Functional interactions between the *Arabidopsis* homologs of spindle assembly checkpoint proteins MAD1 and MAD2 and the nucleoporin NUA

THESIS

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Abstract

In eukaryotes, the mitotic spindle assembly checkpoint (SAC) ensures the fidelity of chromosome segregation. The SAC acts through monitoring the attachment of microtubules (MTs) to chromosomes at the kinetochores. When not all chromosomes are attached with MTs in a bi-polar manner, components of the SAC on the kinetochores constitutively initiate signals to delay sister chromatid separation. Recently, it has been shown that the SAC components Mitotic Arrest Deficient 1 and 2 (MAD1 and MAD2) associate with the nuclear pore complex (NPC) during interphase and require certain nucleoporins, such as Tpr in animal cells, to properly localize to kinetochores during mitosis. In plants, although some of the SAC components including MAD2, BUR1, BUB3 and Mps1 have been isolated, the roles of SAC proteins during interphase and mitosis are still poorly understood and it is unknown whether they require plant nucleoporins for proper localization.

In this study, we characterized the Arabidopsis homologs of human Mad1 and Mad2 (hMad1 and hMad2), AtMAD1 and AtMAD2, and their interactions with the Arabidopsis homolog of Tpr, NUA. An AtMAD2 T-DNA insertion mutant with lower expression of mRNA transcripts exhibits a shorter primary root and a smaller root meristem, very
similar to a NUA null allele. Two other AtMAD2 T-DNA insertion lines with higher levels of mRNA transcripts exhibit a longer primary root, and an extended root meristem. AtMAD1 and AtMAD2 are localized at the nuclear envelope (NE) in interphase Arabidopsis root cells and their NE localization requires the plant nucleoporin NUA. This requirement for NUA appears specific, since depletion of another nucleoporin (Nup160) does not affect the localization of AtMAD2. AtMAD2 localizes to kinetochores during prophase and prometaphase and disassociates from kinetochores as the cell cycle proceeds in live BY-2 cells. Yeast two-hybrid assays demonstrate binding of AtMAD1 to AtMAD2 and NUA, as well as AtMAD1 dimerization. No direct interaction between AtMAD2 and NUA was detected. The interactions of AtMAD1 and AtMAD2, as well as between AtMAD1 and NUA were confirmed by co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC), respectively. Together, these data suggest that, in Arabidopsis, NUA scaffolds AtMAD1 and AtMAD2 at the nuclear envelope to form a functional complex and that both NUA and MAD2 are required for root development in Arabidopsis.
This document is dedicated to my family.
Acknowledgments

I would like to thank my advisor for her generous support and guidance while working on the project. Thanks go to Dr. Sivaramakrishnan Muthuswamy who started this project and also go to my previous and current coworkers who contribute to the success of this project. I would like to express special gratitude to Dr. Rebecca Lamb for her writing correction and other matters. Additionally, I am also appreciative to all my committee members who encouraged me and advised me.
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Chapter 1

Introduction

In eukaryotic cells, maintaining the genomic integrity and fidelity of chromosomes is critical for cell cycle progression (Nasmyth, 2005). One mechanism to ensure genomic fidelity are cell cycle checkpoints, such as the Spindle Assembly Checkpoint (SAC) (Zhou et al., 2002). The SAC functions during the transition from metaphase to anaphase. In prometaphase, when not all kinetochores are attached to microtubules in a bi-polar manner, the chromosomes move to the metaphase plate through the pulling forces of microtubules (Hoffman et al., 2001; Tanaka et al., 2005; Zhou et al., 2002). As the cell cycle continues to metaphase, all chromosomes are finally aligned at the metaphase plate. Only after all the chromosomes are attached to microtubules in a bi-polar manner does the cell cycle proceed to anaphase (Hoffman et al., 2001). During this metaphase-to-anaphase transition if even a single kinetochore remains unattached, the cell cycle will be arrested in metaphase until the SAC is fulfilled (De Souza et al., 2009a; Nasmyth, 2005). Without microtubule attachment, SAC proteins, such as Mitotic Arrest Deficient proteins (MAD1, MAD2, MAD3) and Budding Unperturbed by Benzimidazole proteins (Bub1, Bub2, and Bub3), all localize to the kinetochores and are initiating signals to inhibit the
activity of the Anaphase Promoting Complex/Cyclosome (APC/C) (Suijkerbuijk and Kops, 2008). Upon proper tethering of microtubules to the kinetochores in a bi-polar manner, MAD1 and MAD2 are removed and the SAC is turned off. This activates the APC and, as a result, the sister chromatids are separated (Howell et al., 2001; Musacchio and Salmon, 2007). The SAC checkpoint was first identified and well characterized in yeast. However, some emerging proteins involved in the SAC pathway do not have homologs in yeast, suggesting that more complex eukaryotes might have evolved a more elaborate mechanism. Metazoan proteins such as Rod, Zw10, and Zwilch, which play crucial but poorly understood roles in cell division, are the best examples (Buffin et al., 2005). This indicates that there exist unexplored mechanisms of SAC pathway exclusive to multicellular eukaryotes.

In plants, the SAC proteins MAD2, BUB3.1, and BUBR1 have been identified and characterized (Caillaud et al., 2009). MAD2 was first identified in maize, where it is associated with kinetochores both in mitotic cells and during meiosis I and II (Yu et al., 1999). During mitosis, ZmMAD2 (maize MAD2) localizes to kinetochores in prometaphase and disappears from the kinetochores during metaphase, correlated with the attachment of k-fibers. In meiosis, the disappearance correlates less with microtubule attachment and more with the distance between the kinetochores, consistent with a tension-based mechanism. Kinetochore association of MAD2 was also reported in wheat (Kimbara et al., 2004). In addition, Caillaud et al. (2009) reported the localization dynamics of Arabidopsis MAD2, BUB3.1 and BUBR1 in tobacco suspension cultured
cells (Caillaud et al., 2009). They showed that Arabidopsis MAD2, BUB3.1 and BURB1 are concentrated at kinetochores if the SAC is activated by the microtubule-destabilizing herbicide propyzamid. Without microtubule destabilization, all three proteins are found diffusely distributed in the nucleus and cytoplasm. In addition, BUB3.1 appeared to have a specific location at the phragmoplast/cell plate during cytokinesis.

