) (Alberts et al., 1994). This results in spindle elongation and further separates spindle poles and chromosomes. Unlike animal and yeast cells, plants lack conspicuous microtubule organizing centres. Plant spindle poles are less focused compared to those of animal cells, which have centriole-based microtubule organizing centres, called centrosomes, at the spindle poles. Centriole-based organizing centres are not required for accurate cell division in animal cells, as illustrated in female meiotic spindles, which form independently of centrosomes (Gadde and Heald, 2004). γ-tubulin is a highly conserved protein in eukaryotes and is a part of microtubule organizing centres. Although plants lack centrosomes, γ-tubulins are immunolocalized in spindle pole regions (Liu et al., 1994). When γ-tubulin is severely depleted by RNAi, the spindle fails to form and remnants of microtubules surround the chromosomes (Binarova et al., 2006).

1.3.3 Phragmoplasts

Phragmoplasts are important for constructing the cell plate between daughter nuclei during cytokinesis. At the early stage of phragmoplast formation, soon after anaphase, the phragmoplast is a thick-bundled barrel-like structure formed between two daughter nuclei (Figure 1.3). Then the phragmoplast develops into two ring-like structures, side by side, composed of short microtubules and grow centrifugally to the periphery of the cell (Figure 1.3). It had long been believed that oppositely oriented microtubules overlap at the mid-zone of the phragmoplast. A study using TEM 3D tomography proved that microtubules do not overlap at the mid-zone (Austin et al., 2005). In the mid-zone, the cell plate is formed by Golgi-derived vesicles fusing to each other in the ring-like structure of the phragmoplast (Samuels et al., 1995). The cell plate has to be constructed in the right position and orientation in the cell. This is achieved by fine coordination of phragmoplast development and some unknown cues left by the preprophase band.
In maize, the tangled mutants cannot guide phragmoplasts to the sites where the preprophase band previously formed (Cleary and Smith, 1998). TAN is suggested to guide phragmoplasts while associating with phragmoplast microtubules (Smith et al., 2001). Two kinesins from the arabidopsis Kin12 family were isolated through a yeast two-hybrid screen for TAN1-interacting proteins and were shown to be involved in spindle and phragmoplast orientation (Muller et al., 2006), but the mechanism of the phragmoplast guidance still remains elusive.

1.3.4 Cortical microtubules

Cortical microtubules are plant-specific arrays that are formed during interphase. Cortical microtubules are often aligned parallel to each other in the area where directional growth is happening. These parallel arrays are perpendicular to the growth axis and are believed to control the direction of cell expansion. The mechanisms of this regulation, however, seems very complex (Himmelspach et al., 2003; Sugimoto et al., 2003; Wasteneys and Fujita, 2006). It has been suggested that cortical microtubules are self-organized into parallel arrays by intermicrotubule interactions (Wasteneys and Williamson, 1989a; Dixit and Cyr, 2004). Many MAPs discussed above are also involved in microtubule dynamics and organization.

1.4 OBJECTIVES

The major goal of this study is to understand functions and mechanisms of the MOR1 protein using the mor1-1 mutant of arabidopsis. MOR1 is a microtubule-associated protein and belongs to MAP215/Dis1 family that is known to be involved in spindle functions.

Objective 1: As a member of the MAP215/Dis1 family, the possibility of MOR1’s involvement in mitosis and cytokinesis should be explored. In chapter 2, I tested if MOR1 is essential for the structural organization and function of mitotic and cytokinetic microtubule array by live cell imaging and immunofluorescence labelling.
Objective 2: It has been shown that the morl-1 mutation, which substitutes a single amino acid near the N-terminus, causes microtubule disorganization. Does the morl-1 mutation affect MOR1's affinity for microtubules or alter other functions to keep microtubules organized? To answer this question, I immunolocalized the wild-type MOR1 protein and the mutant morl-1 protein as described in chapter 3.

Objective 3: To better understand how MOR1 controls microtubule organization, in chapter 4, microtubule dynamics were measured in the morl-1 temperature-sensitive mutant and in wild-type plants using confocal laser scanning microscopy. Analysis of time-lapse images of cortical microtubules were performed by visualizing microtubules with various GFP reporter proteins in leaf cells at morl-1's restrictive temperature.

Objective 4: What mechanism does MOR1 use to regulate microtubule dynamics? MOR1 is comprised of 4 or possibly 5 TOG domains that are situated in series at the N-terminus. By comparing the dimensions of the MOR1 protein and the microtubule protofilament, and based on studies of MOR1's homologues, we hypothesized that each TOG domain interacts with one tubulin dimer. In chapter 5, I propose experimental strategies and some preliminary data to test the hypothesis.
Chapter 2: The Role of MOR1 in Cell Division

1. A version of this chapter has been published.

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Chapter 2: The Role of MOR1 in Cell Division

2.1 INTRODUCTION

Microtubules are essential for all eukaryotic cells to divide. During mitosis, microtubules separate chromosomes to make two daughter cells. In animal and yeast cells, microtubules nucleate from and are anchored to fixed points, microtubule organizing centres (centrosomes in animal and spindle pole bodies in yeast). Higher plants lack microtubule organizing centres but form somewhat focused spindle poles. Instead of fixed nucleating sites, microtubules nucleate from the nuclear surface, at the cell cortex (Wasteneys and Williamson, 1989b; Schmit, 2002; Kumagai et al., 2003; Shaw et al., 2003; Yoneda and Hasezawa, 2003), and along existing microtubules (Wasteneys and Williamson, 1989b; Wasteneys et al., 1993; Murata et al., 2005). Each known member of the MAP215/Disl family, except for the MOR1 protein found in plants, was first characterized as a MAP that assembles spindles and has been shown to be essential for spindle formation.

The following text outlines what is currently known about the function of this family of proteins for processes of cell division.

2.1.1 Yeast MAP215/Disl proteins are important for spindle formation and function

There are three known yeast MAP215/Disl members. In fission yeast, Schizosaccharomyces pombe, there are two proteins, Dis1 (chromosome non-disjunction) and Alp14 (Altered polarity) (Ohkura et al., 1988; Nakaseko et al., 2001), while in budding yeast, Saccharomyces cerevisiae, there is one orthologue, called Stu2 (suppressor of tubulin) (Wang and Huffaker, 1997). All of these proteins have been implicated in spindle function (Gard et al., 2004). When Dis1 is depleted or mutated, prometaphase centromere movement is impaired, chromosome movement
and alignment are abnormal, yet spindle elongation is similar to that in wild-type (Ohkura et al., 1988; Nabeshima et al., 1995; Nabeshima et al., 1998; Nakaseko et al., 2001). The \textit{alpl4} mutants have defects in spindle organization, chromosome separation, cytoplasmic microtubule organization and cell shape (Garcia et al., 2001; Nakaseko et al., 2001). In addition, Dis1 and Alp14 are suggested to link kinetochores and kinetochore microtubules (Garcia et al., 2001; Nakaseko et al., 2001; Garcia et al., 2002). Stu2 was first isolated as a suppressor of a β-tubulin mutant (Wang and Huffaker, 1997). Stu2 is also important for mitotic spindle assembly and chromosome arrangement (Kosco et al., 2001; Severin et al., 2001). \textit{stu2} forms short spindles and delays progression to anaphase (Severin et al., 2001). When Stu2 was depleted by repression of \textit{STU2} mRNA, cytoplasmic microtubules became fewer and less dynamic (Wang and Huffaker, 1997; Kosco et al., 2001).

\textbf{2.1.2 Dictyostelium MAP215/Dis1 protein is important for centrosome duplication and microtubule nucleation and elongation.}

\textit{DdCP224 (Dictyostelium discoideum centrosomal protein 224)} is a member of the MAP215/Dis1 family. Interestingly, the structure of DdCP224 is closer to that of higher eukaryote homologues that it is to yeast homologues. DdCP224 is important for duplicating centrosomes and for cytokinesis (Graf et al., 2000; Graf et al., 2003). Replacing the endogenous DdCP224 with a low expressing DdCP224-GFP inhibited cell growth, and reduced the number and length of cytoplasmic microtubules, which were hypersensitive to nocodazole. The recovery from nocodazole was delayed and impaired, suggesting that DdDP224 was involved in microtubule nucleation and elongation (Graf et al., 2003).

\textbf{2.1.3 Caenorhabditis elegans MAP215/Dis1 protein, Zyg-9}

The MAP215/Dis1 member in \textit{C. elegans} is Zyg-9, first identified by the temperature-sensitive
strict maternal effect lethal mutations (Kemphues et al., 1986). The zyg-9 mutants formed
defective mitotic spindles with short microtubules and incorrect positioning and orientation
(Matthews et al., 1998). Meiotic spindles which normally do not require long microtubules were
also impaired (Matthews et al., 1998).

2.1.4. Drosophila MAP215/Dis1 protein, Msps

MSPS (mini spindles) was originally identified in a cytological screen for mitotic defects. The
mutant msps formed spindles that were multipolar or that were composed of short bundled
microtubules, with an increased mitotic index mainly due to the metaphase arrest (Cullen et al.,
1999). Depletion of Msps by RNAi also caused multipolar spindles and induced bundling of
interphase microtubules (Brittle and Ohkura, 2005).

2.1.5 Xenopus MAP215/Dis1 protein, XMAP215

XMAP215 was first isolated by biochemical purification from oocytes and identified as a MAP
because it promoted microtubule assembly in vitro (Gard and Kirschner, 1987). Its critical role in
mitosis was demonstrated by microinjection of anti-XMAP215, which disrupted spindle
assembly and organization during oocyte maturation (Becker et al., 2003). XMAP215-depleted
cells have been shown to form multi-polar spindles, which resemble the msps mutant phenotype
(Gard et al., 2004). XMAP215 can nucleate and anchor microtubules at their minus ends, and the
activity requires γ-tubulin in pure tubulin solution but not in Xenopus egg extracts (Popov et al.,
2002).

2.1.6 Human MAP215/Dis1 protein, ch-TOG

The human member of MAP215/Dis1 family, ch-TOG (colonic and hepatic tumor over-
expressed gene), was originally selected during a screen for cytochrome p450 and was found to
be highly expressed in hepatic and colonic tumours and in brain tissues (Charrasse et al., 1995;
Charrasse et al., 1998). Depletion of ch-TOG by RNAi disrupts the tight-focussing of microtubule minus-ends at spindle poles, and causes centrosome fragmentation and multi-polar spindles (Conte et al., 2003; Gergely et al., 2003; Cassimeris and Morabito, 2004; Holmfeldt et al., 2004). However, none of these studies detected major changes in microtubule stability during mitosis. This is very interesting because significant effects of microtubule stability have been noted in mutational analysis and depletion experiments with most other homologues.

2.1.7 MOR1 was suggested to be involved in cell division.

Does the plant member of the XMAP215/Dis1 family, MOR1, also play a role in cell division? In the initial study of the mor1 mutants, obvious defects in cortical microtubule arrays of interphase and terminally differentiating cells developed rapidly at 29°C, but mitotic and cytokinetic microtubule arrays did not show obvious defects, and cell division continued (Whittington et al., 2001). Tissue patterns and cell numbers in the root elongation zone were the same in mor1-1 and the wild-type, suggesting that the enlarged diameter of the mor1-1 root tip was generated entirely by radial expansion and not through addition of extra cell layers. Furthermore, the effects of treatment at the restrictive temperature for several weeks were reversible, suggesting that apical meristems were well preserved (Whittington et al., 2001). As outlined above, these results were puzzling, given that all MOR1 homologues previously identified in animal and yeast cells have been shown to be essential for spindle formation and function (Gard et al., 2004).

MOR1's likely function in cell division was first supported by the finding that MOR1 gene expression peaks during the M phase of arabidopsis suspension culture cells (Menges et al., 2002), and the discovery that the gemini1 mutants, which affect cell plate formation in haploid microspores, were allelic to MOR1 (Twell et al., 2002). The apparent lack of obvious division defects in the temperature-sensitive mor1 mutants after short treatments at 29°C (Whittington et
al., 2001) prompted us to consider the possibility that the specific amino acid substitutions near the N-terminus may be inconsequential for microtubule function during cell division. On closer examination, however, morl-1 root tips swell more severely above the threshold restrictive temperature of 29°C, and after prolonged (in the order of 24 to 48 h) incubations. After root tips of morl-1 were treated at 30°C for 48 h, incomplete cross walls and other indications of aberrant cell division were observed (Himmelspach et al, 2003a). Recently, these results have been confirmed (Eleftheriou et al., 2005).

To better understand MOR1’s role in cell division, I re-examined cell division events in detail in morl-1 at the restrictive temperature. Using live cell imaging and immunolabelling approaches, I explore in this chapter the effects of the morl-1 mutation on the structure and function of the mitotic and cytokinetic microtubule arrays, and show that MOR1 is important for spindle and phragmoplast organization, cell plate formation and the completion of cell division.

2.2 RESULTS

2.2.1 Mitotic index is higher in morl-1 mutants at the restrictive temperature.

I first investigated MOR1’s function in dividing cells by comparing the proportion of cells undergoing mitosis in wild-type and morl-1 root tips after 24 h at morl-1’s restrictive temperature of 31°C (Figure 2.1). I detected a significant (significance by t-test, p < 0.05) increase in the proportion of mitotic cells in morl-1 for both the cortex and endodermis, and a slight but statistically non-significant (p < 0.15) increase in the mitotic index in the epidermis. These findings indicate that either mitosis proceeds more slowly in morl-1 or that the cell production rate is increased relative to wild-type.
Figure 2.1. High mitotic indices in the *mor1-1* mutant suggest MOR1 is involved in cell division.

The proportion of cells in mitosis is increased in the *mor1-1* mutant after 24h at the restrictive temperature of 31°C, suggesting that either mitosis is more frequent in the mutant or that mitotic progression is impaired.
2.2.2 Mitosis and cytokinesis persist longer in morl-1 at the restrictive temperature

To compare the duration of mitosis and cytokinesis in morl-1 and wild-type, I examined microtubules in living root tips of plants stably transformed to express the green fluorescent protein – microtubule binding domain of mammalian MAP4 (GFP-MBD; Granger and Cyr, 2001a), a reporter fusion protein that decorates microtubules. Plants were cultured with their roots growing through agarose-solidified medium spread along a coverslip attached to the base of culture dishes. Use of these chambers enabled individual cells in the division zone of roots to be observed for several hours, and the periodic collection of fluorescent images to record the progression of these cells through mitosis and cytokinesis. A temperature-controlled stage maintained plants at restrictive temperature. This procedure confirmed that mitosis and cytokinesis were significantly slower in morl-1 mutants. As shown in Figures 2.2, spindles and phragmoplasts observed between 2 and 8 hours after transfer to the restrictive temperature persisted longer in morl-1 (averages of 25.3 and 38.2 min respectively) than in wild-type cells (13.9 and 23.3 min respectively). Interestingly, neighbouring cells, both within cell files and in adjacent files, tend to enter mitosis and complete cytokinesis together in both wild-type and morl-1 plants (Figure 2.2C). This indicates that the signals triggering and regulating cell division may extend beyond the confines of single cells.
Figure 2.2. Live cell imaging of GFP-MBD in roots

Analysis of microtubules in living cells using GFP-MBD fusion protein reveals that cell division takes longer and microtubule organization is aberrant in mor1-1 at restrictive temperature. A and B, Frequency distribution histograms showing the ranges of time that individual spindles (A) and phragmoplasts (B) persist in wild-type (white bars) and mor1-1 (shaded bars) cells. C and D, Progression of mitosis and cytokinesis shown in series of images from the root epidermal cells from wild-type (C) and mor1-1 roots (D) stably expressing the GFP-MBD fusion protein. Bars = 5 μm.
2.2.3 *morl-1* preprophase bands, spindles and phragmoplasts are disrupted at the restrictive temperature.

**Live Cell Analysis**

The appearance of microtubule arrays in living and dividing *morl-1* cells also differed from that in wild type as shown in Figures 2.2C and 2.2D. In *morl-1*, approximately half of the observed spindles appeared without prior PPB formation. Spindles were at first very disorganized with no clear axis (Figure 2.2D, 9min), yet were eventually able to organize chromosomes into metaphase configurations. Spindles were clearly shorter than normal and mis-aligned (Figure 2.2D, 21min). Despite this, cells consistently progressed through to telophase, as judged by eventual spindle degradation and phragmoplast formation. Phragmoplasts were also of abnormal appearance in *morl-1* (Figure 2D, 27 to 60min) compared to wild-type (Figure 2.2C, 18 to 27min). In some cases, phragmoplasts were mis-oriented and formed discontinuous arrays, which would likely be deleterious for completion of cytokinesis.