Recently, an intriguing connection between the SAC proteins MAD1 and MAD2 and the nuclear pore complex has emerged in several organisms (Buffin et al., 2005; De Souza et al., 2009b; Lee et al., 2008). The common theme of these findings is that MAD1 and MAD2 are found associated with the nuclear periphery during interphase and that the inner nuclear basket nucleoporin Tpr (mammals), Mtor (Drosophila), or Mlp1/2 (yeast, Aspergillus) is involved in this association (De Souza et al., 2009a; Lee et al., 2008; Lince-Faria et al., 2009; Scott et al., 2005). However, differences exist between the studied systems. For example, while in mammalian cells Tpr binds both MAD1 and MAD2, Mtor binds only MAD2 and yeast Mlp1/2 are required for MAD1/2 localization, but without direct interaction (Lee et al., 2008; Lince-Faria et al., 2009; Scott et al., 2005). Depletion of Tpr leads to a loss of MAD2 from the kinetochores, reduced MAD1 at kinetochores (Lee et al., 2008) and to chromosome segregation defects. Mtor depletion leads to a reduction of the Mad2 signal at kinetochores and an accelerated entry into anaphase, consistent with a weakened checkpoint (Lince-Faria et al., 2009). Depletion of Mlp1/2 leads to absence of MAD1/2 association with the nuclear rim (Niepel et al., 2005;
Scott et al., 2005). These data indicate that the presence of the SAC proteins are at the pore in interphase somehow primes them for their function during mitosis.

In plants, the connection between the nuclear pore and the spindle assembly checkpoint has not been addressed. Here, we show that the Arabidopsis protein NUA, the homolog of Tpr, Mtor, and Mlp1/2, binds to Arabidopsis MAD1 (AtMAD1) and that AtMAD1 is associated with the nuclear periphery. AtMAD1 binds to AtMAD2, which is located in the nucleus and cytoplasm with some enrichment at the NE. In a NUA mutant, AtMAD1 is displaced from the NE and the AtMAD2 NE signal also disappears. Importantly, in the NUA null allele nua-4, root length and meristematic size are affected in a way similar to the AtMAD2 allele mad2-2, in which no full length AtMAD2 transcript was detected. These data indicate that the interaction of SAC proteins with the nuclear pore is conserved beyond the opistokonts, and that NUA and MAD2 are involved in root development in plants.
Chapter 2

Materials and Methods

2.1 Plant materials

T-DNA insertion mutant lines nua-1 (SALK_057101), nua-4 (WiscDsLox297300_17E), and atnup160-3 (SAIL_877_B01) have been characterized previously (Muthuswamy and Meier, 2011; Xu et al., 2007a; Xu et al., 2007b). T-DNA insertion mutant lines mad2-2 (SAIL_191_G06), mad2-3 (SALK_125904) and mad2-4 (SALK_136419) were acquired from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University, Columbus, OH). Homozygous mutants for the T-DNA insertion were identified by PCR-based genotyping. Arabidopsis thaliana wild type (Columbia ecotype, WT-Col) and T-DNA insertion lines were grown in soil under standard long-day condition (16 hour light and 8 hour dark) at 22°C, or on Murashige and Skoog (MS) (Murashige T., 1962) plates under constant light at 22°C. MS plates with 2% sucrose were used unless indicated otherwise.

2.2 Protein sequence analysis
To identify putative *Arabidopsis* homologs of human MAD1 (hMad1) (Genebank ID: Q9Y6D), MAD2 (hMad2) (Genebank ID: Q13257), and yeast YRA1 (Genebank ID: CAY78884), full-length amino acid sequence of these genes were searched using PSI-BLAST and PHI-BLAST in Genbank (performed by Dr. Sivaramakrishnan Muthuswamy) (Muthuswamy, 2009). The identified amino acid sequences with significant similarity to hMAD1, hMAD2, and yeast YRA1 were then confirmed as putative AtMAD1, AtMAD2 and AtYRA1 using WU-BLAST at TAIR with the same full-length amino acid sequences of hMAD1, hMAD2, and yeast YRA1.

### 2.3 Genotyping of T-DNA insertion lines

Genomic DNA was extracted according to a published protocol (Krysan et al., 1999). Primers were designed using IDT iSci tools ([http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/](http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/)). The T-DNA insertion sites were determined by aligning the sequence of each T-DNA insertion line from SALK ([http://signal.salk.edu/cgi-bin/tdnaexpress?JOB=TEXT&TYPE=GENE&QUERY=At3g25980](http://signal.salk.edu/cgi-bin/tdnaexpress?JOB=TEXT&TYPE=GENE&QUERY=At3g25980)) and the genomic sequence of *AtMAD2*. The T-DNA insertion site of *mad2-2* was also confirmed by sequencing the product amplified with the T-DNA specific primer pCSA110-LB and gene specific primer MAD2-1GpR. For *mad2-3* and *mad2-4*, primer pairs MAD2-3GpF/MAD2-3GpR or MAD2-3GpF/MAD2-4GpR and MAD2-3GpF/LBa4 were used to genotype the T-DNA insertion lines. For *nua-4*, primer pairs CS850695FP/p745-primer and CS850695RP/p745-primer were used as previously described (Xu et al., 2007b) (all
primers are in table 2.1).

2.4 Root length, meristem size and meristematic cortex cell number analysis

Seeds of WT-Col, *nua-4*, *mad2-2*, *mad2-3*, *mad2-4*, and lines over-expressing GFP-AtMAD2 (GFP fused to the N-terminus of AtMAD2) were grown on MS plates with either 2% or 0% sucrose under constant light at 22°C. After 8 days of growth, the primary root lengths of a minimum of 45 seedlings were measured. To check the division zone (DZ) size, eight-day old seedlings were stained with 10 µg/µl propidium iodide (PI) for 7 min. Confocal images were taken with a Nikon Eclipse 90i confocal microscope. The joint connection of the division zone and the elongation zone was determined based on Perilli *et al.* (Perilli and Sabatini, 2010). The size in the division zone was measured using the Nikon NIS-Elements Microscope Imaging Software (Figure 3.5D) and the cell number of the meristematic zone was counted. Statistic significance of differences was calculated by Student’s t-test using the Microsoft Excel software package.

2.5 RNA extraction and quantification

Total RNA was extracted with the RNeasy Plant Mini kit (Qiagen, U.S.A.) from 5-day-old *Arabidopsis* seedlings grown on MS plates with 2% sucrose. One microgram of the total RNA was incubated with 1 µl DNase I (Invitrogen, U.S.A.). After digestion, cDNA synthesis was performed using oligo-dT primers and the SuperScript III First-Strand Synthesis System (Invitrogen). The cDNAs were used as templates in PCR (RT-PCR) and Quantitative Real Time PCR (Q-PCR) amplification with gene-specific primers (See
Table 2.1 for primers). For RT-PCR, one microliter reverse-transcribed cDNA was used as template in a total of 50 µl amplification mixture and 30 PCR cycles were run. PCR products were separated on 1% agarose gels and band intensities were quantified with the software ImageJ developed by the National Institutes of Health (Collins, 2007). All band intensities were first normalized against the Actin II control and then expressed relative to the WT level set to one. The experiment was repeated five times, three of which are biological repeats. Statistical significance was tested with a T-test at the significance level of 0.01 using the Microsoft Excel software package. Q-PCR was performed using the iQ™ SYBR® Green Supermix system (Bio-Rad). 1 µl cDNA solution was used as template in 20 µl assay volume. Forty cycles were run with CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Standards were run in triplicate on each plate and two biological repeats were conducted.

2.6 Plasmid Vector Construction

Construction of pENTR/D-TOPO vectors bearing AtMAD1, AtMAD2, and AtYRA1 cDNAs have been previously described (Muthuswamy, 2009). The inserts were confirmed by sequencing. To construct the N-terminal GFP fusion proteins, each cDNA was inserted into the Gateway binary vector pGWB6 (Nakagawa et al., 2007) by LR recombination cloning (Invitrogen). To construct the vector expressing HA-AtMAD1, the AtMAD1 full coding region was moved from pENTR/D-TOPO into pEarlyGate102 by LR recombination. For Bimolecular Fluorescence Complementation (BiFC) constructs (NUA-CGFP, AtMAD1-NGFP, AtMAD2-NGFP, and RanGAP-NGFP) driven by CaMV
35S promoter, a multisite Gateway reaction was conducted to generate translational fusions driven by the CaMV 35S promoter in the pH7m34GW and pK7m34GW destination vectors according to available protocols (Boruc et al., 2010).

2.7 Yeast two-hybrid assays
Yeast two-hybrid (Y2H) vectors pDEST22 and pDEST32 (Invitrogen) harboring AtMAD1, AtMAD2, and AtYRA1 full coding regions were constructed according to published protocols (Dohmen et al., 1991; James et al., 1996) and Y2H assays were conducted as described in the Clonetech Yeast Protocols Handbook (1996). This experiment was performed by Dr. Sivaramakrishnan Muthuswamy (Muthuswamy, 2009).

2.8 Generation of transgenic plants
Expression vectors were transformed into Agrobacterium tumefaciens (Agrobacterium) strain GV3101 by electroporation. Arabidopsis WT-Col were transformed by floral dip (Clough and Bent, 1998) with pGWB6 harboring AtMAD1 or AtMAD2 and selected by Kanamycin (50µg/ml) resistance for primary transformants. The T1 generation of transformants was subjected to further analysis, such as immunolabeling, primary root growth and fluorescence analysis.

2.9 Agrobacterium infiltration, BiFC and immunoprecipitation
Agrobacterium strains containing constructs expressing NUA-CGFP, AtMAD1-NGFP, AtMAD2-NGFP, and RanGAP-NGFP were incubated overnight in LB liquid cultures
with the corresponding antibiotic at 28 °C. Agrobacterium was then collected by centrifugation and re-suspended with infiltration buffer (50 mM MES, 2 mM MgCl₂, and 100 mM acetosyringone) with a final OD₆₀₀ of 0.4. For BiFC analysis, Agrobacterium mixture containing constructs NUA-CGFP and AtMAD1-NGFP, NUA-CGFP and AtMAD2-NGFP or NUA-CGFP and RanGAP-NGFP were infiltrated into *Nicotiana benthamiana* epidermal leaves as described (Boruc et al., 2010; Yang et al., 2000). Three days later, the epidermal cells were examined for GFP fluorescence by Nikon Eclipse 90i confocal microscopy. RanGAP1-NGFP was used as an unrelated protein to serve as negative control.

For immunoprecipitations (IPs), combinations of GFP-AtMAD2 and HA-AtMAD1 (GFP-AtMAD2/HA-AtMAD1) or GFP and HA-AtMAD1 (GFP/HA-AtMAD1) were transiently co-expressed in *N. benthamiana* epidermal leaves for 3 days with RNA silencing suppressor P19 (Zhao et al., 2006). The following steps were conducted at 4 °C. About four hundred microliter of tissue powder from the *N. benthamiana* leaves containing combinations of GFP-AtMAD2/HA-AtMAD1 or GFP/HA-AtMAD1 were suspended with 1 ml IP buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 3 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (1:100 dilution, Sigma-Aldrich, St. Louis, USA)). After centrifugation at 16,000 g for 10 min, the supernatant was incubated with antibody anti-HA (Sigma-Aldrich) (1:200 dilution) or anti-GFP (Invitrogen) (1:100 dilution) bound to protein A-Sepharose for 3 h. After centrifugation at 1000 g for 3 min, the immunoprecipitates were then washed for 3 times.
with IP buffer and suspended in 50 µl 1× SDS-PAGE loading buffer and subjected to 12% SDS-PAGE and immunoblotting with anti-GFP antibody (A11122, 1:2000; Molecular Probes, Eugene, OR) and HRP-conjugated anti-HA antibody (1:2000). The HRP-conjugated anti-rabbit secondary antibody (1:15,000, GE healthcare, Piscataway, NJ) was used to probe the anti-GFP antibody. The Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL, U.S.A.) for the HRP system was used for further detection.

2.10 BY-2 cell culture and transformation

Bright Yellow-2 (BY-2) tobacco (Nicotiana tabacum) cell suspension cultures were maintained and transformed according to protocol as described by Joubès et al. (2001) (Joubes et al., 2004) with slightly modification. BY-2 cell culture were grown in modified Murashige and Skoog medium (BY-2 medium) (David and Perrot-Rechenmann, 2001) at 25°C in the dark and were transferred to fresh medium every 7 days. To generate BY-2 cells stably expressing GFP-AtMAD2 driven by the 35S promoter, 5 ml of a suspension of stationary-phase cells grown for 7 d were transferred to 50 ml of fresh medium. After 2 days, 5 ml of BY-2 cells and 500 µl of Agrobacterium (OD600 =0.8) bearing the plasmid pGWB6 containing AtMAD2 were co-inoculated in Petri dishes in the dark for 3 d without agitation. Then, the cells were plated on BY-2 medium with 0.8% agar containing 50mg/ml kanamycin and 500mg/ml carbenicillin. After 3 weeks, individual resistant calli were transferred into fresh solid BY-2 medium containing 50mg/ml kanamycin and 500mg/ml carbenicillin for maintenance or were re-
suspended in liquid BY-2 medium containing 50mg/ml kanamycin and 500mg/ml carbenicillin to obtain liquid suspension of transgenic cells expressing GFP-AtMAD2. To investigate the localization of GFP-AtMAD2 during mitosis, 5 ml of BY-2 cells expressing GFP-AtMAD2 was transferred to fresh BY-2 medium for 3 days and cells were stained for 30 min with Draq 5 (1:50 dilution, Biostatus Limited, UK), a live cell DNA dye. For the detection of GFP in the green channel, the 488-nm excitation line of an Argon laser was used in combination with a 515/30-nm band-pass emission filter. To detect the DNA stained with Draq 5 in the far red channel (blue color in Figure 3.10), the 635-nm excitation of a modulated diode laser was used in combination with a 650LP long-pass emission filter. The Nikon EZ-C1 software was used for image capture.
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Table 2.1. Primers used in this study
Chapter 3