As shown in previous studies (Whittington et al., 2001), interphase microtubules were disrupted in *morl-1* at the restrictive temperature. In addition, some microtubules took on a thick, heavily bundled appearance in the *morl-1* mutant cells (Figure 2.3B). Similar but less extensively developed structures were also observed in interphase wild-type cells (Figure 2.3A), suggesting that the thick bundle-like elements are generated by the GFP-MBD fusion protein, and not specific to the *morl-1* remodelling of microtubules at high temperatures. No such structures were observed in root tip interphase cells by immunofluorescence (shown in Figures 2.4A, 2.4B, 2.4F and 2.4G) or in living hypocotyl cells of *morl-1* expressing a GFP-α-tubulin fusion protein (Whittington et al., 2001). Unfortunately, GFP-tubulin constructs available during this research do not incorporate into microtubules in root tissues (Ueda et al., 1999; Wasteneys and Yang, 2004) so I was unable to probe microtubules in living root cells by alternate means.
Figure 2.3. Apparent microtubule bundles expressing GFP-MBD fusion proteins become prominent in morl-1 at 31°C. (A) and (B) MBD-GFP labelling identifies bright, bundle-like structures (arrowheads) at 30°C. Although some evidence for this is observed in wild-type cells (A), microtubule remodelling is much more extensive in morl-1 (B). These thickened microtubule structures are not observed with immunofluorescence labelling (shown in Figure 2.4A, 2.4B, 2.4F and 2.4G ), suggesting this putative bundling of microtubules is dependent on the GFP-MBD fusion protein. Bars = 10 μm.
Immunofluorescence Analysis

To assess microtubule organization quantitatively during cell division, I used tubulin immunofluorescence of fixed material. As shown in Figure 2.4, all microtubule structures, including the PPB, spindles and phragmoplasts, showed some degree of disorganization in morl-1 after seedlings were grown for 24 hours at the restrictive temperature. Some PPBs were partially split instead of forming continuous ring-like structures (Figure 2.4B). Some spindles were severely disorganized, with misaligned short microtubules, resulting, in some instances, in obliquely distributed chromosomes (Figures 2.4D). Other spindles had short microtubules but normally arranged chromosomes (Figure 2.4E), while in other instances, spindles were fragmented so that one or more pair of chromosomes was separated from the others (Figure 2.4F). Phragmoplasts were typically crooked and fragmented (Figure 2.4H). These results confirm that the MOR1 protein has an important role in organizing not only cortical microtubules but also the microtubule arrays involved in cell division.

To investigate microtubule organization in detail, we compared the arrangement of PPBs in morl-1 and wild-type cells after 24 hours at the restrictive temperature using root tips prepared by gentle squashing to separate cells from one another (Figures 2.4A to 2.4H). Compared to wild-type cells, in which 96% of PPBs formed ring-like structures encircling the cell at the position of the nucleus, only 80% of morl-1 PPBs were arranged this way (Figure 2.4I). Wild-type and morl-1 had the same proportion of acentric PPBs (3%), while morl-1 had an increased incidence of crooked (see Figure 2.4I, left-most aberrant PPB drawing) PPBs (wild-type, 1%; morl-1, 7%). Six percent of morl-1 PPBs were discontinuous and 4% were branched, whereas aberrant PPBs were not observed in wild-type cells. In total, only 17% of PPBs detected in morl-1 cells were considered to be aberrant.

Phragmoplast organization was analyzed in cells prepared by root tip squashing after 24 hours at
the restrictive temperature (Figure 2.4J). In wild-type cells, phragmoplasts were observed in three typical configurations. Early on they were barrel-like and at later stages, formed double ring-like structures that were either continuous or discontinuous, the latter occurring when phragmoplasts reached the parent cell wall. In morl-1, only 41% of phragmoplasts were deemed similar in appearance to wild-type phragmoplasts while the remainder were considered aberrant. Approximately 40% of morl-1 phragmoplasts were crooked (Figure 2.4J, first three aberrant phragmoplast drawings), compared to only 1% of wild-type phragmoplasts and 9% of morl-1 phragmoplasts were severely fragmented. Other aberrant configurations accounted for another 10% of morl-1 phragmoplasts.
Figure 2.4. Immunofluorescence comparison of microtubule arrangement in root tip cells of wild-type and mor1-1 after culture at 31°C for 24 hours.

(A) to (H) Representative confocal images show anti-tubulin (green) and DAPI-stained nuclei and chromosomes (blue). Bars = 5 μm.

(A) Wild-type preprophase band. Projection of confocal z-series.

(B) mor1-1 split preprophase band. Single optical confocal section.

(C) Wild-type spindle. Projection of confocal z-series. The double headed arrow indicates how spindle length was measured (Figure 4A).

(D) to (F) mor1-1 spindles. Projections of confocal z-series (D and E) and single optical confocal section (F). Arrowhead indicates uncoupled spindle component and associated chromosomes.

(G) wild-type phragmoplast. Single optical confocal section. The double headed arrow indicates how phragmoplast length was measured (Figure 4B).

(H) mor1-1 phragmoplast. Projection of confocal z-series.

(I) and (J) Quantitative analysis of preprophase band (I) and phragmoplast (J) arrangement in wild-type (white bars) and mor1-1 (shaded bars) root tip cells. Frequency distribution histograms are shown for each structural category, which are indicated in line drawings depicting normal and aberrant microtubule patterns (I) and (J), with blue square depicting nuclei (I).
2.2.4 Spindle and phragmoplast lengths are reduced in morl-1.

Typical morl-1 spindles were very short and not as focused as in wild-type cells (Figures 2.4C to 2.4F). To compare spindle microtubule lengths, I measured metaphase and anaphase spindles in wild-type and morl-1 root tip cells after 24 hours at the restrictive temperature (Figure 2.5A). The morl-1 spindles, with mean lengths of 3.82 ± 0.93 (SD) μm, were significantly shorter than wild-type spindles (p < 0.01), which had a mean length of 8.82 ± 1.59 (SD) μm. Metaphase and anaphase spindle lengths were combined for these calculations because in morl-1 it was often difficult to distinguish metaphase and anaphase spindles due to the aberrant chromosome arrangements. Metaphase and anaphase spindle lengths differ significantly in wild-type cells (Figure 2.6) but I found that spindles in morl-1 were significantly shorter than even the shortest metaphase spindles of wild-type (p < 0.01). I considered the possibility that cell length regulates spindle size. In morl-1 it was not possible to accurately measure cell length owing to the crooked cross walls, but in wild-type cells I found no correlation between spindle and cell length (Figure 2.7). The reduction in spindle length in morl-1 mutants at the restrictive temperature can therefore be attributed to the specific defect in the mutant protein.

Consistent with shorter spindles and disorganized cortical microtubules (Whittington et al., 2001), I found that phragmoplast microtubule lengths were reduced in morl-1 (Figure 2.5B). I limited phragmoplast measurements to epidermal cells from roots grown for only 2 h at the restrictive temperature. This short time at restrictive temperature produced wild-type and morl-1 epidermal cells of similar diameter and volume. This strategy allowed us to avoid measuring phragmoplasts in radially swollen cells of morl-1, in which an obligate increase in phragmoplast diameter may dilute the tubulin pool, and thereby reduce the average length of microtubules. Phragmoplast length was measured as the combined length of microtubules in both halves of the phragmoplast array plus phragmoplast mid-zone (double-headed arrow in Figure 2.4G).
Preliminary analysis detected no significant difference in the length of early and late stage phragmoplasts in wild-type cells (Figure 2.8), so mean phragmoplast length was calculated from all phragmoplasts measured in both morl-1 and the wild-type controls. The mean phragmoplast length in morl-1 of 3.87 ± 0.45 (SD) μm was significantly less than the wild-type mean of 5.15 ± 0.51 (SD) μm (p < 0.01). These data indicate that MOR1 is important for maintaining the length of microtubules in phragmoplasts.

Of significance, all morl-1 phragmoplasts observed in the larger epidermal cells after this shift to restrictive temperature formed discontinuous, crooked structures, and never formed the continuous ring-like configurations typical of wild-type phragmoplasts. This is in contrast to the 60% of cells sampled from all tissues with phragmoplast disruption after 24h at the restrictive temperature. This finding suggests that controlling phragmoplast structure is a greater challenge in larger cells.
Figure 2.5. Immunolabelled spindles and phragmoplasts are significantly shorter (p < 0.01) in morl-1 roots at restrictive temperature.

Frequency distribution histograms compare wild-type (open bars) and morl-1 mutants (shaded bars), after culturing at 31°C for 24h (spindle) and 2h (phragmoplast). At least 20 spindles and 16 phragmoplasts were measured for each treatment, using image data collected from confocal laser scanning optical sections.

(A) Spindle lengths, defined as the maximum distance from pole to pole, are significantly shorter in morl-1.

(B) Phragmoplast length, defined as the greatest distance from the one side of phragmoplast array to the other, is almost consistently lower in morl-1.
Figure 2.6. The mor1-1 metaphase spindles are shorter than metaphase spindles of wild-type.

mor1-1 metaphase and anaphase spindles cannot be distinguished but are consistently shorter than the metaphase spindles of wild-type (p < 0.01). Plants were grown for 5 days at 21°C followed by 1 day at 31°C. Spindles were immunolabelled with anti-α-tubulin antibody. Bars indicate standard deviations.
Figure 2.7. Spindle length has no direct correlation with cell length.

According to Pearson product moment analysis, spindle length has no direct correlation with cell length in wild-type ($r = 0.27$). Wild-type Arabidopsis plants were grown for 5 days at 21°C followed by 1 day at 31°C. To measure cell lengths, cross walls were labelled with anti-callose antibody. Spindles were labelled using anti-soy tubulin antibody.
Figure 2.8. Phragmoplast lengths at early stage and late stages in wild-type.

Phragmoplast length does not vary between the early and late stage of phragmoplast development in wild-type epidermal cells of similar diameter (\( p < 0.01 \)). Wild-type arabidopsis seedlings were grown for 5 days at 21°C followed by 2 h at 31°C. Phragmoplasts were immunolabelled with anti-\( \alpha \)-tubulin antibody and the maximum lengths measured from phragmoplasts. These measurements excluded the earliest and rare barrel-shaped phragmoplasts, which were never observed in morl-1. Bars indicate standard deviations. These measurements demonstrate that it is valid to combine measurements of phragmoplasts at early and late stages.
2.2.5 *morl-1*’s microtubule defects cause chromosome rearrangements, multi-nucleate cells and aberrant cell plate formation.

Having determined that *morl-1* produces significant defects in mitotic and cytokinetic arrays, I investigated the downstream consequences of these defects. Consistent with the very short spindles formed at the restrictive temperature, chromosome arrangements were often disorganized in *morl-1* (Figures 2.9C to 2.9E). Typically, chromosome alignment in one plane during metaphase was impaired (Figures 2.9D and 2.9E). Separation of two or more chromosomes from the main cluster was also commonly observed (Figures 2.9C and 2.9E). In most cases, however, mitosis appeared to progress to completion.

The severe defects observed for *morl-1* phragmoplasts in dividing root tip cells are consistent with defects in cell plate formation. To follow cell plate anatomy in *morl-1* at the restrictive temperature, I followed callose accumulation in the newly formed cell plate during telophase. Callose, a major polysaccharide component of cell plates, accumulated at apparently normal levels in both the wild type and *morl-1*. Callose distribution patterns in *morl-1* were abnormal (Figures 2.10), suggesting that cell plate formation was altered. Consistent with the cell plate formation defects, at the restrictive temperature, multi-nucleated cells were commonly observed (Figures 2.11). Often four or five nuclei were clustered together in *morl-1* cells (Figure 2.11B), confirming cytokinesis defects.
Figure 2.9. Chromosome alignment was impaired in the root tip of mor1-1 at restrictive temperature after 1 day.

DAPI staining of whole mount roots showing condensed chromosomes in wild-type (A) and (B) and mor1-1 (C) to (E). Projections of confocal z-series.
Figure 2.10. Cell plate formation was abnormal in the root tip of *morl-1* at restrictive temperature after 1 day.

(A) and (B) Immunolabelling with anti-callose (red), and anti-tubulin (green) and DAPI staining of DNA (blue) reveals normal pattern of cell plate formation in wild-type (A) and crooked, incomplete cell plate in *morl-1* (B). Single optical sections of confocal. Bars = 5 μm
Figure 2.11 Multinucleate cells were observed in *morl-1* root tips after growing at the restrictive temperature for 1 day.

DAPI staining of whole mounted intact roots showing a single nucleus in wild-type (A) and a cluster of nuclei in *morl-1* (B). Projections of confocal z-series.
2.3 DISCUSSION

Analysis of the temperature-sensitive morl-1 mutant revealed that MOR1 is required for the production of the long microtubules involved in mitotic spindle and cytokinetic phragmoplast construction and that loss of this function slows the progression of mitosis and cytokinesis or prevents completion of cell division. These results demonstrate an important role for MOR1 in the organization of all microtubule arrays in expanding and dividing cells.

2.3.1 The MOR1 protein may promote long microtubules.

My data support the idea that MAP215/Dis1 proteins promote relatively long microtubules. As with the previous discovery that cortical microtubules become short in the morl-1 mutant (Whittington et al., 2001), I found here that spindle and phragmoplast microtubules were quantitatively shorter at morl-1’s restrictive temperature. The other array examined in this study, the PPB, might also have had shorter microtubules in the morl-1 mutant, though its organization as a concentrated band of overlapping microtubules is not amenable for measuring microtubule length. Taken together, these observations suggest that MOR1 has a general role in controlling the length of microtubules, and that this fundamental property of microtubules is critical for the organization and function of each array. Previous work supports the concept that the XMAP215 family members are primarily important for microtubule growth (Gard et al., 2004).

2.3.2 Spindle Structure

The morl-1 mutant forms fragmented, short spindles that either do not focus or develop with multiple poles. These defects explain the higher mitotic indices recorded and the significant increase in the time required for morl-1 cells to complete mitosis at the restrictive temperature. Live cell recordings of microtubule arrays in dividing cells confirmed that spindles persisted for considerably longer in morl-1 cells at the restrictive temperature in comparison with wild-type
cells. Lateral interactions of spindle microtubules have been shown to be essential for spindle organization and function (Ambrose et al., 2005). Presumably shorter microtubules comprising the morl-1 spindle might have reduced lateral microtubule interactions and require longer time and in some cases might fail to capture kinetochores and align chromosomes at the metaphase plane. Shorter spindle microtubules could reduce sliding of interpolar microtubules of anaphase B and result in shorter anaphase spindles. Collectively, these spindle-associated defects could reflect a general consequence of reduced microtubule length, which alone might impede spindle structure and function. However, these observations also support the idea that MOR1 participates directly in overall spindle organization. Members of the MAP215/Dis1 family of proteins seem to be essential for spindle pole function, and are found along with γ-tubulins and other proteins in centrosomes, which acquire spindle pole status during mitosis (Gard et al., 2004). The Minisprindles protein, a member of this family from Drosophila, was identified in a mitotic mutant that produced one or more additional spindles (Cullen et al., 1999). In vitro experiments have demonstrated that XMAP215 contributes, along with γ-tubulin, to the nucleation of microtubule assembly (Popov et al., 2002). In HeLa cells, depletion of TOGp increases the number of γ-tubulin foci per aster, indicating that TOGp controls centrosome integrity (Cassimeris and Morabito, 2004). TOGp-depleted cells also have aberrant chromosome arrangements, which have been described as being in a ‘prometaphase-like state (with no discernible metaphase plate)’ and as a ‘metaphase plate with lagging chromosomes’ (Gergely et al., 2003). I observed these same two aberrant chromosomal arrangements in morl-1 at restrictive temperature. Plant cells lack mitotic centrosomes as well as tightly focussed spindle poles but γ-tubulins have been immunolocalized in spindle pole regions (Liu et al. 1994) and are likely to have important functions in microtubule assembly (Schmit, 2002). Therefore, MOR1 might function in controlling nucleation of spindle microtubules, possibly in concert with γ-tubulin, may be conserved in higher plants.
2.3.3 Phragmoplast structure and cytokinesis

Phragmoplasts are unique to plant cells and necessary for the completion of cytokinesis. The results of my study show that the morl-1 mutation disrupts phragmoplast organization in vegetative cells, leading to incomplete cell plate formation during telophase and the production of multinucleate cells. These changes match reports for the morl-1 mutant (Himmelspach et al., 2003a; Eleftheriou et al., 2005). Importantly, these abnormalities resemble the aberrant cell wall formation observed in microspores from plants heterozygous for the geml alleles of MOR1 (Park and Twell, 2001; Twell et al., 2002). Some of the microspores inheriting the geml alleles fail to produce a generative cell as a result of defective nuclear migration prior to pollen mitosis I and/or incomplete or aberrant (i.e., symmetrical) cell plate formation following pollen mitosis I (Park et al., 1998). Although the arrangement of phragmoplasts was not examined in the geml microspores, it seems likely that their disruption would be a primary cause of the cell plate defects documented (Twell et al., 2002).

Based on immunofluorescence data from vegetative tissue culture cells, Twell et al (2002) suggested that MOR1/GEM could be essential for regulating the phragmoplast mid-zone, explaining in part the failure of geml microspores to complete pollen mitosis I. My results, however, do not support the idea that the abnormal cell plates in the root tip were formed by an irregular phragmoplast mid-zone. The gap between microtubules at the mid-zone of the phragmoplast is similar in morl-1 and wild-type cells, and as discussed below, there is no evidence that MOR1 plays a role in cross-linking microtubules. Instead, my data suggest that these abnormal cell plates are produced by crooked, mis-oriented and fragmented phragmoplasts. The susceptibility of morl-1 phragmoplasts to disruption appears to be greater in larger cells, with more severe disruption documented much sooner in the larger epidermal cells. Other cytokinesis-defective mutants like knolle (Lukowitz et al., 1996) and keule (Assaad et al., 1996), which both affect vesicle fusion at the cell plate, and hinkel (Strompen et al., 2002) and the
MAP65 mutant *pleiade* (Muller et al., 2004), which both affect the microtubule cytoskeleton, also form aberrant cell plates during cytokinesis, resulting in multinucleate cells or polyploid nuclei. The *pleiade* (*ple*) mutant produces phragmoplasts that are wider than normal, with an increased ‘clear zone’ between the two opposing microtubule arrays and increased microtubule lengths, resulting in incomplete cell plate formation and multinucleate cells. The general arrangement of *ple* phragmoplasts is not, however, altered (Muller et al., 2004). The different effects of *morl-1* and *ple* mutants on phragmoplast formation underscore the very distinct functions of these two microtubule-associated proteins. MAP65-3/PLE’s role in microtubule cross-linking is supported by the substitution in the *ple-4* mutant of a conserved residue, shown to be critical for microtubule binding activity in the MAP65-1 paralogue (Smertenko et al., 2004). A tobacco homologue of MOR1, TMBP200, isolated from telophase culture cells, was also considered to have microtubule bundling properties (Yasuhara et al., 2002). More recently, the ability of TMBP200 to bundle microtubules was ruled out, and the earlier observation (Yasuhara et al., 2002) explained by co-purification of MAP65 protein in the MAP200 fraction (Hamada et al., 2004).