Results

3.1 Identification of Arabidopsis AtMAD1 and AtMAD2

The nucleoporins Nup153 and Tpr have been functionally implicated in cell cycle and development though their interaction with the SAC proteins MAD1 and MAD2 in mammalian cells, Aspergillus and yeast (De Souza et al., 2009a; Lee et al., 2008; Lussi et al., 2010; Scott et al., 2005). To investigate a potential connection between plant nucleoporins and the SAC, homologs of the known MAD1 and MAD2 proteins were identified from Arabidopsis. Using full length amino acid sequences of human MAD1 and MAD2 (hMAD1 and hMAD2, Genebank Accession No. NP_001013859 and CAA89321, respectively), a BLAST search against the translated Arabidopsis genome was performed. The translated open reading frame with the highest similarity to hMAD1 (e-value 4e-11) was encoded by the gene At5g49880, which had been annotated as potential Arabidopsis MAD1 homolog in the Arabidopsis Information Resource (TAIR) database (Muthuswamy, 2009). AtMAD1 had also been identified as a long coiled-coil protein with over 50% coiled-coil coverage in the ARABI-COIL Arabidopsis coiled-coil protein database (Rose et al., 2004). The open reading frame with the highest similarity to
hMAD2 was encoded by the gene At3g25980 (e-value 2.5e-35). This gene was annotated as *Arabidopsis* MAD2 in both Genebank and TAIR.

### 3.2 Characterization of AtMAD2 T-DNA insertion lines

Three *Arabidopsis* lines with T-DNA insertions in *AtMAD2* were selected from the SALK T-DNA insertion line collection (Alonso et al., 2003; Sessions et al., 2002) (Figure 3.1A) and given the mutant allele names *mad2-2*, *mad2-3*, and *mad2-4*. The allele *mad2-2* has a T-DNA insertion at nucleotide +579 (with +1 being the A of the start codon ATG), which is within exon 4 (Figure 3.1A). Flanking sequences of the *mad2-2* T-DNA insertion site were amplified by PCR and sequenced to confirm the insertion site (data not shown). Although the insertion sites of *mad2-3* and *mad2-4* were mapped to the same site at -392 (with +1 being the A of the start codon ATG), *mad2-3* and *mad2-4* are still shown as two separate alleles on the SALK and TAIR website with different e-values indicating the accuracy of the mapped site. Moreover, genes contained in the T-DNA regions of different alleles may be silenced after generations, affecting the expression of downstream genes in different alleles. Therefore, in this study we characterized them as separate alleles.

To identify whether full-length transcripts of *AtMAD2* were present in these lines, RT-PCR was performed with primer pair 3 (Figure 3.1). The results showed that no full-length *AtMAD2* transcript was detected in *mad2-2* after 30 PCR cycles (Figure 3.1B and 3.1C) or 40 PCR cycles (data not shown), indicating that most likely no full-length
AtMAD2 protein is present in mad2-2. When using quantitative real time RT-PCR (Q-PCR) to quantify the amount of AtMAD2 transcripts with a primer pair amplifying exon 6 and exon 7 (Figure 3.1A), a significantly higher amount of transcript was detected in mad2-2 than in WT (Figure 3.2). Together, these data indicate that - consistent with the site of the T-DNA insertion - no full length AtMAD2 transcript can be detected but that the region of the AtMAD2 gene 3’ of the T-DNA insertion is transcribed, likely as a run-through transcript initiated in the T-DNA.

For mad2-3 and mad2-4, slightly higher AtMAD2 transcript levels compared with WT were detected with RT-PCR using primer pair 3 (Figure 3.1B and 3.1C). Quantification of band intensities from 5 experiments suggests that the relative AtMAD2 mRNA levels in mad2-3 and mad2-4 are indeed higher than in WT (p<0.01) (Figure 3.1C). Consistent with these results, Q-PCR analysis also detected significantly higher levels of AtMAD2 transcripts in mad2-3 and mad2-4 (Figure 3.2). In summary, we conclude that no full-length AtMAD2 mRNA accumulates in mad2-2 and that somewhat increased levels of full-length AtMAD2 mRNA accumulate in mad2-3 and mad2-4.

3.3 Phenotypic analysis of AtMAD2 T-DNA mutants

It has been shown that MAD2 regulates cell cycle progression through inhibiting anaphase entry until all chromosomes are attached to microtubules in a bi-polar manner (Skinner et al., 2008). In mice, MAD2 down-regulation initiates apoptosis (Dobles et al., 2000), whereas MAD2 over-expression triggers tumor formation (Sotillo et al., 2007). To
investigate does the cell cycle proceed in *Arabidopsis* cell division and growth, we focus on the phenotypes related to the primary root of *AtMAD2* T-DNA insertion alleles that are either null for *AtMAD2* (*mad2-2*) or slightly over-accumulating *AtMAD2* mRNA (*mad2-3 and mad2-4*), since established techniques are available for us to study further the mechanisms at the cellular level if any phenotypes are observed from roots. At 8 days post germination, roots of *mad2-2* seedlings grown in the absence of sucrose were significantly stunted (Figure 3.3A, and 3.3G), whereas *mad2-3* and *mad2-4* roots were slightly longer than WT seedling roots (Figure 3.3C, 3.3E and 3.3G). Addition of 2% sucrose partially rescued the *mad2-2* growth inhibition (Figure 3.3B and 3.3G), while it had only a marginal effect on *mad2-3* and *mad2-4* (Figure 3.3D, 3.3F, and 3.3G). To investigate whether the small, but significant increase in root growth observed in *mad2-3* and *mad2-4* is due to the overexpression of *AtMAD2*, lines expressing GFP-*AtMAD2* driven by a CaMV 35S promoter were also investigated. T1 transgenic seedlings from five independent lines expressing GFP-*AtMAD2* (shown by GFP fluorescence, data not shown) also had longer roots after growth on MS plates with 2% sucrose for 8 days (Figure 3.4), similar to *mad2-3* and *mad2-4*. These data suggest that overexpression of *AtMAD2* might promote primary seedling root growth, while loss of *AtMAD2* full-length transcripts clearly inhibits primary seedling root growth in *Arabidopsis*.