There is good evidence that interactions with the actin microfilament cytoskeleton are important for both spindle alignment and for guiding the phragmoplast to the site of fusion with the parent wall (Granger and Cyr, 2001b; Yoneda et al., 2004). Granger and Cyr (2001b) reported that tobacco BY-2 cells treated with the actin-targetted drug, latrunculin B, could position the preprophase nucleus but not the spindle or phragmoplast. My data suggest that MOR1 is involved in aligning and shaping the spindle and phragmoplast by regulating the length of microtubules, which in turn interact with actin filaments.
2.3.4 Cues for positioning the cell plate

In comparison to spindles and phragmoplasts, PPB disorganization was less obvious in the morl-1 mutant at the restrictive temperature. Immunofluorescence experiments showed that only 17% of PPBs looked abnormal. This relatively normal appearance, however, may reflect the difficulty in resolving details of this tightly packed array of cortical microtubules. Furthermore, in the GFP-MBD-expressing plants, about half of the spindles observed formed in cells with no prior PPB formation, whereas in the wild-type, all mitotic cells observed developed PPBs before spindles. The immunofluorescence data therefore underestimate the severity of the morl-1 mutant on PPB structure, and also its function. PPBs mark the site of attachment of the future cell plate (Murata and Wada, 1991; Mineyuki, 1999) and some studies have found novel proteins that are involved in the spatial control of cytokinesis (Smith et al., 1996; Buschmann et al., 2006; Muller et al., 2006), though it remains unclear how this is achieved. Previous studies have shown that although spindle orientation can affect subsequent phragmoplast position and orientation (Granger and Cyr, 2001b), considerable correction can take place as the cell plate is built, leading to fusion at the site originally marked by PPBs (Mineyuki and Gunning, 1990). In our live cell experiments, the early orientation of phragmoplasts in morl-1 cells consistently followed the preceding spindle orientation but later stage phragmoplast positioning was highly variable and unpredictable. Even in the 50% or so of cells in which PPBs were documented, there was no clear relationship between PPB orientation and the eventual site of cell plate attachment. This could indicate that late phragmoplast misalignment is so severe that cues left by the PPB are irrelevant. In addition, it remains possible that the relatively normal looking PPBs observed in morl-1 may not always function effectively in marking the cell plate attachment sites.

In conclusion, the MOR1 protein plays an important role in organizing microtubule arrays at all stages of the cell cycle. The three homozygous-viable mutant alleles of MOR1 described so far,
including morl-1, morl-2 and rid5, all have single amino acid substitutions near MOR1's conserved N-terminal TOG domain, and generate conditional phenotypes (Whittington et al., 2001; Konishi and Sugiyama, 2003). In this study, careful analysis of cells in the primary root division zone demonstrates that spindles, phragmoplasts, and PPBs are disorganized when morl-1 seedlings are cultured at restrictive temperature. In the original description of the morl-1 and morl-2 mutant phenotypes, it was noted that the first obvious morphological effect was left-handed twisting of organs (Whittington et al., 2001), which initiates in the elongation zone (Sugimoto et al., 2003). This is followed after 24h by an almost complete loss of growth anisotropy, resulting in severely swollen roots and other cylindrical organs. The gradual onset of this second phase phenotype could reflect the cumulative effects of cell division anomalies, which will impair axialization (Wasteneys and Collings, 2004), a process that relies on auxin transport through well-defined tissue files. The defective microtubule patterns leading to the disruption of cell plate formation may therefore contribute to the loss of anisotropy and dwarfing that is characteristic of the morl and other cytokinesis-defective mutants.

2.4 Materials and Methods

2.4.1 Plant material and growth conditions

The A. thaliana (ecotype Columbia) morl-1 mutant, (GenBank accession no. AF367246; Whittington et al., 2001), was backcrossed 8 times to the parental Columbia ecotype. Control lines were segregated after the eighth backcross and the F5 and F6 generation of both wild-type and morl-1 homozygous segregants were used in this study. Seeds were surface-sterilized (3% (v/v) hydrogen peroxide and 50% (v/v) ethanol, 2 min) and then rinsed 3 times with sterile distilled water and planted on plates containing Hoagland's media (2 mM KNO3, 5 mM Ca(NO3)2, 2 mM MgSO4, 1 mM KH2PO4, 90 μM EDTA, 46 μM H3BO3, 9.2 μM MnCl2,
0.77 μM ZnSO₄, 0.32 μM CuSO₄, 0.11 μM MoO₃), 3% (w/v) sucrose, 530 μM myo-inositol, 50 μM thiamine hydrochloride and 1.2% (w/v) agar (Bacto Agar, Difco Laboratories, Detroit, MI, USA). Plates were sealed with surgical tape (Micropore, 3M, St. Paul MN, USA), stored at 4°C for 2 days, before being transferred to a growth cabinet to grow under constant light (80 μmol m⁻² sec⁻¹) 10° off-vertical at 21°C for 5 days. For temperature shift experiments, 5-day-old seedlings were transferred to a 31°C cabinet with similar light conditions.

2.4.2 Immunofluorescence labelling of root tips

For immunolabelling intact roots, specimens were prepared as described (Collings et al., 2006). To prepare root tip squashes, seedlings were fixed and processed according to Sugimoto et al. (2000) with the following modifications. The fixation buffer was preheated to the temperature at which seedlings were growing. Cell walls were digested for 30 min in an enzyme mixture comprising 0.1% (w/v) pectolyase Y-23 (Kikkoman, Tokyo, Japan), 0.1% (w/v) cellulysin (ICN, Irvine CA, USA), 1% BSA and 0.25 M sorbitol in PME buffer without Triton-X. Seedlings were washed with PMET and the root tips (approximately 1 cm) were attached to microscope slides coated with 0.1% polyethyleneimine. Root tips were gently squashed in a solution of PME buffer containing 0.25 M sorbitol by applying a glass coverslip and applying downward pressure while observing the root tip under a low power dissection microscope. The coverslip was then removed by placing the slide on dry ice, and then prying off the coverslip with a razor blade. Slides with adherent tissues were incubated in PBS (130 mM NaCl, 5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) containing 1% Triton-X100 (1 to 3 h) to permeabilise membranes, and washed in detergent-free PBS (10 min). To reduce autofluorescence and non-specific antibody binding, samples were first incubated in 1 mg/ml NaBH₄ in PBS (2 washes over 30 min), then washed in 50 mM glycine in PBS (30 min). After antibody labelling, DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) diluted in PBS (1 μg/ml) was applied for 10 min. Then samples were washed
with PBS (2 washes over 30 min). Root tips were mounted in Citifluor AF1 antifade agent (Citifluor, London, UK).

2.4.3 Antibodies used and combinations for double labelling experiments

Primary antibodies included mouse anti-α-tubulin (clone B-5-1-2 diluted 1/1000; Sigma-Aldrich); mouse anti-β-tubulin (clone N357 diluted 1/100; Amersham, Amersham, UK); rabbit anti-soy tubulin (1/200; generously provided by Dr. Richard Cyr, Pennsylvania State University, University Park, PA, USA) and mouse anti-β-1,3-glucan (callose) (1/50; Biosupplies, Parkville, Victoria, Australia). Secondary antibody conjugates and the dilutions at which they were used included fluorescein-isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody, FITC-conjugated sheep anti-rabbit IgG and Cy5-conjugated goat anti-mouse IgG (diluted 1/80, 1/50 and 1/200 respectively; Silenus/Chemicon, Boronia, Victoria, Australia).

2.4.4 DAPI staining for chromosome and nuclei in intact roots

The wild-type and morl-1 plants were grown for 5 days at 21 °C followed by 31 °C for 1 day. Plants were fixed as described above and washed with PEMT (3 rinses over 30 min). DNA was stained by incubating whole roots with DAPI (0.1 μg/ml) for 10 min. Roots were washed 3 times for 10 min and mounted in Citifluor. Mitotic indices were calculated as the percentage of mitotic figures in a set number of cell files from 15 different roots. Epidermal, cortical and endodermal files of wild-type and morl-1 were measured separately.

2.4.5 Immunofluorescence microscopy

Fluorescence images were collected with a Leica TCS-SP2 confocal microscope equipped with a UV laser or with a Bio-Rad Radiance 2000 confocal microscope (Carl Zeiss, Jena, Thuringia, Germany) equipped with a MaiTai sapphire laser (Spectra-Physics, Mountain View, CA, USA). The 488-nm line of an Ar laser and the 633-nm line of a HeNe laser were used for FITC and Cy5
excitation respectively with the Leica system, along with a 63x NA1.2 water-immersion lens and eight-fold line averaging. For the Bio-Rad system, the 488-nm line of the Kr laser and the 647-nm line of red diode were used for FITC/GFP excitation and Cy5 and A647 excitation respectively, along with a 60x NA 1.4 oil-immersion lens and Kalman 2 averaging. Images were processed with image processing software, including Leica Confocal Software (Leica) to construct 3D animations, ImageJ (http://rsb.info.nih.gov/ij/) for measurements and creation of movies from time lapse imaging and Adobe Photoshop 7.0 (Adobe, San Jose, CA, USA) to adjust contrast, to switch colours of images collected from green channel to red and from red channel to green for MOR1 and microtubule double labelling analysis and to overlay the coloured images.

2.4.6 Spindle and phragmoplast measurements

Measurements of spindle lengths and cell size were made on roots kept for 24 h at 31°C that were processed using the root squashing immunolabelling method to isolate cells so that metaphase and anaphase figures could be identified. To ensure accurate measurements, any spindles and phragmoplasts oblique to the axis of Z-scan were discarded. For spindle length measurements, roots kept for 24 h at 31°C were processed using the root squashing immunolabelling method to isolate cells in metaphase and anaphase. Measurements of phragmoplasts were recorded from epidermal cells kept for 2 h at 31°C. Analysis of PPB and phragmoplast arrangement was carried out by the root tip squashing method, with plants kept for 24 h at 31°C prior to fixation.

2.4.7 Live cell imaging of GFP-MBD plants

Wild-type and mor1-1 plants expressing GFP-MBD under the control of the cauliflower mosaic virus Pro 35S (original seeds generously provided by Dr. Richard Cyr, Pennsylvania State
University, University Park, PA, USA) were cultured as described above. Four to 5 day-old seedlings were transferred to a coverslip, which formed the bottom of a culture dish (Electron Microscopy Sciences, Hatfield, USA) and was coated with a drop of media described above with modifications of the exclusion of sucrose and agar, and the inclusion of 0.7% type VII agarose (Sigma-Aldrich). Culture dishes were sealed with surgical tape and placed in a growth chamber (21°C). The dishes were positioned 45° off-vertical so that roots would grow under the agarose and along the cover slip. After one day, culture dishes were transferred to 31°C. For live cell imaging of microtubules at 31°C, a heated stage, Bionomic controller BC-100 (20-20 Technology Inc, Wilmington, NC, USA), was used. Images were collected with a Bio-Rad Radiance 2000 confocal microscope as described above for FITC, using Kalman 1 averaging. Z-series of images were collected every 5 min for general measurements and every 3 min for Figure 2 and the supplemental movie files 1 and 2, for up to 3 hours. Image data were collected from individual samples that were at restrictive temperature for more than 2 but less than 8 h at the restrictive temperature. Orienting cells in horizontal positions appeared to generate a temporary reduction in the incidence of cell division between 2 to 4 h in the wild-type roots but interestingly, I did not encounter this problem in morl-1. I suspect that the temporary reduction in the incidence of cell division may be generated because the wild-type root tip’s continued rapid elongation at high temperature generates a more efficient bending response when roots are placed in a horizontal position for viewing on the microscope stage. Contact of the wild-type root tip with the coverslip may generate a touch signal that temporarily reduces cell division. Cell division resumed after 4 to 7 hours, so image data were collected during this period.
Chapter 3: Localization of MOR1 in Plant Cells through the
Cell Cycle

2. A version of this chapter has been published.
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Chapter 3: Localization of MOR1 in Plant Cells Through the Cell Cycle

3.1 INTRODUCTION

MAP215/Dis1 proteins in animal and yeast cells are generally found on microtubules and at microtubule organizing centres. However, the precise distribution patterns and degree of association with microtubules apparently vary from one orthologue to another, and are dependent on phases in the cell cycle.

3.1.1 Localization patterns of Dis1, Alp14 and Stu2 in yeasts

All three yeast orthologues, Dis1 and Alp14 in *S. pombe* and Stu2 in *S. cerevisiae* are important for mitotic spindle assembly. Dis1 is associated with interphase microtubules, spindles and kinetochores and strongly with the spindle pole body (SPB) (Nabeshima et al., 1995; Nakaseko et al., 2001). Alp14 is localized to interphase microtubules in a punctate manner, to the entire length of spindle microtubules and to SPBs (Garcia et al., 2001; Nakaseko et al., 2001). Stu2 is found on interphase, astral and spindle microtubules, and SPBs (Wang and Huffaker, 1997; Kosco et al., 2001). Stu2 is also known to accumulate at plus ends of interphase and astral microtubules, at SPBs and at the kinetochores (Wang and Huffaker, 1997; He et al., 2001, Kosco et al., 2001; Usui et al., 2003).

3.1.2 Localization patterns of DdCP224 in Dictyostelium

DdCP224 is localized to centrosomes strongly and to microtubules weakly throughout the cell cycle. DdCP224 is also found at kinetochores during metaphase and at spindle microtubules, especially at the midbody region during anaphase and telophase. The C-terminal half of DdCP224 is required for targeting to centrosomes, kinetochores and spindle microtubules.
Neither the C-terminal nor the N-terminal half is sufficient for interphase microtubule localization, suggesting that full length is required for interphase microtubule binding (Graf et al., 2000).

3.1.3 Localization patterns of ZYG-9 in *Caenorhabditis elegans*

Immunofluorescence has shown that ZYG-9 is strongly associated with centrosomes during mitosis (Matthews et al., 1998; Srayko et al., 2003) but that the association is diminished to an undetectable level between telophase and interphase (Matthews et al., 1998). ZYG-9 is partially localized to spindle microtubules. There is some labelling at the central region of the mitotic spindle and at the kinetochore/microtubule region of chromosomes, indicating that ZYG-9 is localized to microtubule plus ends (Matthews et al., 1998; Srayko et al., 2003). However, it is not clear if ZYG-9 connects kinetochores and microtubules (Srayko et al., 2003).

3.1.4 Localization patterns of MSPS in *Drosophila*

Immunolocalization has shown that MSPS is concentrated at the centrosomal region throughout the cell cycle except during interphase. During metaphase and anaphase, Msps is found along mitotic spindles. In telophase, the spindle midbody is weakly labelled (Cullen et al., 1999). During female meiosis, in which spindles form independently of centrosomes, MSPS is found at the acentrosomal poles of meiotic spindles (Cullen and Ohkura, 2001). In interphase, the MSPS protein is concentrated in the cell centre, where microtubules are enriched, and also at the microtubule plus ends situated at the periphery of the cell, and also distributed in a punctate pattern along microtubules (Brittle and Ohkura, 2005).

3.1.5 Localization patterns of XMAP215 in *Xenopus*

Like other homologues, XMAP215 associates with centrosomes (Tournebize et al., 2000; Popov et al., 2001). Immunological detection indicates that XMAP215 colocalizes with interphase
microtubules, and that it associates strongly with spindle microtubules and weakly with astral microtubules during mitosis (Tournebize et al., 2000). During *Xenopus* oocyte maturation, XMAP215 is found at the meiotic spindles without obvious accumulation at the spindle poles (Becker et al., 2003).

### 3.1.6 Localization patterns of TOGp in human

Immunofluorescence using TOGp antibody shows that TOGp also has cell cycle-dependent localization patterns. During interphase, TOGp is distributed in the perinuclear cytoplasm, and is partially colocalized with the ER, as confirmed by examining the staining of trans-Golgi (NBD-ceramide) and vesicle-like organelles, the plasma membrane and the ER (DiOC6), along with drug treatments targeting the Golgi apparatus and rough ER. During prophase, TOGp is localized at the centrosome. During prometaphase and metaphase, TOGp is found at centrosomes and spindles and in anaphase, at the spindle poles. During cytokinesis, TOGp accumulates between nuclei and centrosomes where cleavage-furrows form (Charrasse et al., 1998). A recent immunogold transmission electron microscopy study shows that TOGp preferentially localizes to microtubule ends but that it is also distributed along the walls of microtubules (Bonfils et al., 2006).

### 3.1.7 Localization patterns of MOR1 in plants

There have been two reports on MOR1’s localization pattern in plants. In arabidopsis, MOR1 has been found to localize to preprophase bands, and to partially localize to spindles and phragmoplasts in vegetative cells (Twell et al., 2002). More recently, it has been shown that the tobacco homologue of MOR1, MAP200, which was originally purified from tobacco BY-2 cells in telophase (Yasuhara et al., 2002), localizes along and in the vicinity of microtubules through the cell cycle (Hamada et al., 2004).
In chapter 2, I demonstrated that the mor1-1 mutation results in disorganized microtubule arrays and shorter microtubule-based spindles and phragmoplasts. To determine if those changes in microtubules are caused by dissociation of the mutant mor1-1 protein from microtubules, I examined localization patterns using an antibody raised against a synthetic peptide identical to an N-terminal sequence of MOR1 found in both the mutant and wild-type forms.

3.2 RESULTS

3.2.1 MOR1 associates with microtubules throughout the cell cycle.

To produce antibodies, 5 different regions of MOR1, which were predicted by the amino acid sequences to be exposed to the surface of the protein, were synthesized and five rabbits were inoculated. I tested the five sera by immunofluorescence microscopy of arabidopsis root tips to see if they showed specific labelling patterns (Figures 3.1 and 3.2). Only one serum out of the five showed microtubule-like structures. This serum was raised by an amino acid sequence in the N-terminal MOR1 residues 235-249. Another serum (a.a. 1298-1312) showed a chromosomal labelling pattern but did not label microtubules. Considering that the 235-249 serum produced a microtubule labelling pattern with no labelling of chromosomes, the latter is likely to be non-specific. To confirm that the microtubule-like structures recognized by the serum raised against a.a. 235-249 were microtubules, double labelling of MOR1 and tubulin was performed (Figures 3.1A to 3.1D). MOR1 was colocalized with all microtubule arrays throughout the cell cycle. There was also cytoplasmic labelling by the serum. The pre-immune serum did not recognize anything resembling microtubules (Figure 3.1E).
Figure 3.1 Double immunolabelling of anti-tubulin and anti MOR1
Double immunolabelling using anti-tubulin and anti-MOR1 (a.a. 235-249) serum without purification or preimmune serum of the rabbit that produced the anti-MOR1, in wild-type.

(A) to (D) Immunofluorescence using anti-tubulin (green) and anti-MOR1 (red). MOR1 labelled along the length of microtubules and cytoplasm.

(E) Immunofluorescence using tubulin (green) and preimmune serum (red). The pre-immune serum did not recognize any microtubule-like objects at any stage of cell cycle.
**Figure 3.2 Immunofluorescence screening of sera from rabbits inoculated with various MOR1 peptides.**

Left column shows DAPI-staining of DNA (blue). The middle column shows the labelling of each serum at the dilution indicated in the methods. Bars=5μm. None of the sera labelled microtubule-like structures.

(A) Serum from a.a. 373-385 peptide inoculation.
(B) Serum from a.a 807-821 peptide inoculation.
(C) Serum from a.a. 1133-1146 peptide inoculation.
(D) Serum from a.a. 1298-1312 inoculation. The serum did not recognize microtubules but apparently labelled chromosomes, which are also labelled with DAPI (blue).
Western blotting with anti-MOR1_{pep235-249} serum (hereafter anti-MOR1), after affinity purification, identified a high molecular weight protein band of about 220 kDa (Figure 3.3), which is close to MOR1’s predicted molecular weight of 217kD. Double labelling of the anti-MOR1 serum and anti-β-tubulin showed that MOR1 is closely associated with cortical microtubules in interphase cells, and PPBs, spindles and phragmoplasts in dividing cells of the wild-type (Figures 3.4A to 3.4D). This antiserum recognized epitopes along the full length of microtubules in all arrays, and showed no preference for the mid-zone of the phragmoplast, as was reported in a previous study (Twell et al., 2002). The antiserum weakly labelled additional cellular contents distributed in punctate cytoplasmic patterns. MOR1’s colocalization with microtubules was also confirmed in leaf tissues. Both before and after purification, the anti-MOR1 recognized microtubules along their length in wild-type (Figures 3.5A and 3.5C). After purification, the punctate cytoplasmic labelling of anti-MOR1 was reduced in wild-type (Figures 3.1, 3.4 and 3.5).
Figure 3.3 Immunoblotting analysis of wild-type arabidopsis protein extracts with anti-MOR1_{pep235-249} serum

The immunoblotting shows that the serum recognizes a protein band at MOR1's predicted molecular mass. Immunoblotting from a large gradient gel shows that anti-MOR1 purified with a peptide-specific column (lane 1) and anti-MOR1 purified with a protein G-column (lane 3) recognize a high-molecular mass band at approximately 220 kD (arrow) plus several low-molecular mass bands. Secondary antibody control (lane 2) demonstrates that the labelling of one of the low-molecular mass bands (arrowhead) is non-specific.
Figure 3.4 MOR1 is colocalized with microtubules throughout the cell cycle.

MOR1 protein associates along the entire length of microtubules throughout the cell cycle, and this association is not lost after 24h at 31°C despite disruption of microtubule organization in morl-1 root tip cells.

Single optical sections from confocal z-series are used to demonstrate strict co-localization of anti-MOR1 (red) and anti-tubulin (green) in wild-type (A to D) and morl-1 (E to H). Yellow colour in merged images reveals MOR1 and microtubule co-localization. Nuclei and chromosomes were DAPI-stained and appear blue after UV-excitation. Bars = 5 μm (A) and (E) Cortical microtubules. (B) and (F) Preprophase bands. (C) and (G) Spindles. (D) and (H) Phragmoplasts.
Figure 3.5 Double immunofluorescence labelling of MOR1 and microtubules in leaf epidermal cells.

(A) and (B) Anti-MOR1 before purification (red) labelled along the length of microtubules (green). Bars = 10 μm

(C) and (D) Anti-MOR1 after protein G purification (red) also labelled along the length of microtubules (green). The background labelling is reduced compared to anti-MOR1 before the purification. Bars = 10 μm
3.2.2 The association of MOR1 with microtubules is not lost in *mor1-1* at the restrictive temperature.

In *mor1-1* cells at the permissive temperature, MOR1's co-localization with microtubules throughout the cell cycle was identical to that found in wild-type (data not shown). After culturing seedlings at the restrictive temperature prior to fixation, there was no apparent reduction in MOR1's association with microtubules, in either wild-type (Figures 3.4A to 3.4D, 3.5 A and 3.5C) or *mor1-1* (Figures 3.4E to 3.4H, 3.5B and 3.5D), even after 24 hours at the restrictive temperature when considerable disorganization of microtubules was apparent. This result indicates that disorganization of microtubules at *mor1-1*'s restrictive temperature is neither caused by reduction in the amount of MOR1 present nor the dissociation of the mutant form of the protein from microtubules.

3.2.3 anti-MOR1 is likely to recognize the MOR1 peptides

Using immunofluorescence and immunoblotting analysis, I tested the specificity of the anti-MOR1 serum by preabsorbing with the antigen peptide (residues 235-249) to block MOR1-specific binding sites. Preabsorption completely removed all microtubule-specific labelling and also greatly reduced cytoplasmic fluorescence (Figure 3.6), demonstrating that the majority of labelling and the microtubule co-localization in particular is MOR1-specific. Immunoblotting with anti-MOR1s purified with peptides or protein G identified a high molecular mass band close to MOR1's predicted molecular mass of 217 kDa (Figure 3.3). The peptide-purified anti-MOR1 worked well in immunoblotting (Figure 3.3 lane 1) but not immunofluorescence labelling. The protein G-purified anti-MOR1 did not work well in immunoblotting but labelled microtubules well in immunofluorescence assays (Figures 3.4, 3.5 and 3.3 lane 3). For further analysis the protein G-purified anti-MOR1 was therefore used. Our antiserum, however, also
consistently labelled several lower molecular mass bands. To determine whether these bands were proteolytic fragments of MOR1, they were further resolved on 10% SDS polyacrylamide mini gels and blotted with the same antigen peptide-preabsorbed anti-MOR1 serum used for immunofluorescence controls (Figure 3.7A). This eliminated labelling of the ~30 and 60 kDa bands, suggesting that these polypeptides are degradation products of MOR1 (Figure 3.7A). Preabsorption did not, however, eliminate the labelling of 2 other bands that ran at ~50 and 24kDa, suggesting that these polypeptides are not recognized by the MOR1-specific immunoglobulin in the serum. The ~60kDa bands were further separated by longer electroporation and blotted with anti-α-tubulin, anti-β-tubulin and anti-MOR1 to confirm that the ~60kDa band disappeared with peptide preabsorption was neither α-tubulin nor β-tubulin. Neither of these tubulins migrated to the same position as the ~60kDa band that disappeared with preabsorption (Figure 3.7B). This demonstrates that the anti-MOR1 labelling of microtubules is MOR1 peptide-specific. The α-tubulin band was located close to the ~50kDa lower band recognized by anti-MOR1. However, given the fact that the preabsorbed serum produced no microtubule-like labelling pattern in immunofluorescence assays (Figure 3.6), it is unlikely that the ~50 kDa band could be tubulin. Nevertheless this major band was confirmed to not be tubulin by determining that the anti-MOR1 did not label purified tubulin on western blots (Figure 3.7C). Taken together, these analyses indicate that the microtubule labelling by the anti-MOR1 serum is specific to MOR1.
Figure 3.6 Immunofluorescence of wild-type arabidopsis root tips with anti-MOR1 shows that the antibody recognizes microtubules in a MOR1-specific manner.

(A) Overnight preabsorption of the polyclonal anti-MOR1 serum with the antigen peptide completely removed all microtubule-like fluorescent labelling in root-tip cells. Both images were taken from the same cells using different camera exposure times to demonstrate that even at the 1,400-ms exposure time required to detect fluorescence there is no microtubule-specific pattern. Bars = 5 μm.

(B) A control overnight incubation of the anti-MOR1 serum without the antigenic peptide demonstrates that the labelling of microtubules is not lost by this treatment. An exposure time of 250 ms was sufficient to detect microtubule labelling. Bar = 5 μm.
Figure 3.7 Immunoblotting analysis of lower molecular mass bands with anti-MOR1\textsubscript{pep235-249} serum shows that the serum recognizes MOR1.

(A) Immunoblotting the lower molecular mass bands, which were recognized with anti-MOR1\textsubscript{pep235-249}, resolved on a 10% mini gel demonstrates that two of these bands are likely to represent proteolytic degradation products of MOR1. Lane 1 shows four prominent bands. The secondary antibody control in this case included a blocking reagent that eliminated nonspecific binding (lane 2). Lanes 3 and 4 show the results of preabsorption with the antigen peptide. After overnight incubation of anti-MOR1 with no added peptide (lane 3), all four bands are still recognized. Anti-MOR1 preabsorbed overnight with the antigen peptide (lane 4), however, did not label the bands of approximately 60 and approximately 30 kD (arrows). Two bands at approximately 50 and 20 kD are still present, but, as indicated in Figure 3.6, these polypeptides are not microtubule-localized.

(B) To confirm the ~60kDa band that disappeared with peptide preabsorption was neither α-tubulin nor β-tubulin, the bands were further separated by longer electrophoresis and blotted with anti-α-tubulin (lane 1), anti-β-tubulin (lane 2) and anti-MOR1 (lane 3). Both tubulins did not migrate similar to MOR1’s ~60kDa that disappeared with preabsorption. The α-tubulin, however, migrated to a similar but not identical position as the lower band recognized by the anti-MOR1.

(C) To confirm that the lower ~50kDa band was not α-tubulin, immunoblotting of purified tubulin with anti-MOR1 was performed. Lanes 1 and 5 were loaded with arabidopsis extract, while lanes 2 to 4 (5μg, 2.5 μg and 1.25 μg respectively) and lanes 6 to 8 (5μg, 2.5 μg and 1.25 μg respectively) were loaded with purified tubulin. Anti-α-tubulin and anti-MOR1 were applied to lanes 1 to 4 and lanes 5 to 8 respectively. The anti-MOR1 did not recognize the purified tubulin or the tubulin found in the arabidopsis extract. Note that the higher molecular mass band is lost after preabsorption with the 235-249 peptide (B; lane 4).
3.3 DISCUSSION

Using an immunological approach, I demonstrated in this study that the MOR1 protein is situated along the entire length of microtubules at all stages of the cell cycle. I also showed that the protein encoded by the mor1-1 mutant allele remains associated with microtubules despite being unable to maintain microtubule organization at the restrictive temperature. The specificity of the anti-MOR1 was confirmed by immunoblotting of arabidopsis protein extract and purified tubulin, and by immunoblotting and immunolabelling using anti-MOR1 preabsorbed with antigen peptides. This indicates that the mor1-1 mutant’s leucine to phenylalanine substitution at residue 174 in an N-terminal HEAT repeat does not prohibit the mutant protein, mor1-1, from binding to microtubules under restrictive conditions.

3.3.1 MOR1 distributes along the entire length of microtubules throughout the cell cycle.

The demonstration by immunolabelling that MOR1 associates along the entire length of microtubules is important in light of the wide variety of distribution patterns reported for non-plant MOR1 homologues. Depending on the method of labelling, cell type or stage of the cell cycle, MAP215/Dis1 proteins have been observed at centrosomes and spindle pole bodies, distributed along the lengths of microtubules, or concentrated at microtubule plus ends (Gard et al., 2004). My results suggest that MOR1 is less selective in its distribution along microtubules, a finding that may reflect the more dispersed nature of plant cortical microtubules, and their tendency to initiate new assembly at points along existing microtubules rather than at fixed organizing centres (Wasteneys and Williamson, 1989b; Wasteneys et al., 1993; Wasteneys, 2002; Murata et al., 2005).

Two previous studies have used immunolabelling approaches to investigate MOR1 and its tobacco homologue’s association with microtubules in dividing cells but in comparison to my
results, their labelling patterns along microtubules were less clear. Twell et al. (2002) used an antibody generated against the C-terminal region of AtMOR1 expressed in *Escherichia coli*, and described MOR1 distribution in spindles and phragmoplasts of isolated culture cells as being concentrated to the midline where “oppositely orientated microtubules overlap”. Recent electron tomographic studies on cell plate formation, however, have revealed very little overlap of oppositely oriented phragmoplast microtubules (Segui-Simarro et al., 2004), except at the very early stages of phragmoplast formation (Austin et al., 2005). The interpretation that labelling in the mid-zone is related to a preference for or an involvement in microtubule overlap may therefore be incorrect. The antibody used in our study, which was raised against a synthetic peptide from the N-terminal region of MOR1, showed no preferential binding to microtubule ends. I only occasionally detected fluorescence in the cell plate, whose identity was confirmed by labelling with anti-callose. Hamada et al. (2004) used an antibody raised against purified MAP200, a tobacco homologue of MOR1, in tobacco BY-2 cells. Like our antiserum, this monoclonal antibody labelled all microtubule arrays in dividing tobacco BY-2 cells, with no preference for the phragmoplast mid-zone. Precise localization, however, was not clear, and there was considerable label in the cytoplasm surrounding microtubules. Accessibility of the targeted epitope could explain the strikingly different labelling patterns produced by the two MOR1- and one MAP200-derived antibodies discussed here, and, given the large size of these proteins, such variation is not unexpected. Perhaps as a result of our peptide design strategy, the N-terminal epitope of MOR1 recognized by the antiserum may be relatively exposed for binding. In contrast, the C-terminal epitope recognized by the Twell et al. (2002) antiserum may be less accessible, and it is tempting to speculate that it may be more exposed when MOR1 is associated with the plus ends of microtubules. This explanation is supported by the recalcitrance of the antibodies used in the previous studies to recognize interphase cortical microtubules, and the apparent requirement for use of protoplasts (Twell et al., 2002) or membrane ghosts (Hamada et
al., 2004) to generate partial antibody decoration of cortical arrays.

3.3.2 The N-terminal region of MOR1 may have microtubule stabilizing properties.

My results demonstrate that MOR1 is an integral part of functioning microtubules. I have shown that morl-1’s mutation in the N-terminal HEAT repeat does not abolish the protein’s ability to bind microtubules. Although Popov et al., (2001) demonstrated that the N-terminal region of XMAP215 can bind pure microtubules, there is as yet no clear evidence that the N-terminal region of MAP215/Dis1/MOR1 homologues is essential for microtubule binding. Consistent with the observation that a C-terminal fragment of MOR1 can bind to microtubules in vitro (Twell et al., 2002), experiments with the XMAP215 homologue suggest that its C-terminal region is critical for microtubule binding, while the N-terminal region has stabilizing properties (Popov et al., 2001). How then does the morl-1 mutation generate the disrupted microtubule phenotype? MOR1’s apparent stabilizing function could be mediated through its ability to protect microtubules from destabilizing or catastrophe-causing factors such as kin-1 kinesins, and this function would necessitate remaining in close contact with microtubules (Hussey and Hawkins, 2001; Wasteneys, 2002). To understand MOR1’s functions on microtubule organization, I measured microtubule dynamics in the morl-1 mutant, which is described in Chapter 4.

3.4 MATERIALS AND METHODS

3.4.1 Immunolabelling of microtubules and MOR1 in root squash and leaf

Root squashes were prepared as described in chapter 2 and (Kawamura et al., 2006). For immunolabelling leaf tissues, plants were grown for 11 days at 21°C followed by 31°C for 1 day. First leaves were excised from seedlings directly into fixative as for roots. Leaves were processed for immunofluorescence by freeze shattering in liquid nitrogen as described
3.4.2 Peptide-specific antibody production

Production of the MOR1-specific antibody was carried out in collaboration with Kevin Gale (CSIRO Plant Industry). The MOR1 amino acid sequence was first scanned for high surface probability regions using 'Peptidestructure' on WebANGIS (www1.angis.org.au). A BlastN analysis was used to check that peptides designed were specific for MOR1. On this basis, 5 peptides representing different regions of the MOR1 protein sequence were synthesized and purified by HPLC (Biomolecular Resources Facility, Australian National University). The 5 peptide sequences from MOR1 selected were a.a. 235-249, a.a. 373-385, a.a. 807-821, a.a. 1133-1146 and a.a. 1298-1312. Peptides were synthesised with an additional GC dipeptide at the C-terminus and coupled to keyhole limpet haemocyanin (KLH; Sigma-Aldrich, St Louis MO, USA) using the heterobifunctional cross-linker m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Pierce Chemical Co., Rockford, IL, USA). Peptide (5 mg) was dissolved in 10 mM phosphate buffer pH 7 and mixed with MBS-activated KLH (5 mg) for 3 h at room temperature. Coupled peptides were dialysed exhaustively against phosphate buffered saline (PBS, pH 7) and stored at 4°C with 0.1 % sodium azide as preservative. New Zealand white rabbits were immunised using 1 mg coupled peptide (in 0.5 ml PBS plus an equal volume of Freund's Complete Adjuvant). Booster immunisations (0.5 mg coupled protein in 0.5 ml PBS plus 0.5 ml Incomplete Freund's Adjuvant) were administered at monthly intervals. Blood was collected from the ear vein for serological testing 7 days after the second booster immunisation.