The postembryonic growth of the plant root occurs from localized regions, the meristems. In the primary *Arabidopsis* root, stem cells in the quiescent center generate cells which divide a finite number of times in the proximal meristem (called the division zone (DZ)).
They then enter a zone of rapid cell elongation and differentiation in the elongation-differentiation zone (EDZ). Elongation of the primary root is thus regulated by the rate of cell division in the meristem and the rate of elongation/differentiation in the EDZ (Dello Loio et al., 2008). The altered root length of AtMAD2 T-DNA insertion alleles could thus be attributed either to altered meristematic division or to root elongation abnormalities. Since AtMAD2 was shown to be primarily expressed in meristematic tissues in Arabidopsis (Caillaud et al., 2009), it was most likely that a meristematic abnormality would be the primary cause for the altered primary root length in the AtMAD2 T-DNA insertion alleles.

The size of the Arabidopsis seedling root meristem can be quantified by measuring the length from the root tip to the cell wall immediately below the first cortex cell undergoing cell elongation, or, alternatively, by counting one row of cortex cells up to this point (Perilli and Sabatini, 2010). As shown in Figure 3.3, in the absence of sucrose, mad2-2 has a very shorter root meristem and fewer meristematic cortex cells, while mad2-3 and mad2-4 have slightly longer division zones, but not significant more cells in the meristematic cortex zone (Figure 3.3H and 3.3I). These suggest that mad2-2 might have defects of cell division in the division zone, and that mad2-3 and mad2-4 have similar cell division rate as WT and larger cell size. In the presence of 2% sucrose, mad2-2 still has shorter root meristem and mad2-3 and mad2-4 still have slightly longer division zone (Figure 3.3H). However, mad2-2, mad2-3 and mad2-4 have similar cell numbers in the meristematic cortex zone in the presence of 2% sucrose (Figure 3.3I),
suggesting that they might have very similar cell division rate in these mutant alleles, and that \textit{mad2-2} has smaller cell size and \textit{mad2-3} and \textit{mad2-4} have larger cell size in the division zone. (Figure 3.3H and 3.3I). These data suggest that the primary effect of AtMAD2 on seedling root growth occurs in the division zone, consistent with its expression pattern in \textit{Arabidopsis} root tissues as AtMAD2 is only expressed in the root division zone (Caillaud et al., 2009).

Homologs of NUA, Tpr/Megator/Mlp1p/Mlp2/An-Mlp1 have been demonstrated as NE anchors of SAC proteins in mammalian cells, \textit{Drosophila}, yeast and \textit{Aspergillus}, suggesting a functional involvement in the proper function of SAC proteins (De Souza et al., 2009a; Lee et al., 2008; Lince-Faria et al., 2009). Thus, the primary root growth, root meristematic zone size and meristematic cortex cell number were also checked in \textit{nua-4}, a knock out line of NUA (Xu et al., 2007b). Consistent with the implication of their functional connection, \textit{nua-4} and \textit{mad2-2} have such similar phenotypes in seedling roots with stunted primary root development (no development beyond the cotyledon stage) in the absence of sucrose, shorter primary roots in the presence of 2% sucrose, and smaller DZ size in the absence and presence of 2% sucrose (Figure 3.5A, 3.5B, 3.5C and 3.5E).

### 3.4 Physical interactions among NUA, AtMAD2 and AtMAD1

The mammalian homolog of NUA (Tpr) directly interacts with MAD1 and MAD2 in immunoprecipitation assays and MAD1 interacts with MAD2 at the nuclear rim and at the kinetochores (Lee et al., 2008). To investigate the physical interactions between
Arabidopsis NUA, MAD1, and MAD2, yeast two-hybrid (Y2H) assays were performed (Muthuswamy, 2009). The Y2H data supports AtMAD1-AtMAD1, AtMAD1-NUA, and AtMAD1-AtMAD2 interactions, but no NUA-AtMAD2 interaction. To confirm the interaction between AtMAD1 and AtMAD2 in planta, co-immunoprecipitation (CoIP) assays were carried out. Either GFP-AtMAD2 and HA-AtMAD1 or GFP and HA-AtMAD1 were transiently expressed in Nicotiana benthamiana leaf epidermis (Figure 3.6A and 3.6B, left lane). After immunoprecipitation with a GFP antibody, both GFP-AtMAD2 and GFP were detected in the precipitates from GFP-AtMAD2/HA-AtMAD1 and GFP/HA-AtMAD1 infiltrated leaves (Figure 3.6B, middle lanes). However, HA-AtMAD1 was only detected in the precipitates from GFP-AtMAD2/HA-AtMAD1 infiltrated leaves (Figure 3.6A, top panel right lane), but not from GFP/HA-AtMAD1 infiltrated leaves (Figure 3.6A bottom panel right lane). When the anti-HA antibody was used to precipitate proteins from GFP-AtMAD2/HA-AtMAD1 and GFP/HA-AtMAD1 infiltrated leaves, GFP-AtMAD2 was detected in the precipitate (Figure 3.6B top panel right lane), but not GFP (Figure 3.6B bottom panel right lane). These data indicate that AtMAD1 and AtMAD2 associate in planta.

Full-length GFP-NUA or NUA-GFP could not be immunoprecipitated to a detectable level in our hands, likely due to extreme protein instability during the IP protocol (data not shown). Therefore, the in planta interactions between NUA and AtMAD1 or NUA and AtMAD2 were investigated by BiFC analysis (Figure 3.7). Co-expression of NUA-CGFP (NUA fused to the C-terminal half of GFP) and AtMAD1-NGFP (AtMAD1 fused
to the N-terminal half of GFP) resulted in the reconstituted GFP complexes in the cytoplasm of *N. benthamiana* epidermal cells. No GFP fluorescence was detected in the negative-control combination NUA-CGFP and RanGAP1-NGFP (RanGAP1 fused to the N-terminal half of GFP) (Figure 3.7B). Additionally, no GFP fluorescence was detected in co-expression of NUA-CGFP and NGFP-AtMAD2 (AtMAD2 fused to the N-terminal half of GFP, Figure 3.7C)). These data suggest that, consistent with the yeast two-hybrid data, NUA interacts with AtMAD1 but not AtMAD2. The fact that reconstituted GFP fluorescence was detected only in the cytoplasm of *N. benthamiana* epidermal cells suggests that the NUA-AtMAD1 complex does not associate with the nuclear envelope in this heterologous system.