From these inoculations, I tested all sera and determined that only one serum, raised against the TRKIRSEQDKEPEAE peptide sequence found in the N-terminal region (amino acids 235-249) of MOR1, produced a promising labelling pattern. This serum, designated as anti-MOR1_{pep235-249}, was affinity purified using HiTrap Protein G HP (Amersham Biosciences, Uppsala, Sweden).
3.4.3 Antibodies used and combinations for double labelling experiments

For double labelling of microtubules and MOR1 protein, mouse anti-β-tubulin (clone N357 diluted 1/100; Amersham, Amersham, UK) and rabbit anti-MOR1 (1/30) were applied together, followed by the Cy5- goat anti-mouse IgG (1/200) and FITC- sheep anti-rabbit IgG (1/50) secondary antibody respectively.

3.4.4 Immunofluorescence of anti-MOR1 preabsorbed with the MOR1 peptide 235-249

Root tip squashes were prepared from six day old seedlings as described above. Anti-MOR1 (1/30) was incubated overnight with the peptide235-249 (anti-MOR1:peptide, 1μl:1.2mg) or without the peptide as a control, before applying to root tips. As a secondary antibody, goat anti-rabbit A488 (1/200) was used (Molecular Probes, Eugene, OR, USA).

3.4.5 Immunoblotting

Seedlings were ground in liquid nitrogen and boiled for 3 min in sample buffer (final concentrations, 125mM Tris, 0.8mM EDTA, 20mM DTT, 10% glycerol, 4% SDS, 0.001% bromophenol blue, pH6.8). Extract was centrifuged at 22,000g for 5 min and the supernatant was applied to a polyacrylamide gel for separation by electrophoresis. A 4%-20% gradient gel was used to detect the full range of proteins including the high molecular mass bands and a 10% gel was used to better resolve low molecular mass bands recognized by components of the serum. Proteins were blotted onto PVDF membrane using a 12.5mM Tris, 96mM glycine and 20% MeOH transfer buffer. Anti-MOR1 was diluted to 1/100 and 1/5000, and was applied to blots from 4%-20% gradient gels and 10% gels respectively. For the anti-MOR1 peptide preabsorption assays, anti-MOR1 serum was incubated overnight with the original peptide antigen, peptide235-249 (anti-MOR1:peptide, 1μl:1.2mg) or without the peptide as a control, before applying to blots or use in immunofluorescence control experiments. Horseraddish
peroxidase conjugated-anti-rabbit IgG (Amersham BioSciences, Buckinghamshire, UK) was used as a secondary antibody. For tubulin blots, tubulin (cytoskeleton Inc., Denver, CO, USA) was dissolved in 1x sample buffer (0.1µg/µl) and boiled immediately. Anti-α-tubulin (B512, Sigma) was used to detect α-tubulin and Horseraddish peroxidase-conjugated-anti-mouse IgG (Amersham BioSciences) was used as a secondary antibody. Blots from 4%-20% gradient gels and 10% gels were developed using ECL plus and advance (Amersham BioSciences, Buckinghamshire, UK) respectively according to the manufacturer’s instructions.
Chapter 4: Quantitative Analysis of Microtubule Dynamics
in the morl-1 Mutant

Kawamura E, Wasteneys, GO. The MAP215/Dis1 family of MICROTUBULE ORGANIZATION 1 in Arabidopsis thaliana has functions to keep microtubules highly dynamic.
Chapter 4: Quantitative Analysis of Microtubule Dynamics in the *mor1-1* Mutant

4.1 INTRODUCTION

4.1.1 Microtubules are highly dynamic

Microtubules are highly dynamic structures, undergoing transitions between states of growth, shrinkage and pause. Microtubules are formed by 13 linear protofilaments in a hollow cylinder structure. Each protofilament is composed of repetitive α- and β-tubulin heterodimers. The polymerization of tubulin dimers at microtubule ends that results in an increase in microtubule length is called growth. The end where αβ-tubulin dimers are exchanged more rapidly is called the 'plus-end', and the other end which is less dynamic is called the 'minus-end'. The tubulin dimers situated at the growing ends of microtubules are mostly GTP-bound, so this region is called the GTP cap. The GTP cap stabilizes microtubule ends and promotes microtubule polymerization. As tubulin dimers are added to a growing end, the GTP bound to β-tubulin gets hydrolyzed to GDP. Microtubules composed of GDP-bound tubulins are less stable. The hydrolysis of GTP in β-tubulin causes conformational changes and weakens the bonds between tubulin dimers. Loss of the GTP cap causes microtubules to depolymerize. EB1 (EB1) is a microtubule associated protein that is mainly found at the growing microtubule ends (Bisgrove et al., 2004). It is suggested that EB1 associates with special structures or chemicals at the growing microtubule end (Tirnauer et al., 2002). Ultrastructural studies have suggested that stably growing microtubule ends have a sheet-like structure with protofilament extension. Depolymerization of tubulin dimers from microtubule ends results in shortening of microtubules, which is referred to as shrinkage. Shrinking microtubules are believed to have a ram’s-horn-like structure with curled microtubule protofilaments peeling off microtubule, based on ultrastructure
studies. Pause is a phase where polymerization and depolymerization of tubulin dimers at one end happen at a similar rate and there is no net microtubule growth or shrinkage. The structure of a pausing microtubule end is indicated to be blunt. The transition from growth to shrinkage is called catastrophe and from shrinkage to growth is called rescue. When addition of tubulin dimers at one end and removal of tubulin dimers at the other end take place at a similar rate, microtubules show a treadmilling behaviour (Shaw et al., 2003). Those microtubule dynamics are regulated by various microtubule interacting proteins and concentrations of free tubulin dimers in the environment.

4.1.2 Cortical microtubule organization in plants

Unlike microtubules in animal cells, which generally emerge from fixed points called centrosomes to form radial arrays, microtubules in non-flagellated algal and plant cells either nucleate at the nuclear surface or at the cell cortex (Wasteneys and Williamson, 1989b; Schmit, 2002; Kumagai et al., 2003; Shaw et al., 2003; Yoneda and Hasezawa, 2003), and also along the surface of existing microtubules, forming an acute (approx. 40°) branching angle (Wasteneys and Williamson, 1989b; Wasteneys et al., 1993; Murata et al., 2005). Although there are numerous microtubule nucleation sites in plant cells, cortical microtubules in rapidly elongating cells are somehow organized into parallel arrays that are perpendicular to growth axis of the cells. Studies in living cells support the model that cortical microtubules are organized into parallel arrays by selective stabilization of dynamic microtubules (Wasteneys and Williamson, 1989a; Dixit and Cyr, 2004; Dixit et al., 2006). Microtubules physically encountering other microtubules at steep angles tend to depolymerise. When the angle is shallow, however, microtubules are more likely to grow along the existing microtubule; promoting microtubule coalignment and bundling (Dixit and Cyr, 2004). Bundling of microtubules is promoted by the activity of cross-bridging MAPs of the MAP65 family (Smertenko et al., 2004). In a recent study,
parallel arrays of microtubules have been shown to be promoted through biased growth polarity. Soon after cytokinesis in BY-2 cells, microtubules had multiple directionality with 30-40% coalignment. As the cells matured, up to 80% of microtubules had uniform polarity. Even when microtubules are coaligned, microtubules with the non-predominant microtubule growth polarity tended to become depolymerized (Dixit et al., 2006). These data suggest that cortical microtubules self-organize through dynamic intermicrotubule interactions.

### 4.1.3 Role of MAP215/Dis1 in microtubule dynamics

**in vitro analysis**

MAP215/Dis1 proteins generally stabilize and promote polymerization of microtubules with the exceptions that Stu2 in yeast can destabilize microtubules and XMAP215 can destabilize pausing microtubule ends, *in vitro* (Shirasu-Hiza et al., 2003; van Breugel et al., 2003; Gard et al., 2004). *In vitro* analysis showed that XMAP215 promoted both growth and shrinkage of microtubules (Vasquez et al., 1994; Tournebize et al., 2000). Studies on the tobacco homologue of MOR1, MAP200, demonstrate that it can increase the number and length of microtubules *in vitro* (Hamada et al., 2004). A recent *in vitro* study on the molecular dynamics of microtubules showed that MAP215 speeds up both growth and shrinkage rate in a step-like manner in 40-60nm increments (Kerssemakers et al., 2006). These data suggest that MAP215/Dis1 proteins generally have a role in keeping microtubules more dynamic.

**in vivo analysis**

Analysis on microtubule dynamics using MSPS-depleted cells in Drosophila demonstrated that MSPS can suppress pausing of interphase microtubules and promote rapid shrinkage but does not control microtubule growth rate (Brittle and Ohkura, 2005). Immunofluorescence microscopy shows that depletion of MSPS also promotes microtubule bundling. Depletion of
Stu2 induces less dynamic cytoplasmic microtubules, with suppression of rescue and catastrophe and increased pausing time (Kosco et al., 2001).

Studies on the morl-1 temperature-sensitive mutant of arabidopsis demonstrate that MOR1 is important for organizing cortical microtubules (Whittington et al., 2001) and keeping microtubule structures like mitotic spindles and phragmoplasts long (Chapter 2; Kawamura et al., 2006). The analysis of microtubule organization described in chapter 2, however, mainly utilized immunofluorescence of fixed material, and I was therefore unable to determine just how the dynamic properties of microtubules are altered at restrictive temperature (Kawamura et al., 2006). This chapter therefore focuses on comparing the dynamic properties of microtubules in living cells of morl mutants and wild-type. GFP fusion proteins that label microtubules were used to measure microtubule dynamics in leaf epidermal cells. Although live cell imaging can provide unprecedented information, it is also known that the introduction of GFP fusion proteins can cause abnormal development and give misleading results (Wasteneys and Yang, 2004; Abe and Hashimoto, 2005). For instance, the expression of α-tubulin with a GFP reporter linked at its N-terminus causes right handed organ twisting (Ueda et al., 1999). Recently, this twisting phenotype has been attributed to interference by the GFP tag at the N-terminal region of α-tubulin with GTP hydrolysis, causing microtubules to become more stable, with extended GTP caps (Abe and Hashimoto, 2005). To get a more balanced idea of how the morl-1 mutation alters microtubule dynamics, in this study, I used several different GFP fusion reporter proteins, including GFP-TUA, CMV35S::GFP-EB1 and ProEB1::EB1-GFP.
4.2 RESULTS

4.2.1 Microtubules labelled with GFP-TUA grew and shrunk more slowly in the \textit{morl-1} mutant

Microtubule dynamics in the first leaf were measured by tracking the changes in length of microtubules, which were fluorescently labeled by the stable expression (under the CMV 35S promoter) of GFP-\(\alpha\)-tubulin6 (GFP-TUA), a reporter protein that incorporates into and labels the entire length of microtubules (Fig. 4.1). Confocal images were recorded every 8 seconds for the GFP-TUA reporter and every 7.2 seconds for the EB1 reporter proteins. A temperature-controlled stage kept the specimens at restrictive temperature throughout imaging. Since microtubules were highly dynamic and capturing one frame took several seconds, only one optical section was collected with a wider pinhole to allow greater depth of focus. From a set of time lapse images, the position of each microtubule end was manually plotted for all time frames using the manual tracking plugin of ImageJ, and parameters for microtubule dynamics were calculated. In the \textit{morl-1} mutant at the restrictive temperature, microtubule growth and shrinkage rates were significantly reduced (\(p<0.001\)) compared to the wild-type (Fig. 4.2). This indicates that MGR1 normally promotes both rapid growth and shrinkage of microtubules. In the \textit{morl-1} mutant at the restrictive temperature, the GFP-TUA cytoplasmic fluorescence was greatly increased in comparison to wild-type (Fig. 4.1), consistent with a previous report for hypocotyl cells (Whittington et al., 2001). This indicates that significant amount of GFP-TUA was unpolymerized. However, microtubules visible with the expression of GFP-TUA looked longer in \textit{morl-1} when compared to microtubules visualized after chemical fixation and immunofluorescence imaging (Figure 3.5 and Figure 4.1). This suggested that either the chemical fixation reduces, or that the GFP-TUA reporter protein increases the length of microtubules.
Figure 4.1 Time lapse images of microtubules labelled with GFP-TUA at the restrictive temperature.

Microtubule ends were marked with coloured dots. Images were collected with Bio-Rad Radiance confocal microscope equipped with a temperature-controlled stage to keep specimens at the restrictive temperature. First leaves from 11-12 day-old plants were used. Images were collected every 8 sec. Bars=5µm.

(A) wild-type. Microtubules grew and shrank faster than in morl-1.

(B) morl-1. Microtubules were less dynamic and appeared “indecisive”. Long periods of growth and shrinkage, as seen in wild-type, were not observed. Background fluorescence is increased in morl-1, indicating less GFP-tagged tubulin was in the polymer state.
Figure 4.2 Growth rate and shrinkage rate are reduced in *morl-1* at the restrictive temperature.

From the time lapse images of microtubules expressing GFP-TUA in both wild-type and *morl-1* at the restrictive temperature, growth rates and shrinkage rates were calculated. Epidermal cells on the abaxial surface of first leaves from 11-12 days old seedlings were used. Growth rates and shrinkage rates were significantly decreased in *morl-1* (both p<0.001). Bars are SD. Data was collected from 41 microtubules from 3 cells from 3 different plants for wild-type and 61 microtubules from 4 cells from 2 different plants for *morl-1*. 
It has been shown that the expression of the GFP-TUA itself alters microtubule dynamics and can affect morphology and development (Abe and Hashimoto, 2005). It is therefore probable that this particular reporter protein altered the microtubule dynamics. Rather than relying on only one reporter protein, I compared microtubule dynamics using a range of fluorescent reporter proteins. These included a high expression line, \textit{CMV35S::GFP-EB1}, in which the GFP-tagged EB1 showed strong labelling of the growing microtubule ends but also had a weak association along the length of microtubules, and a low expression line driven by the endogenous promoter, \textit{Pro\(_{EB1}\)::EB1-GFP}, which labeled strictly the growing ends of microtubule. In both lines, microtubule growth rates in the \textit{morl-1} mutant were reduced compared to the wild-type (Fig. 4.3), confirming the results obtained with GFP-TUA. Unfortunately, in \textit{morl-1} mutants at restrictive temperature even the highly expressed GFP-EB1 labelling along microtubules as well as labelling of comets was reduced and therefore I was not able to compare shrinkage rate using these lines.
Figure 4.3. Time lapse images of Pro<sub>EB1</sub>::EB1-GFP in leaves of wild-type and morl-1 at 31°C.

Images were collected with Zeiss Meta510 confocal microscope with a temperature-controlled stage to keep specimens at the restrictive temperature. First leaves from 12 day-old plants were used for these figures. Images were collected every 7.2 sec. Bars are 5μm.

(A) Transgenic Pro<sub>EB1</sub>::EB1-GFP line. Comets were distinct, lasted for a longer period of time and traveled faster than in morl-1. Arrowheads follow same comet between time frames.

(B) morl-1 expressing Pro<sub>EB1</sub>::EB1-GFP. Even with increased laser power, comets were hard to detect and are less frequent than in wild-type. Comets often disappeared within few time frames (white and red arrowheads) or were detected in only one time point (blue arrowhead). Comets traveled more slowly than in wild-type.
4.2.2 Microtubule growth is suppressed in the *morl-1* mutant expressing EB1 reporter proteins.

GFP-EB1 strongly associates with the growing plus ends of microtubules so that its fluorescence forms a comet-like shape when viewed in time-lapse confocal microscopy. In *morl-1*, GFP-EB1 comets were smaller and the frequency of GFP-EB1 (86.6 comets/400µm²) was less (p<0.001) than in the wild-type control line (127.8 comets/400µm²) expressing the same construct (Fig. 4.4). In the low expressing *ProEB1::EB1-GFP* line, there was an even more severe reduction in the frequency of comets in *morl-1* (Figure 4.3). Despite the differences in the level of expression and/or the placement of the GFP at the N-terminal (high expression) and C-terminal (low expression) of EB1 in these two reporter protein lines, the mutant morl-1 protein had the same general effect on microtubule growth. These results also support the idea that MOR1 has a role in stabilizing growing microtubule ends.
Figure 4.4 Expression of CMV35S::GFP-EB1 in wild-type and *morl-1* at the restrictive temperature.

Samples were imaged with a Bio-Rad radiance confocal microscope equipped with a temperature control stage. Abaxial sides of first leaves of 11 days old seedling were used.

(A) wild-type. GFP-EB1 comets were longer and GFP-EB1 were also found along microtubules. Bar is 5μm.

(B) *morl-1*. GFP-EB1 comets were much smaller and the intensity of fluorescence was reduced compared with wild-type. The frequency of comets appeared to be reduced. Bar is 5μm.

(C) Frequency of GFP-EB1 comets. The number of GFP-EB1 comets per 400μm² was counted. The frequency of comets was significantly reduced in *morl-1* (p<0.001). Bars are SD. Data were collected from 22 cells from 6 leaves from 6 different plants for wild-type and from 12 cells from 7 leaves from 7 different plants for *morl-1*.
4.2.3 Microtubules labelled with GFP-TUA spent more time in pause in *morl-1*.