In summary, NUA interacts with AtMAD1, but not AtMAD2 in an Y2H assay and in BiFC analysis. AtMAD1 interacts with AtMAD2 in both Y2H and CoIP assays. These data are consistent with a complex consisting of NUA, AtMAD1 and AtMAD2 (with AtMAD1 acting as a bridge between NUA and AtMAD2) or with two separate complexes, one containing AtMAD1 and NUA and the second containing AtMAD1 and AtMAD2.

### 3.5 Subcellular localization of AtMAD1 and AtMAD2

MAD1 and MAD2 in mammalian cells are associated with the nuclear envelope (NE) in interphase (Lee et al., 2008). Given that AtMAD2 and NUA mutants have similar root growth defects and NUA, AtMAD1, and AtMAD2 physically interact, we questioned if
AtMAD1 and AtMAD2 are associated with the NE and whether this interaction requires NUA. To determine the localization of AtMAD1 in interphase, transgenic *Arabidopsis* lines expressing GFP-AtMAD1 driven by the CaMV 35S promoter were generated. When roots of the T1 seedlings were imaged by confocal microscopy, GFP-AtMAD1 was concentrated at the NE (Figure 3.8A and 3.8B). Co-immunolocalization utilizing anti-GFP and anti-NUA antibodies to simultaneously detect GFP-AtMAD1 and NUA showed clear co-localization of the two signals at the NE (Figure 3.8C). Consistent with the interaction between AtMAD1 and NUA in Y2H and BiFC assays, this suggests that AtMAD1 associates with NUA at the NE in *Arabidopsis*.

To investigate the localization of AtMAD2, transgenic *Arabidopsis* Col-0 lines were created that express GFP-AtMAD2 driven by the CaMV 35S promoter. Fluorescence signals from roots of live T1 transgenic seedlings containing GFP-AtMAD2 showed a distribution between the nucleus and the cytoplasm and a concentration at the NE (Figure 3.9A). GFP-AtMAD2 was also found distributed between the nucleus and the cytoplasm in the epidermal cells of *N. benthamiana* transiently expressing GFP-AtMAD2 (Figure 3.9B and 3.9C) and in BY-2 cells stably expressing GFP-AtMAD2 (Figure 3.10A to 3.10C). In both cases, no concentration at the NE was observed. This is consistent with the prior report of AtMAD2 localization in the nucleus and cytoplasm in epidermal cells of *N. benthamiana* and BY-2 cells (Caillaud et al., 2009). To further address the localization of AtMAD2 in *Arabidopsis*, the MAD2 (C19) (anti-hMAD2) antibody against human Mad2 (SC-6329; Santa Cruz Biotechnology Inc.) was used in an
immunofluorescence experiment using wild type Col-0 seedlings. Using BLASTP analysis from NCBI, the alignment between Human Mad2 and AtMAD2 showed 47% identity and 68% similarity at the amino acid level over the entire length of the protein, suggesting that this polyclonal serum might successfully detect AtMAD2. The antibody decorated the nuclear rim co-labeled by the anti-NUA antibody (Figure 3.9D to 3.9F), suggesting that a fraction of AtMAD2 and NUA co-localize at the NE. However, the anti-hMAD2 antibody did not detect any protein in immunoblots of whole Arabidopsis seedlings extracts (data not shown). It is possible that the concentration of AtMAD2 is too dilute in a whole-seedling protein extracts to be observed by immunoblotting, since the promoter activity of AtMAD2 is primarily confined to the meristematic regions (Caillaud et al., 2009; Muthuswamy, 2009). Alternatively, the antibody might detect AtMAD2 in fixed cells but not after SDS-PAGE. Additionally, using immunofluorescence microscopy with the anti-GFP and anti-NUA antibodies, co-localization of AtMAD2 and NUA at the NE could also be shown in the root tip cells of transgenic seedlings expressing GFP-AtMAD2 (Figure 3.9M to 3.9O). These data indicate that AtMAD2 is located in the nucleus and the cytoplasm and that a fraction of AtMAD2 co-localized with NUA at the Arabidopsis NE. For reasons currently not clear, this fraction is more clearly revealed by immunofluorescence microscopy than by live imaging of GFP.

3.6 Requirement of NUA for the nuclear envelope localization of AtMAD1 and AtMAD2

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In HeLa cells, the nuclear rim localization of human MAD1 and MAD2 requires the proper NE localization of NUA’s homolog Tpr through their direct interaction with Tpr (Lee et al., 2008). To investigate whether NE localization of NUA is also required for the proper NE localization of AtMAD1 in *Arabidopsis*, we attempted to create transgenic *nua-1* and *nua-4* lines expressing GFP-AtMAD1 under the control of the CaMV 35S promoter. For *nua-1*, primary transformants were obtained, however, they did not develop past the cotyledon stage (Muthuswamy, 2009). For *nua-4*, no primary transformants expressing GFP-AtMAD1 were obtained. When heterozygous *nua-4* plants expressing GFP-AtMAD1 were self-segregated, no homozygous *nua-4* stably expressing GFP-AtMAD1 could be obtained in the progeny. Therefore, one possible reason for these data could be that over-expression of GFP-AtMAD1 in *NUA* null mutants might be lethal. Using immunolabeling with anti-GFP and anti-NUA antibodies, GFP-AtMAD1 could be detected in the nucleoplasm of cells of the cotyledon-stage primary transformants of *nua-1* stably expressing GFP-AtMAD1 (Muthuswamy, 2009), suggesting that the NE association of GFP-AtMAD1 requires NUA (Muthuswamy, 2009).

Because AtMAD2 might indirectly interact with NUA through AtMAD1, AtMAD2 NE localization might also depend on NUA. Immunolabeling analysis using anti-hMAD2 and anti-NUA indicated that the anti-hMAD2 antibody did not decorate the NE in *nua-1* (Muthuswamy, 2009) or *nua-4* (Figure 3.9G to 3.9I). To test if the disappearance of AtMAD2 from the NE was specific for the loss of NUA, or was caused by a more generic
defect in mutant nuclear pores, we also tested AtMAD2 localization in *nup160-3*, a mutant of the scaffold nucleoporin Nup160, which has very similar developmental and molecular phenotypes as *nua-1* and *nua-4* (Muthuswamy and Meier, 2011). Both anti-NUA and anti-hMAD2 still decorated the NE in *nup160-3* (Figure 3.9J to 3.9L). Together, these data suggests that NUA is also required for the proper NE localization of AtMAD2.

### 3.7 Kinetochore localization of AtMAD2

In metazoan cells, when the SAC was activated by preventing the spindle assembly with microtubule poisons, MAD2 was specifically associated with the kinetochores (Chen et al., 1996; Lee et al., 2008). In maize, wheat and *Arabidopsis* MAD2 was also shown to localize to kinetochores when the SAC were chemically activated (Caillaud et al., 2009; Kimbara et al., 2004; Yu et al., 1999). However, maize ZmMAD2 was also shown to localize to kinetochores in prometaphase without any microtubule poison treatment (Yu et al., 1999). To further gain insights into the dynamic localization of AtMAD2 during mitosis, a BY-2 suspension culture cell line was created that stably expressed GFP-AtMAD2 (GFP fused to the N-terminus of AtMAD2) under the control of the CaMV 35S promoter. This orientation of fusion was shown to not disrupt the function of MAD2 in human cells (Howell et al., 2000).

The mitotic stages were determined based on the condensation of DNA visualized by live staining with Draq5 (Biostatus) (Martin et al., 2005). GFP-AtMAD2 showed a strong
nucleoplasmic and a weaker cytoplasmic signal in interphase cells (Figure 3.10A to 3.10C). In prophase, GFP-AtMAD2 was found somewhat concentrated in bright spots (possibly the kinetochores) when DNA began to condense (Figure 3.10D through 3.10F). In prometaphase, when DNA began to align at the metaphase plane, but trailing chromosomes were still detected, GFP-AtMAD2 was concentrated in bright spots on the condensed chromosomes (Figure 3.10G through 3.10I). When cells were in metaphase (chromosomes perfectly aligned at the metaphase plane, no trailing chromosomes detected), GFP-AtMAD2 was not detected at the kinetochores (Figure 3.10J through 3.10L). No further concentration of GFP-AtMAD2 was observed during anaphase and telophase, defined by the beginning and progressing separation of the chromosomes (Figure 3.10M to 3.10O). The same localization of GFP-AtMAD2 in bright spots resembling kinetochores was also observed in cells not counterstained with Draq5 (data not shown), suggesting that it is not due to Draq5 treatment. These data indicates that AtMAD2 is likely associated with the kinetochores and suggest that the plant SAC is activated in prophase and remains active until metaphase, consistent with the predictions based on the other model organisms.
Figure 3.1. Characterization of *AtMAD2* T-DNA Insertion Lines.

(A) Position of T-DNA insertions and PCR primers. The confirmed insertion sites of *mad2-2*, *mad2-3*, *mad2-4* are shown by vertical arrows above and below the schematic genomic structure of the *AtMAD2* gene. Primer pairs 1 and 2 were used for *mad2-3*, *mad2-4* and *mad2-2* mutant genotyping. Primer pair 3 was used for full length *AtMAD2* RT-PCR. Primer pair 4 was used for quantitative real-time PCR. (B) RT-PCR products amplified with primer pair 3 indicated in (A) and *Actin II* primer pair (See primer summary in table 1). The *Arabidopsis* genotypes are indicated on the top. WT, *Arabidopsis* ecotype Col-0 wild type (C) Quantification of band intensity of five RT-PCR experiments (as shown in B) using the software ImagJ. Asterisks indicate a significant difference from WT (at least p<0.05).
Figure 3.1. Characterization of *AtMAD2* T-DNA Insertion Lines.
Figure 3.2. Quantitative analysis of AtMAD2 expression in wild-type Arabidopsis and AtMAD2 mutant alleles.

Five-day-old Arabidopsis seedlings grown on MS with 2% sucrose were obtained and total RNA was extracted. Quantitative real-time PCR was performed with primer pair 4 (Figure 3.1) to analyze the expression level of AtMAD2 transcript. Error bars represent standard deviation (n = 6).
Figure 3.2. Quantitative analysis of the AtMAD2 expression in wild-type *Arabidopsis* and *AtMAD2* mutant alleles.
Figure 3.3. Phenotypic characteristics of *AtMAD2*-mutant seedling roots.

(A, C and E) Seedlings at 8 days after germination on MS plate with 0% sucrose, grown under long-day conditions. (B, D and F) Seedlings at 8 days after germination on MS plate with 2% sucrose, grown under long-day conditions. (G) Length quantification of seedling roots grown as in (A through F). A minimum of 45 seedlings each were measured. (H) Length quantification of the division zone of seedling roots grown as in (A through F). Ten seedlings each were randomly picked from the seedlings used in (G) to measure the length of the division zone. Asterisks indicate significant difference from WT (p<0.05).
Figure 3.3. Phenotypic characteristics of *AtMAD2*-mutant seedling roots.
Figure 3.4. Phenotypic characteristic of seedling roots of GFP-AtMAD2 expressing lines.

(A) Primary root length of at least 25 seedlings each was measured and recorded. Asterisk indicates significant difference to WT (p<0.05).

(B) RT-PCR products amplified with primer pair 3 and Actin II (ACT2) primer pair (see Table 2.1). Plant materials are indicated on the top.
Figure 3.4. Phenotypic characteristic of seedling roots of GFP-AtMAD2 expressing lines.
Figure 3.5. Phenotypic characteristics of *nua-4* seedling roots.

(A and B) Representative 8-day old seedlings grown on MS with 0% and 2% sucrose under long-day conditions. (C) Primary root length of 8-day old seedlings grown on MS with 0% and 2% sucrose under long-day conditions. Mean values and standard deviation for at least 45 seedlings each are shown. (D) An 8-day old WT seedling stained with propodium iodide. Length of the division zone and position of the first elongating cell in the cortex (inset) are indicated. (E) Length of the division zone of 8-day old seedlings grown on MS with 0% and 2% sucrose under long-day conditions. Ten seedlings each were randomly picked from the experiment shown in (C). Asterisk indicates significant difference compared to WT (at least p<0.05).
Figure 3.5. Phenotypic characteristics of nua-4 seedling roots.
Figure 3.6. AtMAD2 interacts with AtMAD1 in a Co-Immunoprecipitation (CoIP) assay.