One of the overall impressions of microtubules labelled with GFP-TUA in *morl-1* was that microtubules were static compared to wild-type (Fig. 4.1). To verify this observation, the percentage of overall time spent in each phase, which included growth, shrinkage and pause, was calculated. The sum of time every microtubule end spent in each phase was divided by the total time for all GFP-TUA-labelled microtubule ends recorded. Microtubules in *morl-1* spent 35.4% of their time in pause compared to 25.1% for wild-type (Fig. 4.5). Time spent for growth was reduced in *morl-1* (37.5%) compared with wild-type (45.1%). These data match the observation that microtubules appeared static. Time spent in shrinkage was only slightly affected (*morl-1*: 27.2% and wild-type: 29.9%).
Figure 4.5 Time spent in growth, shrinkage and pause at the restrictive temperature

Based on time lapse imaging of microtubules expressing GFP-TUA in both wild-type and *morl-l* at the restrictive temperature, time spent in growth, shrinkage and pause were calculated. The epidermal cells from the abaxial surface of first leaves from 11-12 day-old seedlings were used. The sum of time every microtubule end spent in each phase was divided by the total time all microtubule ends were measured for. Microtubules spent more time in the paused state in *morl-l* and spent less time in growth. Data were collected from 41 microtubules from 3 cells from 3 different plants for wild-type and 61 microtubules from 4 cells from 2 different plants for *morl-l*.
4.2.4 Frequencies of catastrophe and rescue were increased in the *morl-1* mutant expressing GFP-TUA

Another general characteristic of microtubule dynamics in *morl-1* was that microtubules appeared “indecisive”. This means that the *morl-1* microtubules grew for a few time frames, then shrunk or paused for a few time frames, then grew again a little bit and repeated this behaviour. Transition from growth to shrinkage (catastrophe), and transition from shrinkage to growth (rescue) are also important parameters in measuring microtubule dynamics. Catastrophe frequency and rescue frequency were calculated by dividing the total events of catastrophe or rescue by the total time spent for growth or shrinkage respectively (Walker et al., 1988; Howell et al., 1999). As expected, the frequency of catastrophe was increased in *morl-1* (Figure 4.6). However, the frequency of rescue was also increased (Figure 4.6). These data confirm the observation that microtubule appeared “indecisive” but are rather surprising given that microtubule growth rate is also suppressed in *morl-1*, as demonstrated by the GFP-TUA measurements (Figures 4.2 and 4.5).
Figure 4.6 Frequencies of rescue and catastrophe were increased in \textit{morl-1} at the restrictive temperature.

Based on time lapse imaging of microtubules expressing GFP-TUA in both wild-type and \textit{morl-1} at the restrictive temperature, frequency of rescue and catastrophe were calculated. Epidermal cells from the abaxial surfaces of the first leaves from 11-12 days old seedlings were used. The total number of rescue events was divided by the total time spent for growth to calculate the frequency of rescue. Similarly, the total number of catastrophe events was divided by the total time spent for shrinkage to obtain the frequency of catastrophe. The frequencies of rescue and catastrophe were increased in \textit{morl-1}. Data was collected from 41 microtubules from 3 cells from 3 different plants for wild-type and 61 microtubules from 4 cells from 2 different plants for \textit{morl-1}.
4.3 DISCUSSION

4.3.1 MOR1 promotes and maintains microtubule dynamic behaviour.

The data obtained from the microtubule dynamics analysis in the mor1-1 mutant suggest that the normal function of MOR1 is to promote constant and rapid growth and shrinkage and to prevent pausing of microtubules. In the mor1-1 mutant, microtubule growth and shrinkage rates were both reduced, the frequency and size of EB1 comets were reduced, and the time spent in pause was increased.

These results are in many respects consistent with functional analyses of MOR1 homologues in a range of eukaryotic organisms. In an experiment in which the Drosophila MOR1 homologue MSPS protein was depleted using RNAi, microtubules paused significantly longer and shrank more slowly, yet growth rate was not changed (Brittle and Ohkura, 2005). The difference in growth rate changes between MOR1 and MSPS could mean MOR1 and MSPS have slightly different roles in controlling microtubule dynamics. Indeed, localization patterns of these two proteins appear to be different. MOR1 is localized along the length of interphase microtubules (chapter 3; Kawamura et al., 2006), whereas MSPS is mainly concentrated at the plus ends of interphase microtubules, and only distributed along interphase microtubules in a punctate manner (Brittle and Ohkura, 2005). It is also possible that the estimated 30% of the normal level of MSPS protein still present in RNAi-treated cells at the time of observation (Brittle and Ohkura, 2005) was enough to maintain growth rate but not shrinkage rate.

The Xenopus homologue of MOR1, XMAP215, promotes both microtubule growth and shrinkage. Both purified XMAP215 protein, and XMAP215 supplemented with interphase egg extract promotes in vitro microtubule growth and shrinkage rates, resulting in long and dynamic microtubules (Vasquez et al., 1994; Tournebize et al., 2000). Biochemical studies suggest that
XMAP215 acts antagonistically with XKCM1 (Tournebize et al., 2000), a destabilizing factor and a member of the kinesin superfamily (Walczak et al., 1996). It is later shown that XMAP215’s antagonistic activity on XKCM1 is found in the N-terminal region that includes the two N-terminal-most TOG domains (Popov et al., 2001). A possible molecular mechanism for XMAP215’s regulation of microtubule assembly is recently presented in a study that utilized optical tweezers to measure the velocity of microtubule plus end growth and shrinkage at extremely high temporal resolution. In this study, adding recombinant XMAP215 to tubulin solutions sped up growth and shrinkage rates of microtubules growing from isolated axonemes in 40-60 nm increments, which is equivalent to 4-7.5 tubulin dimers (Kerssemakers et al., 2006). This suggested that XMAP215 either carries tubulin oligomers to microtubule plus ends or binds to microtubule plus ends, and then accelerates polymerization along the length of XMAP215 to allow rapid growth. The data also suggest that XMAP215 removes several tubulin oligomers at one time promoting rapid shrinkage (Kerssemakers et al., 2006). If the growing microtubule end has a ‘sheet-like’ structure with protofilament extensions as has been suggested by TEM, the measured step-like increments must have been a result of one XMAP215 adding tubulins along its length at the very tip of the sheet like structure. If a growing microtubule end does not always form a sheet like structure, how could a microtubule composed of 13 protofilaments with multiple XMAP215 interactions show these increments? When shrinking, microtubules are believed to show a ‘ram’s-horn-like’ structure with curled microtubule protofilaments peeling off microtubule like a banana skin. Unless the XMAP215 proteins are aligned precisely with their ends positioned close to each other around microtubule periphery, it is hard to imagine step-like shrinkage of microtubules. XMAP215’s antipausing activity is also supported by experiments in which full-length and N-terminal fragments of XMAP215 were both able to destabilize microtubules that had first been stabilized by treatment with the slowly hydrolysable GTP analogue, GMPCPP, a (Shirasu-Hiza et al., 2003). Although the structure and nucleotide
hydrolysis state, either GTP or GDP, of pausing microtubule end is not known, electron micrographs of GMPCPP-stabilized microtubules showed blunt ends, typical of microtubule ends proposed to be pausing (Shirasu-Hiza et al., 2003). My study of microtubule dynamics in the mor1-1 mutant supports the idea that, like XMAP215, MOR1 acts as an antipause factor that promotes rapid microtubule growth and shrinkage in order to keep microtubules highly dynamic.

4.3.2 Growth is suppressed in mor1-1 and microtubules possibly form a smaller GTP-cap

The size of EB1 comets, as measured by GFP-tagged EB1, was significantly reduced in mor1-1 at restrictive temperature. This indicates that the GTP cap at the growing microtubule end is smaller than normal. EB1 is classified as a microtubule plus end-tracker (+TIP), and is thought to associate with structures or chemicals found specifically at the growing microtubule ends (Tirnauer et al., 2002). Delayed GTP hydrolysis, which will generate longer GTP-caps, is expected to increase the size of GFP-EB1 comets. Experimental support for this correlation has been shown by mutating the putative GTPase-activating and nucleotide binding domains of α-tubulin, or by epitope tagging the N-terminus of α-tubulin, which is thought to be critical for activating hydrolysis of the β-tubulin-bound GTP. In either case, GFP-EB1 labelling was more extensive (Abe and Hashimoto, 2005). There is some evidence for interaction between EB1 and the MOR1 homologues DdCP224 from Dictyostelium discoideum and STU2 from Saccharomyces cerevisiae (Chen et al., 1998; Rehberg and Graf, 2002; Bisgrove et al., 2004). However, whether EB1 and MOR1 interact directly or indirectly in plants still needs to be explored.

4.3.3 The frequencies of catastrophe and rescue were increased in mor1-1

In mor1-1, microtubules underwent catastrophic disassembly events more often compared to wild-type microtubules under identical conditions. This might have been caused by the smaller
GTP-cap, as discussed above, that simply weakens a microtubule growing end, making it more prone to catastrophe. The increased catastrophe occurrence is consistent with previous reports that the morl-1 microtubules are short (Whittington et al., 2001) and that XMAP215 suppresses catastrophe through antagonistic activity toward the destabilizing XKCM1 (Tournebize et al., 2000; Popov et al., 2001). In arabidopsis, there are two kinesins, kinesin-13A and -13B, that belong to the kinesin-13 subfamily including KINI XKCM1 (Reddy and Day, 2001; Lee and Liu, 2004; Lu et al., 2004). However, it has been suggested that KINESIN-13A has a role in microtubule-dependent Golgi distribution, and may therefore lack specific microtubule depolymerizing activity (Lee and Liu, 2004; Lu et al., 2004). Nevertheless, there is still no direct evidence that the arabidopsis kinesin-13s do not depolymerize microtubules. It would be interesting to see if kinesin-13a and -13b mutants have altered microtubule dynamics, and whether double and triple mutants with morl-1 can modify or rescue the morl-1 phenotype.

In comparison to the increased catastrophe, it was rather surprising that microtubule rescue frequency was increased in morl-1. One simple explanation for this effect might be the higher free tubulin concentration in the cytoplasm caused by increased microtubule depolymerization. This cytoplasmic free tubulin was apparent by higher diffused fluorescence in morl-1 cells expressing the GFP-TUA reporter. It has been demonstrated that higher concentrations of free tubulin favour rescue events in vitro (Pedigo and Williams, 2002).

4.3.4 Effects of GFP fusion proteins on microtubule dynamics

It has been shown that fusing a reporter tag to the N-terminus of ο-tubulin, alters microtubule dynamics, resulting in conspicuous right-handed twisting of organs in arabidopsis (Abe and Hashimoto, 2005). Recent evidence suggests that tagging the ο-tubulin N-terminus inhibits GTP-hydrolysis and that this favours microtubule polymerization (Abe and Hashimoto, 2005). If so, the GFP-TUA reporter protein used for my microtubule dynamics data is likely to have
ameliorated the effects of the \textit{morl-1} mutation. To get a more accurate picture of \textit{morl-1}'s effects on microtubule dynamics, it would be ideal to use variety of microtubule reporter proteins. Like the GFP-TUA line, a GFP-TUB line labels microtubules along their full length, and has been shown to have no or minimal affects on plant morphology (Nakamura et al., 2004; Wasteneys and Yang, 2004; Abe and Hashimoto, 2005). Unfortunately, the expression of GFP-TUB in this line was too low to measure microtubule dynamics using the confocal laser scanning microscopes available during this research. With the recent acquisition of a Quorum spinning disc confocal microscope, it should now be feasible to extend these studies. Two additional transgenic lines expressing GFP-tagged EB1, which marks the growing ends of microtubules were used. The low expressing \textit{Pro}_{EB1}::EB1-GFP line (Dixit et al., 2006) and the highly expressing \textit{CMV 35S::GFP-EB1} (Mathur et al., 2003; Abe and Hashimoto, 2005) line, however, showed microtubule growth suppression in the \textit{morl-1} background. This means the data collected using the GFP-TUA reporter is at present the most reliable measure of the \textit{morl-1} phenotype. Interestingly, GFP-EB1 with higher expression labelled more EB1 comets than in EB1-GFP with lower expression.

Clearly there is a need to explore in more detail how each reporter protein affects microtubule dynamics. Recent studies indicate that leaf epidermal pavement cell morphology is a reliable indicator of changes in microtubule dynamics (Fu et al., 2005). Typical leaf pavement cell shape is like a piece from a jigsaw puzzle. The projecting part of a jigsaw puzzle piece is called lobe in the pavement cell. An arabidopsis pavement cell shows three typical shapes throughout development. At the beginning of development, pavement cells are small and pentagonal or hexagonal. Then the cell expands and shows localized outgrowth. In the final stage, the cell finally forms the jigsaw puzzle shape (Fu et al., 2002). The formation of this final shape requires orchestrated organization of cortical microtubules and microfilaments (Fu et al., 2005). A preliminary study using fractal dimension analysis of pavement cells was recently conducted by
an undergraduate student, Llewellyn Surajballi, under my supervision. In this study, we measured the complexity of pavement cells, which reflected both the depth and the number of lobes. This work has shown us that the high-expression *CMV 35S::GFP-EB1* line suppresses lobing of pavement cells, both in wild-type and *mor1-1*. In contrast, the native promoter-driven *Pro*$_{EB1}$::EB1-GFP showed no significant effects on pavement cell morphology. This study also revealed that the *CMV 35S::GFP-EB1* expression reduced pavement cell size, while the GFP-TUA and GFP-TUB lines produced significantly larger pavement cells than non-transgenic control plants. It is possible that overexpression of GFP fusion proteins altered microtubule dynamics and affected self-organization mechanisms (Wasteneys and Williamson, 1989a; Dixit and Cyr, 2004; Dixit et al., 2006) causing morphological changes. It is also possible that the position of the GFP reporter, whether N-terminus or C-terminus, might have affected the localization patterns of EB1 and microtubule dynamics.

### 4.3.5 Possible mechanisms of microtubule dynamics regulation by MOR1

How does MOR1 regulate microtubule dynamics? To identify a precise mechanism, it is important to consider all available evidence. My immunofluorescence study indicated that the mutant mor1-1 protein remains bound to microtubules even at restrictive temperature (Kawamura et al 2006). Twell et al. (2002) reported that the C-terminal 855 amino acids of MOR1 can bind to microtubules *in vitro*. Taken together, it is likely that the binding of MOR1 protein to microtubules via the C-terminal region is not sufficient to confer function. The *mor1-1* mutation lies in one of the HEAT repeats, which are implicated in protein-protein interaction, in the N-terminal-most TOG domain. There are thus three plausible hypotheses to explain MOR1’s regulation of microtubule dynamics. First, the N-terminal domain of MOR1 may work antagonistically with as yet unidentified destabilizing factors, equivalent to XKCM1 in XMAP215 (Tournebize et al., 2000; Popov et al., 2001). Second, the binding of the N-terminal
domain of MOR1 to microtubules may specifically inhibit pausing of microtubules (Shirasu-Hiza et al., 2003). However, this does not explain how MOR1 encourages rapid growth and shrinkage. A third alternative is that MOR1 may interact with the growing end of microtubules to accelerate the addition of tubulin dimers, but also associate with tubulin dimers along the length of microtubules to physically block the access of destabilizing factors, and accelerate shrinkage when required. In addition to the C-terminal microtubule binding site, another microtubule binding site located somewhere in the second and third TOG N-terminal TOG domains, 1B and 2A, has been identified in the frog homologue XMAP215 (Gard et al., 2004). To test this third possibility, I have initiated in vitro experiments that are discussed in the next chapter.

4.4 MATERIALS AND METHODS

4.4.1 Plant material and growth conditions

Seeds were planted on petri plates containing Hoagland's medium as described in Chapter 2 with the exception that plates were stored at 4°C for 3-5 days before being transferred to a growth cabinet, and that plants were grown at 21°C for 10 days. The A. thaliana (ecotype Columbia) morl-1 mutant, (GenBank accession no. AF367246; Whittington et al., 2001), was backcrossed 8 times to the parental Columbia ecotype. Control lines were segregated after the eighth backcross and wild-type and morl-1 homozygous segregants were used in this study. Transgenic plants of GFP-TUA (Ueda et al., 2003), GFP-TUB (Nakamura et al., 2004), CMV35S::GFP-EB1 (Mathur et al., 2003; Abe and Hashimoto, 2005) and ProEB1::EB1-GFP (Dixit et al., 2006) were crossed to the 8 times backcrossed morl-1. F3 segregants that were homozygote for both GFP and morl-1 or wild-type MOR1 were used in this study.
4.4.2 Live Cell Imaging

The first true leaves of 11 to 12 day old seedlings were excised and placed on a coverslip that formed the bottom of a culture dish (Electron Microscopy Sciences, Hatfield, USA). The leaves were mounted in water with their abaxial side facing a coverslip and a piece of 2% agar was placed on top to stabilize the leaves. Culture dishes were kept at 30°C for 0.5-2 hours before observation. A Bio-Rad Radiance Plus confocal microscope (Carl Zeiss, Jena, Thuringia, Germany) with a 63x NA 1.4 oil-immersion lens or a Meta510 confocal microscope (Carl Zeiss) with a 63xNA1.4 oil-immersion les were used to collect time lapse images. The Bio-Rad Radiance microscope was used for collecting images of GFP-TUA and the Meta 510 microscope was used for CMV35S::GFP-EB1 and ProEB1::EB1-GFP. The 488-nm line of an Ar laser was used for GFP excitation. To record microtubule dynamics at the restrictive temperature, a Bionomic controller BC-100 (20-20 Technology Inc, Wilmington, NC, USA) heated stage was used. Images were taken every 8 s over 3 minutes for GFP-TUA and every 7.2 s over 5 minutes for GFP-EB1 and ProEB1::EB1-GFP. Images were processed with ImageJ (http://rsb.info.nih.gov/ij/) for contrast adjustment and creation of movies from time lapse imaging and measurements were done with ImageJ Manual Tracking.
Chapter 5: Investigating the Mechanism of MOR1’s Regulation of Microtubule Dynamics
Chapter 5: Investigating the Mechanism of MOR1’s Regulation of Microtubule Dynamics

5.1 INTRODUCTION
In the previous chapter, I show that MOR1 promotes both rapid microtubule growth and shrinkage, and prevents the pausing of microtubules, keeping them highly dynamic. In this chapter, I consider the evidence from my own and other studies to propose possible ways MOR1 can control microtubule organization and to outline experimental strategies to test one hypothesis. I also present the preliminary work that I have conducted that will enable future investigations to identify the mechanism of MOR1’s regulation of microtubule dynamics.

5.1.1 A hypothesis on how MOR1 regulates microtubule dynamics
Based on negative staining electron microscopy, the MAP215/Dis1/ proteins are predicted to be long and flexible proteins approximately 60 nm in length (Cassimeris et al., 2001). Structural analyses show that MAP215/Dis1 proteins contain two to five repetitive domains. These domains are referred to as TOG domains because they were first identified in the human Tumour Over expressed Gene protein. Each TOG domain, in turn, contains up to five HEAT repeats, motifs that are generally thought to mediate protein-protein interaction. TOG domains are believed to have evolved through duplications of one ancestral domain. Bioinformatic analyses revealed that TOG-1A and TOG-2A are the most conserved domains (Gard et al., 2004) (Figure 5.1A). In the plant and animal homologues, four N-terminal TOG domains are found at the N-terminus (Gard et al., 2004). There is a fifth putative TOG domain comprised of HEAT repeats (Brittle and Ohkura, 2005), however, this domain lacks the TOG signature sequence and it remains unclear if it retains TOG function (Gard et al., 2004).
Figure 5.1. Strategy for testing the hypothesis that each TOG domain of MOR1 interacts with one tubulin dimer in regulating microtubule dynamics.

(A) When the lengths of a microtubule protofilament and MOR1 are compared, each TOG domain coincides with one tubulin dimer.

(B) An N-terminal fragment containing TOG-1A and -1B. This fragment has been cloned, expressed and purified. This fragment is designated as TOG1A-1B.

(C) C-terminal fragments with and without TOG-3 region. Both fragments have been cloned, expressed and purified. Each of them is designated as TOG3-R4 and R4 respectively.

(D) Fragments without TOG-2A and TOG-2B. If the hypothesis is correct, these fragments should bind to microtubules and have partial MOR1 function.

(E) Fragments with duplicated TOG-1A and -1B and C-terminal fragment bearing microtubule binding ability. The fragment should bind to microtubules and have more effective MOR1 function than in (D).
Interestingly, the N-terminal region containing the five TOG domains of MOR1/MAP215 spans a distance that is equivalent to 5 tubulin dimers (Figure 5.1A). When one MAP215 protein is superimposed schematically and to scale on a tubulin protofilament, it becomes apparent that each TOG domain coincides closely with one αβ-tubulin heterodimer. This remarkable similarity in size of TOG domains and tubulin dimers has led us to develop a plausible hypothesis to be tested on how the TOG domains contribute to the function of the MOR1 protein. The hypothesis, illustrated in Figure 5.1A, is that each TOG domain interacts with one αβ-tubulin heterodimer or two adjacent ones, and that this interaction may be of key importance for maintaining microtubule stability. Multiple TOG domains may increase the efficiency of MAP215 protein function. The model may also help to explain the repeated duplication of TOG domains in the early evolution of the MAP215/Dis1 proteins. No similar hypothesis has emerged so far in the literature, though various reports, outlined in the following paragraphs, provide compelling evidence that TOG domains interact with tubulin dimers.

5.1.2 TOG domains might interact with tubulin dimers in plants.

There is mounting in vitro evidence that TOG domains can interact directly with tubulins. In Stu2, Stu2-tubulin dimer complex formation is shown by the complex size shift using size-exclusion chromatography (Al-Bassam et al., 2006). Interestingly, one TOG-1A can bind to one tubulin dimer but when Stu2 forms a homodimer, it still binds to only one tubulin dimer in vitro (Al-Bassam et al., 2006).

In a recently published article, Kerssemakers et al (2006) used optical tweezers to measure microtubule growth and shrinkage at molecular resolution. Their study provides in vitro evidence that XMAP215 promotes microtubule growth and shrinkage in 40-60 nm increments. Consistent with our hypothesis, they proposed that XMAP215 adds whole tubulin oligomers to microtubule ends, rather than promoting the addition of one tubulin dimer at a time. The binding
of XMAP215 to tubulin oligomers is also consistent with XMAP215's ability to promote rapid microtubule shrinkage since the release of oligomers will lead to faster disassembly. The tobacco homologue of MOR1, MAP200, is shown by in vitro experiments to be able to bind to tubulin oligomers and to promote microtubule polymerization (Hamada et al., 2004). My dynamics data, which showed that MOR1 promotes not only rapid growth, but also rapid shrinkage, further supports the idea that XMAP215/Disl proteins work by the addition to or removal from microtubules of tubulin oligomers.

5.1.3 Properties of MAP215/Disl family proteins

MAP215/Disl proteins are essential for microtubule organization. Extensive functional analyses on XMAP215 in particular have identified microtubule-binding sites and centrosome-targeting sites. The following section summarizes the known properties of the C-terminal and N-terminal regions of the MAP215/Disl proteins.

C-terminal domain

Protein binding sites are often identified by expressing parts of genes to produce fragments of the original full-length protein. In general, C-terminal fragments of MAP215/Disl proteins have the ability to bind to microtubules and are targeted to the centrosome. XMAP215 in the Xenopus is the most studied protein in this family. Microtubule binding studies indicate that the strongest microtubule binding activity in the XMAP215 protein resides in the fragment comprising amino acids 1150 to 1325, a region that includes only two HEAT repeats (Gard et al., 2004). In arabidopsis MOR1, a C-terminal fragment expressed from a cDNA clone, including amino acids 1123-1977, has been shown in one study to co-sediment with taxol-stabilized microtubules in vitro (Twell et al., 2002). This fragment includes the region relevant to the strongest microtubule binding activity in XMAP215. In contrast, the C-terminal fragment of human TOGp has very low affinity to microtubules, whereas a fragment comprising amino acids 1229 to 1972 (the C-
terminal domain) bound only to tubulin dimers (Spittle et al., 2000).

The centrosome-targeting domain is found at the C-terminal fragment of XMAP215, including amino acids 1850-1950. DdCP224 also has a centrosome-targeting domain somewhere in its C-terminal half (amino acids 809-2015) (Graf et al., 2000). The C-terminus of the ascomycete fungal homologues is unrelated in sequence and function to homologues found in most other taxa. Stu2, for example, has a coiled-coil domain near the C-terminus, which is recently suggested to be responsible for forming homodimers (Al-Bassam et al., 2006). However, vertebrate and plant homologues lack this coiled-coil domain, and show no evidence for homodimer formation (Gard et al., 2004).

**N-terminal domain**

In contrast to the C-terminus, the N-terminus, especially the first two TOG domains, of MAP215/Dis1 proteins is highly conserved. The first TOG domain of STU2 (TOG-1A) can bind to one tubulin dimer, while the second TOG domain (TOG-1B) alone, can bind to microtubule polymers *in vitro* (Al-Bassam et al., 2006). In XMAP215, there is a site somewhere in the 250-800 amino acid region that has moderate microtubule-binding activity. This region includes the full second TOG domain (TOG-1B) and nearly the full third TOG domain (TOG-2A) (Gard et al., 2004). Similar to XMAP215, microtubule-binding activity in the human TOGp is also found in the N-terminal region, specifically between amino acids 144-799, which includes half of the first TOG domain (TOG1A), the full second TOG domain (TOG-1B), and nearly the full third TOG domain (TOG-2A) (Spittle et al., 2000). The microtubule-binding regions of all these three orthologues contain TOG-1B, however, it is not clear if TOG-1B alone is sufficient to confer microtubule binding to the human TOGp and *Xenopus* MAP215, or to all orthologues.

The N-terminal region of XMAP215 has another identified function: an antagonistic interaction with the destabilizing factor, XKCM1 (Popov et al., 2001). This antagonistic activity with
XKCM1 has been reported in human cells as well but the responsible domains are not known in TOGp (Cassimeris and Morabito, 2004; Holmfeldt et al., 2004). The N-terminal fragment of XMAP215 itself is suggested to destabilize pausing microtubule ends without XKCM1 *in vitro* (Shirasu-Hiza et al., 2003).

In the next section, I propose strategies to test my hypothesis that one TOG domain interacts with one tubulin dimer. In the following sections, materials and methods, results and discussion, I also present the preliminary work that I have conducted and that will be continued by other investigators in our lab to identify the mechanism of MOR1's regulation of microtubule dynamics. At the end of this chapter, I conclude entire work presented in this thesis.

### 5.2 Experimental Strategies

#### 5.2.1 Cloning strategies to test if one TOG interacts with one tubulin dimer

To test the hypothesis that each TOG domain interacts closely with one tubulin dimer, I have initiated experiments illustrated in Figures 5.1B to 5.1D. My cloning strategies selectively remove and/or duplicate, alone or in combination, the TOG-2A, TOG-2B and TOG-3 domains from MOR1 cDNA, using PCR-engineered restriction sites. Other researchers have shown that the C-terminal fragment of MOR1, including the TOG-3 and the R4 C-terminal domains, binds to microtubules *in vitro*, and truncation of the C-terminal region by mutation generates embryo lethality (Twell et al., 2002). Results from other studies suggest that the 200 or so amino acids in the TOG-3 region have relatively high affinity for microtubules (Gard et al., 2004). However, it is not clear whether R4 alone or in combination with TOG-3 is responsible for microtubule binding in arabidopsis. The following sections outline three strategies to address these questions.

1. An *in vitro* assay will be performed to determine if one TOG can bind to one tubulin dimer. A fragment comprised of the most conserved TOG domains, TOG-1A and TOG-1B (TOG1A-
1B) (Figure 5.1B), will be tested for tubulin dimer binding. TOG domain-depleted and -duplicated fragments (Figures 5.1D and 5.1E) will also be tested.

(2) An in vitro assay will be conducted to assess microtubule-binding for two C-terminal fragments, including the R4 domain alone (R4) or in combination with TOG-3 (TOG3-R4) (Figure 5.1C). If the TOG3-R4 fragment but not the R4 fragment binds microtubules, this will verify that both TOG-3 and R4 are required for microtubule binding activity. If the R4 fragment can bind to microtubules, R4 will be considered sufficient to confer microtubule binding. Once the binding domain is determined, the C-terminal fragment that has microtubule binding activity will be retained for TOG domain depletion and duplication analysis.

(3) For TOG domain depletion analysis, the two TOG-2 domains, and possibly the TOG-3 domain (see above strategy), will be deleted (Figure 5.1D). The TOG-1A and TOG-2A domains will be retained as they are the most conserved among eukaryotes.

(4) If each TOG domain interacts with a tubulin dimer and has similar functions, then duplicating the same TOG domain should restore the efficiency of the MOR1 protein to recruit or remove tubulin dimers. The TOG-2A and 2B domains will be replaced with TOG-1A and 1B (Figure 3E). This is also designed to examine the evolutionary events of TOG duplication that led to the enlargement of MAP215 proteins.

5.2.2 in vitro polymerization and binding assays

Truncated proteins can be expressed in bacteria and purified using tags. The ability of truncated expression proteins to bind to microtubules and free tubulin and to polymerize microtubules will be compared in vitro. Most of truncated MOR1 genes, TOG1A-1B, TOG3-R4 and R4, mentioned above have already been cloned into a pET32b vector (Novagen) and the E.coli BL21(DE3) strain is being used as a host for protein expression (see material and methods). Protein expression can be induced by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG).
If the hypothesis is correct, it is anticipated that truncated MOR1 proteins will associate with fewer tubulin dimers and will polymerize microtubules less efficiently. These predictions will be tested with the following, well established methods.

(1) Tubulin Dimer Binding Assay
Detailed analysis to determine the expression protein to tubulin dimer stoichiometry can be performed by size-exclusion chromatography (Al-Bassam et al., 2006). Expression proteins and unpolymerized tubulin will be incubated at 4°C to allow complex formation and to avoid microtubule polymerization. Reaction mixtures will then be applied to a size-exclusion chromatography column that separates complexes according to their sizes during elution. Fractions of elutant will be analyzed by optical density and SDS-PAGE. The stoichiometry can be calculated by densitometry of Coomassie blue-stained gels. Reaction mixtures of expression proteins only and tubulin only will be used as controls.

(2) Microtubule Binding
To examine if expression proteins bind to microtubules, co-sedimentation assays will be performed. Expression proteins will be incubated with taxol-stabilized microtubules at 30°C and will be pelleted by centrifugation at 100,000g. If expression proteins bind to microtubules, they should co-pellet with microtubules. The contents of pellet and supernatant will be evaluated by SDS-PAGE. Reaction mixtures containing expression proteins only will be used as controls.

(3) Microtubule Polymerization Assays
To compare the ability of my expression proteins to promote microtubule polymerization, two well established methods can be used (Hamada et al., 2004; Smertenko et al., 2004). Using spectroscopy, the degree of microtubule polymerization correlated with the increase in turbidity can be determined by measuring optical density at 350nm (Hamada et al., 2004; Smertenko et al., 2004). Dark-field microscopy can be used to measure the length and number of \textit{in vitro} assembled microtubules (Hamada et al., 2004; Smertenko et al., 2004). Polymerization efficiency will be compared by varying the concentrations of expression proteins while keeping
tubulin concentrations constant. The more efficient a polymerizer is, the lower the concentration at which it will work to produce the same amount of microtubule polymer. If our hypothesis is correct, expression proteins with more TOG domains may polymerize microtubules more efficiently than those with fewer TOG domains. These strategies should generate new information on how MOR1 interacts with each microtubule and how MOR1 regulates microtubule dynamics in greater detail.

5.3 RESULTS

To test my hypothesis that one TOG domain interacts with one tubulin dimer, I have cloned, expressed and purified MOR1 fragments. One of the fragments, TOG1A-1B, has been tested for tubulin binding by performing size-exclusion chromatography.

5.3.1 Protein expression with OrigamiB (DE3) and BL21 (DE3)

Expression of the TOG1A-1B, TOG3-R4 and R4 protein fragments of MOR1 was attempted using OrigamiB(DE3) as a host strain. To induce protein expression, different concentrations of IPTG, including 0.025mM, 0.1mM, 0.4mM and 1mM, were tested, since protein induction with the combination of pET32b and OrigamiB (DE3) is IPTG-dependent. After IPTG addition, cells were cultured at 16°C overnight. Cells were collected and the protein expression level was analyzed by SDS-PAGE. TOG1A-1B and R4 proteins accumulated in the soluble fraction but the largest fragment, TOG3-R4, did not (Figure 5.2A). An alternative incubation condition of 28°C for 3.5 hours, was tested, but TOG3-R4 continued to accumulate in the insoluble fraction. In an attempt to improve expression protein quality, I tried a different host strain, BL21 (DE3). With this host strain, protein induction levels cannot be altered by the IPTG concentration, so a fixed concentration of 1mM was used. After IPTG addition, cells were cultured at 16°C overnight. Protein expression was analyzed on SDS-PAGE. All expression proteins were
expressed in the soluble fraction, although TOG3-R4 accumulation was low (Figure 5.2B).
Figure 5.2 Protein expression of MOR1 fragments in the soluble fraction analyzed on SDS-PAGE.

(A) OrigamiB(DE3) was used as the host strain. Black arrowheads indicate the expected size of expression protein bands. Red arrow indicates the absence of the expected TOG3-R4 band.
1. TOG1A-1B vector after induction with 1mM IPTG
2. TOG1A-1B vector without IPTG
3. TOG3-R4 vector after induction with 1mM IPTG
4. TOG3-R4 vector without IPTG
5. R4 vector after induction with 1mM IPTG
6. R4 vector without IPTG
7. pET32b empty vector with 1mM IPTG

(B) BL21(DE3) was used as a host strain. Black arrowheads indicate the expected size protein bands. All expression proteins were expressed, although the expression level was not high compared to OrigamiB(DE3).
1. pET32b empty vector without IPTG
2. pET32b empty vector with 1mM IPTG
3. TOG1A-1B vector without IPTG
4. TOG1A-1B vector with 1mM IPTG
5. TOG3-R4 vector without IPTG
6. TOG3-R4 vector with 1mM IPTG
7. R4 vector without IPTG
8. R4 vector with 1mM IPTG
5.3.2 Cleaving tags by Enterokinase

The vector was designed so that expression proteins would include epitope tags to assist various applications. Having tags at the end of proteins, however, can interfere with protein-protein interactions. I therefore designed primers so that the tags could be cleaved right before the first amino acid of the expression protein sequence. After purification, the tagged TOG1A-1B protein extract was incubated with Enterokinase at 4°C overnight. The Enterokinase-treated proteins were then subjected to SDS-PAGE, which confirmed by molecular weight that the TOG1A-1B fragment had been reduced by the amount attributable to the tag (Figure 5.3). Cleaving the protein with Enterokinase at room temperature was also tested and gave similar results (data not shown).
Figure 5.3 TOG1A-1B fusion protein was cleaved with Enterokinase to cleave all tags.

The expected molecular weight of the cleaved protein is 58kDa. After incubation with Enterokinase, the molecular weight of the recombinant protein was reduced to the expected size (Lane 1).
1. TOG1A-1B cleaved with Enterokinase
2. TOG1A-1B Enterokinase negative control. The recombinant protein was incubated under the same conditions as in 1, without Enterokinase.
5.3.3 Size-exclusion chromatography showed that TOG1A-1B and tubulin dimers do not interact

To test if the TOG1A-1B fragment could bind to two tubulin dimers, size-exclusion chromatography was performed. Size-exclusion chromatography separates proteins mainly according to their molecular weight. Under this experimental condition, there was no extra peak detected when TOG1A-1B and the tubulin dimer solutions were mixed (Figure 5.4). There was a small earlier hump but it was also found in the tubulin control elution. Therefore this was not an extra peak promoted by the addition of TOG1A-1B. The predicted linear structure of TOG1A-1B made the proteins elute earlier than expected for their actual molecular weight.
Figure 5.4 Size-exclusion chromatography of TOG1A-1B and tubulin showed that the TOG1A-1B fragment did not interact with tubulin dimers.

Samples were thawed, diluted and/or mixed and kept on ice for 2.5 hours before loading onto a column. The molecular weights of TOG1A-1B and tubulin dimers are 97kDa and 110kDa respectively. The predicted linear structure of TOG1A-1B caused it to elute earlier than predicted for its actual molecular weight. The small curve from 8.5 - 11.5 ml elution of fragment TOG1A-1B and tubulin mixture seems to be tubulin, since tubulin alone showed a similar curve. The data indicate that the TOG1A-1B fragment and tubulin did not interact under these *in vitro* experimental conditions.
5.3.4 Summary of results

To test the hypothesis that one TOG domain interacts with one tubulin dimer, I cloned and made expression protein of first 2 N-terminal TOG domains, TOG1A-1B. If the hypothesis is correct, TOG1A-1B should bind to 2 tubulin dimers. The size-exclusion chromatography with TOG1A-1B and tubulin dimer did not generate an extra peak, suggesting that TOG1A-1B does not bind to tubulin dimers, at least not under these in vitro conditions.

5.4 DISCUSSION

5.4.1 Size-exclusion chromatography data suggest that the TOG1A-1B domain of MOR1 lacks in vitro binding to tubulin dimers.

In this study, size exclusion chromatography was used to determine if the two N-terminal TOG domains from MOR1 are able to bind to tubulin dimers. The data indicated that bacterially expressed protein comprising MOR1’s TOG1A-1B domain does not form complexes with tubulin dimers in vitro. This finding is in contrast to a recent report in which the first N-terminal TOG domain of the *Saccharomyces cerevisiae* Stu2 protein, which is the region equivalent to TOG-1A, was shown to be able to bind one tubulin dimer in vitro (Al-Bassam et al., 2006). However, in vitro binding assays with the human TOGp N-terminal region have, as in the present study, failed to show affinity to tubulin dimers, (Spittle et al., 2000).

Three interpretations of the size-exclusion chromatography experiments are listed, and discussed as follows:

1) The TOG-1A domain of MOR1, like TOGp in human but unlike Stu2, does not in fact bind to tubulin dimers.

Considering the diverse functions and localization patterns described for MAP215/Dis1
orthologues, tubulin binding sites in plants may not be conferred in TOG-1A. When Stu2 and MOR1 amino acid sequences are compared, TOG-1A is 24% identical and 53% similar (Rashbrooke, 2005). TOG-1A of TOGp and MOR1 are somewhat more closely related with 33% identity and 60% similarity (Rashbrooke, 2005). The C-terminal region of human TOGp has a tubulin-binding site and the TOG-3 and R4 domains in the C-terminal region of TOGp and MOR1 also are related to similar degrees as their TOG-1A domains (TOG-3: 32% identical and 61% similarity and R4: 30% identical and 61% similarity) (Rashbrooke, 2005). It would be interesting to examine if my C-terminal recombinant proteins, TOG3-R4 and R4, can bind to tubulin dimers, as has been shown for the C-terminal region of human TOGp. Despite the lack of evidence for TOG1A-tubulin binding in animal and plant homologues, there is not enough evidence to rule out this possibility. It has been shown, for example, that tobacco MAP200, when purified from culture cells, can bind to and/or form tubulin oligomers in vitro (Hamada et al., 2004). This certainly favours the idea that full-length protein can bind to free tubulin dimers, but the domain responsible for tubulin binding was not identified (Hamada et al., 2004). In addition, MOR1 was found colocalized with oryzalin- depolymerized microtubules/tubulin aggregates in vivo, indicating that MOR1 binds to tubulin oligomers/dimers (Twell et al., 2002). It is possible that full-length MOR1 is required for the TOG domain-tubulin binding activity, as discussed below.

2) The TOG-1A domain may only bind free tubulin by interacting with the C-terminal MOR1.

As has been discussed above, full length MAP200 and MOR1 are suggested to bind tubulin oligomers (Twell et al., 2002; Hamada et al., 2004). It is possible that full length or at least the presence of both the C- and N-terminal regions might be essential for TOG-tubulin interactions in MOR1. Indeed, DdCP224 was suggested to require cooperative interactions between N- and C-terminal regions to bind interphase microtubules (Graf et al., 2000). The N-terminal fragment (1-813) and C-terminal fragment (809-2015) fused to GFP both failed to localize to interphase
microtubules, but immunolocalization data shows that DdCP224 colocalizes to interphase microtubules (Graf et al., 2000). Binding assays using my TOG depleted recombinant proteins, which has both N- and C-terminal regions (Figure 5.1D), would address these questions.

3) Under *in vitro* conditions, the TOG1A-1B polypeptide may not have the necessary conformation to bind tubulin. This may have occurred because the *in vitro* experimental conditions were not optimal for the TOG domain-tubulin interaction. There are a number of major conditions that could be optimized, as listed below.

3.1) Binding of GTP to tubulin may be essential for interaction with the isolated N-terminal TOG domains. For the tubulin binding assay, I did not include GTP specifically in order to prevent microtubule polymer formation. It seems unlikely, however, that this can explain the lack of interaction with tubulin for the following reasons. First, it has been demonstrated that purified MAP200 from tobacco can bind to and form oligomers from both GTP- and GDP-tubulin (Hamada, T., personal communication). Second, the lack of tubulin binding for the N-terminal region of human TOGp was documented in the presence of GTP (Spittle et al., 2000). Unfortunately, the presence of GTP or GDP was not mentioned in the Al-Bassam et al. (2006) publication that demonstrated the binding of tubulin to the N-terminal-most TOG domain of Stu2. If tubulin needs to be GTP-bound for binding to the TOG1A-1B fragment, that would also explain MOR1’s role in promoting microtubule growth. However, this cannot explain the rapid shrinkage promoted by the wild-type MOR1 protein. Taken together, the presence or absence of GTP did not seem to affect the TOG1A-1B domain’s binding ability. Nevertheless, binding to GTP does cause conformational changes to tubulin dimers, and the possibility should be explored.

3.2) The size-exclusion chromatography experiments were performed at 4°C according to (Al-Bassam et al., 2006). At this temperature, tubulin remains largely depolymerized, which is
important for preventing aggregates of microtubule polymer. Just as tubulin-tubulin interactions are reduced at this temperature, tubulin-TOG1A-1B interactions may also be inhibited.

3.3) Protein expression in heterologous systems may generate protein that is not folded in its normal way, or not allow post-translational modifications needed for function. In future work, the use of alternative eukaryotic expression systems, such as yeast or insect cultures, may be worth considering.

5.5 MATERIALS AND METHODS

5.5.1 Cloning of MOR1 fragment constructs

Fragments of MOR1 were PCR-amplified from MOR1 cDNA with engineered restriction sites for cloning, including an enterokinase cleaving sequence for tag cleavage after purification, and spacing nucleotides to ensure that the fragments would be in-frame. Fragment 1 encodes the region of MOR1 including the TOG1A and TOG1B domains (residues 1-526). Fragment 2 encodes the C-terminal region, deleting the N-terminal 4 TOG domains, TOG1A, 1B, 2A and 2B (residues 1086-1979). Fragment 3 encodes a smaller C-terminal domain, eliminating the four N-terminal TOG domains and the putative fifth TOG domain (residues 1440-1979). The primer sets used for cloning were as follows; For Fragment 1:

5'-CGTCCATGGCAGACGACGACGACGACAAGATGTCGACGGAGGATGAGAAG-3' and 5'-GTCGAATTCCTATGTGGCTGTGCTCCAACTGAAGACTGAACTGTC-3',

For Fragment 2:

5'-ATCGAATTCGGACGACGACGACGACAAGGTGTCAAAAGGTGTTACAAAG-3' and 5'-GGTGCGGCCGCCTACATATGTTCCAGTGATCCACCTTTGAGCCTCTC-3',

For Fragment 3:

5'-TGGGAATTCGGACGACGACGACAAGTATGGTATTTCCGAACAAATG-3' and
5'-GGTGCGGCCGCTACATATGTCCAGTGATCCACCTTTGAGCCTCTC-3'.

The cDNA fragments were cloned into the pET32b vector (Novagen, Madison, WI, USA) using NcoI and EcoRI sites for Fragment 1 and EcoRI and NotI sites for Fragment 2 and 3 (Figure 5.5). The sequences for all fragments were confirmed by DNA sequencing with slab gel system conducted by Nucleic Acid Protein Service Unit at the University of British Columbia.
Figure 5.5 Vector maps for MOR1 fragments.
The pET32b vector (A) was used for all cloning. Sets of restriction sites, NcoI-EcoRI and EcoRI-NotI from multiple restriction sites were used to clone the TOG1A-TOG1B fragment (B), TOG3-R4 (C) and R4 (D). Enterokinase#1 is an original enterokinase recognition site from the pET32b vector. Enterokinase#2 is a PCR-engineered enterokinase recognition site to cut the tags off right before the MOR1 fragment's C-terminus. Trx is Thioredoxin.
5.5.2 Protein Expression and Purification

The expression vectors containing the *MORI* fragments were transformed into BL21(DE3) strains or OrigamiB(DE3). Cells were grown at 37°C for 4-5 hours in 5ml Luria-Bertani (LB) media with ampicillin. The cells were spun down, resuspended in fresh LB with ampicillin, subcultured to a larger scale and grown at 37°C until the OD$_{600}$ reached 0.6-1.0. IPTG was added to make a final concentration of 1mM to induce protein expression. Cells were then grown at 16°C overnight. Cells were collected by centrifugation and stored at -80°C until the purification step. For purification, cells were resuspended in His tag-binding buffer (20mM sodium phosphate buffer, 500mM NaCl, 25mM imidazole, pH 7.4) supplemented with a protease inhibitor cocktail (P9599: Sigma, St Louis MO, USA) and sonicated to extract proteins on ice. Protein extract was centrifuged for 15min at 22,000g. Supernatants were applied to a HisTrap HP 1ml column (Amersham Biosciences, Quebec, Canada) and proteins were purified according to the column manufacturer’s instructions. An elution buffer consisting of 20mM sodium phosphate buffer, 500mM NaCl, 500mM imidazole, pH 7.4 was used. The elutants were applied to a PD-10 column (Amersham Biosciences) in order to exchange the buffer to the enterokinase cleaving buffer (20mM Tris-HCl, pH 7.4, 50mM NaCl, 2mM CaCl$_2$). Proteins were then diluted to 1mg/ml and recombinant Enterokinase (Novagen) was added to a final concentration of 17 units/ml. Cleavage was performed at 4°C overnight. The cleaved protein was applied to a Centricon Ultracel YN-50 centrifugal filter device (Millipore, Billerica, MA, USA) to exchange appropriate buffers for further analysis and to concentrate the purified protein. Proteins were snap-frozen in liquid nitrogen and stored at -80°C.

5.5.3 Size-exclusion Chromatography

The Superdex200 gel filtration column (GE Healthcare) was connected to an ÄKTA system (Amersham Biosciences) and was equilibrated with gel filtration buffer (25mM Tris, 200mM...
NaCl, 1mM MgCl₂, 1mM EGTA, pH7.5) (Al-Bassam et al., 2006). Approximately 20μM of Fragment 1 protein, 20μM bovine brain tubulin (Cytoskeleton Inc., Denver, CO, USA) and a mixture of Fragment 1 protein at a final concentration of approximately 20μM, and tubulin at a final concentration of 20μM were incubated on ice for 30 min before being applied to the column. 25μl of protein solution was loaded onto the column and eluted in 0.5ml fractions at a 0.5ml/min flow rate. OD_{280} and OD_{230} were measured as the elutant came through the column.
Chapter 6: Conclusion and Future Directions
Chapter 6: Conclusion and Future Directions

6.1 SUMMARY OF THE RESULTS

In my PhD studies, I investigated the functions and mechanisms of activity of MOR1, a XMAP215/Dis1 class microtubule-associated protein found in the model higher plant Arabidopsis. In chapter 2, I showed that MOR1 is important for the assembly and function of all microtubule arrays throughout the cell cycle, possibly working by keeping individual microtubules long. In chapter 3, MOR1 is shown to colocalize along the full length of microtubules, and the colocalization is demonstrated not to be lost in the morl-1 mutant at the restrictive temperature. This suggested that the morl-1 mutation near the N-terminus does not abolish microtubule binding ability of the protein but that it affects some other functions that, in the wild type and under permissive conditions in the mutant, keeps microtubules long. To understand how MOR1 promotes long microtubules, I analyzed microtubule dynamics, which is described in chapter 4. This study reveals that MOR1 not only promotes microtubule growth, but also promotes rapid microtubule shrinkage, and prevents the pausing of microtubules, keeping them highly dynamic. Nevertheless, the mechanism by which MOR1 regulates microtubule dynamics remains unclear. Based on my dynamics and immunofluorescence data and other reported evidence, in chapter 5, I propose a hypothesis that one TOG domain interacts with one tubulin dimer. The size-exclusion chromatography with TOG1A-1B and tubulin dimer suggested that TOG1A-1B do not interact with tubulin dimers under these in vitro conditions.
6.2 CONCLUSION AND FUTURE DIRECTIONS

6.2.1 MORI keeps microtubules highly dynamic by promoting rapid growth and shrinkage and preventing microtubule pausing.

Quantitative analysis of microtubule dynamics in a temperature-sensitive mutant morl-1 showed that MORI keeps microtubules highly dynamic, promoting rapid growth and shrinkage, and preventing pausing of microtubules. This finding is consistent with my immunofluorescence data, which indicated that MORI remains along the entire length of microtubules throughout the cell cycle. MORI not only promotes microtubule growth, but when microtubules shrink, MORI, by remaining associated with microtubules, probably assists in the removal of tubulin oligomers to promote rapid microtubule shrinkage. Together with the evidence that tobacco MAP200 and MORI bind to tubulin oligomers (Twell et al., 2002; Hamada et al., 2004), and that XMAP215 speeds up both microtubule growth and shrinkage in 40-60nm increments, which is equivalent to 5-7.5 tubulin dimers (Kerssemakers et al., 2006), it remains plausible that MORI associates with tubulin oligomers when a microtubule grows and shrinks, ensuring rapid growth and shrinkage.

6.2.2 Cell division defects might be caused by short and less dynamic microtubules in morl-1.

In the morl-1 mutant, spindles are disorganized, as determined by live cell imaging experiments, and take a substantially longer time than wild-type spindles to align chromosomes. Dynamic microtubules of sufficient length are essential for spindles to be able to align chromosomes to the metaphase plane. Since interphase microtubules are less dynamic in the morl-1 mutants, spindle microtubules might be less dynamic as well. My analysis demonstrated that phragmoplast arrays were also significantly shorter in the morl-1 mutant. Although the length of individual microtubules within phragmoplast arrays could not be measured by fluorescence microscopy, it
is likely that individual phragmoplast microtubules are shorter in the morl-1 mutant. Phragmoplasts are essential for building the cell plate, and need to be functioning properly to orient the plate in the right position. This is accomplished by finding and following cues left by the preprophase band that formed and then disassembled prior to mitosis. Actin plays an important role in both spindle alignment and in phragmoplast guidance by coordinating with microtubules (Granger and Cyr, 2001b; Yoneda et al., 2004). Long and dynamic microtubules may be essential for interacting with actin filaments and finding those cues and guiding phragmoplasts as the cell plate gets constructed. Therefore all cell division defects in morl-1 are suggested to be downstream effects of altered microtubule dynamics.

6.2.3 MOR1 may interact with an unknown destabilizing factor, equivalent to XKCM1.

Failure of the mutant morl-1 protein to promote rapid microtubule growth would be one of the reasons for shorter microtubules in the morl-1 mutant. It remains unclear if MOR1 has an antagonistic activity with destabilizing factors equivalent to XKCM1 in Xenopus. The N-terminal region of XMAP215 in Xenopus interacts with XKCM1 (Popov et al., 2001). If there is such an interaction with a catastrophic kinesin in plants, the mutation in morl-1, which is near the N-terminus, may reduce the normal inhibition of a destabilizing factor, resulting in less stable, and hence, shorter microtubules. In arabidopsis, there are two kinesins, KINESIN-13A and -13B, that belong to same family as KINI XKCM1 (Reddy and Day, 2001; Lee and Liu, 2004; Lu et al., 2004). One of them, KINESIN-13A, has been suggested not to have microtubule destabilizing activity (Lee and Liu, 2004; Lu et al., 2004). However, there is no direct evidence that KINESIN-13A and -13B do not depolymerize microtubules. It would be interesting to examine if double mutants or triple mutants of kinesin-13a, -13b and morl-1 could alter microtubule dynamics.
6.2.4 The TOG domains in XMAP215/Dis1 may closely interact with tubulin dimers.

One of the most important findings of my research is that the mutant mor1-1 protein still colocalizes with microtubules at restrictive temperature. This indicates that the defective mor1-1 protein can still bind to microtubules, while losing its ability to regulate microtubule dynamics. The region of MOR1 affected by the N-terminal mutation in the mor1-1 allele may not be necessary for binding to microtubules but instead may have other functions that keep microtubules more dynamic and longer. N-terminal regions of all MAP215/Dis1 proteins are comprised of 2 to 4 or perhaps 5 TOG domains. When the sizes of MOR1 and a microtubule protofilament are compared, one TOG domain coincides with one tubulin dimer. To explore the functions of repetitive TOG domains, we hypothesized that one TOG domain interacts with one tubulin dimer. As presented in chapter 5, we have designed biochemical assays that will use modified recombinant proteins of MOR1 fragments in which specific TOG domains have been deleted or duplicated. These future investigations should provide us with new information on how MOR1 regulates microtubule dynamics.
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