Proteins GFP-AtMAD2 and HA-AtMAD1 (as shown GFP-AtMAD2/HA-AtMAD1) or GFP and HA-AtMAD1 (GFP/HA-AtMAD1) were co-expressed in epidermal cells of *Nicotiana benthamiana* leaves and are indicated on the right of each panel (*A* and *B*). HA-AtMAD1, GFP-AtMAD2 and GFP on the left of each panel (*A* and *B*) are used to represent the protein of bands in each panel (*A* and *B*). Input indicates that protein extract was directly detected for HA-AtMAD1, GFP-AtMAD2 and GFP with anti-HA and anti-GFP antibodies. IP indicates that protein extracts (GFP-AtMAD2/HA-AtMAD1 or GFP/HA-AtMAD1) were pulled down with either anti-HA or anti-GFP antibodies shown under IP and the precipitates were detected with the same antibody in Western blotting (*A* and *B*). CoIP indicates that protein extracts (GFP-AtMAD2/HA-AtMAD1 or GFP/HA-AtMAD1) were pulled down with either anti-HA or anti-GFP antibodies shown under CoIP and the precipitates were detected with the other antibody in Western blotting (*A* and *B*).
Figure 3.6. AtMAD2 interacts with AtMAD1 in a Co-Immunoprecipitation (CoIP) assay.
Figure 3.7. NUA and AtMAD1 interact in an in planta BiFC assay.

Imaging of epidermal leaf cells of *N. benthamiana* infiltrated with *A. tumefaciens* co-expressing NUA-CGFP and AtMAD1-NGFP (A), NUA-CGFP and RanGAP-NGFP (B) or NUA-CGFP and NGFP-AtMAD2 (C). Left panels, GFP fluorescence; middle panel, differential interference contrast (DIC); right panel, merge of GFP and DIC images. Arrows in (A), indicate fluorescence from reconstituted split GFP. CGFP, C-terminal fragment of GFP; NGFP, N-terminal fragment of GFP. The confocal images were recorded at the same gain setting. Scale bars = 50 µm.
Figure 3.7. NUA and AtMAD1 interact in an in planta BiFC assay.
Figure 3.8. Localization of GFP-AtMAD1 in the *Arabidopsis* root division zone.

(A and B) Live fluorescence images of GFP-AtMAD1 in root tip cells of *Arabidopsis*. (A) Root tip. (B) A single root-tip cell. (C) Immunofluorescence images of root tip cell files. The green and red channels were probed with first antibodies anti-GFP and anti-NUA, respectively, and with corresponding second antibodies. White arrows in (A), (B), and (C) indicate nuclear envelope. Scale bar in (A) = 50 µm. Scale bar in (B) = 5 µm. Scale bar in (C) = 2 µm.
Figure 3.8. Localization of GFP-AtMAD1 in the Arabidopsis root division zone.
**Figure 3.9. Localization of AtMAD2**

Live fluorescence images of GFP-AtMAD2 in root tip cells of *Arabidopsis* (A) and epidermal cells of *N. benthamiana* (B and C). (B) shows a whole cell and (C) shows the nucleus of the cell in (B). Immunofluorescence images of root tip cell files in interphase (D through O). Green in (D), (G), and (J) indicate proteins decorated by anti-hMAD2 antibody; green in (M) indicate proteins detected by anti-GFP antibody; red in (E), (H), (K) and (N) indicate proteins detected by anti-NUA antibody. (D), (G), (J), and (M) show the green channel only. (E), (H), (K), and N show the red channel only. (F), (I), (L), and (O) show the green and red channels combined. The arrows in (A) indicate the concentrated GFP-AtMAD2 signals. The arrows in (F) and (I) indicate the nuclear envelope. Scale bars (A and C) = 5 µm. Scale bar (B) = 50 µm. Scale Bars (D through O) = 2 µm.
Figure 3.9. Localization of AtMAD2
Figure 3.10. Localization of GFP-AtMAD2 during various stages of mitosis in live tobacco BY-2 cells.

Green channel indicates the GFP signal from GFP-AtMAD2 and blue channel indicates the signal from DNA stained with Draq5. Fluorescence images of GFP-AtMAD2 in interphase (A to C), prophase (D to F), prometaphase (G to I), metaphase (J to L), and anaphase/telophase (M to O). Green in A, D, G, J, and M, GFP; blue in B, E, H, K, and N, DNA stained with Draq5. A, D, G, J, and M show the green channel only. B, E, H, K, and N show the blue channel only. C, F, I, L, and O show the green and blue channels. The arrows in D and G point at the enrichment of GFP-AtMAD2 in the vicinity of the kinetochores. Scale bars = 5 µm.
Figure 3.10. Localization of GFP-AtMAD2 during various stages of mitosis in live tobacco BY-2 cells.
Chapter 4

Discussion

The SAC protein MAD1 is conserved in mammalians, Drosophila, C. elegans, Aspergillus, and yeast (De Souza et al., 2009a; Kastenmayer et al., 2005; Kitagawa, 2009). It has been shown that MAD1 is associated with the nuclear envelope in interphase and re-locates to the kinetochores when the SAC is inactivated (De Souza et al., 2009c; Kastenmayer et al., 2005; Lee et al., 2008). In mice, MAD1 is an essential protein and a MAD1 null mutant is embryo lethal (Iwanaga et al., 2007). MAD1 had not been investigated in plants. Our data now show that Arabidopsis AtMAD1 is also associated with the NE in interphase. Its association with the NE requires NUA, the Arabidopsis homolog of the nuclear basket protein Tpr (Muthuswamy, 2009). Consistent with results in metazoans, AtMAD1 interacts with NUA and AtMAD2 in Y2H (Muthuswamy, 2009), BiFC, and CoIP assays. These data indicate that MAD1 proteins are conserved across species, including plants.

MAD2 proteins have also been shown to be conserved in mammalians, Drosophila, yeast, C. elegans, and plants (Barnhart et al., 2011; Caillaud et al., 2009; Lee et al., 2008;
Orr et al., 2007b; Tarailo-Graovac et al., 2010). Amino acids alignment shows that MAD2 proteins have three conserved motifs (LXGS, VXEFF and NSILYXR) at the N terminus and an evolutionally conserved HORMA domain (Aravind and Koonin, 1998; Kimbara et al., 2004; Nasmyth, 2005). As a key SAC component, MAD2 delays the onset of anaphase until all chromosomes are attached to microtubules in a bi-polar manner by inactivating CDC20, a regulatory subunit of the APC (Elledge, 1998; Fang et al., 1998). Down-regulation of MAD2 proteins promotes transition from metaphase to anaphase and increases the chromosome segregation errors in mammal cells, which initiate apoptosis (a programmed cell death process) (Dobles et al., 2000). Although AtMAD2 was first identified in 2009, its effect on Arabidopsis development has not been characterized (Caillaud et al., 2009). We have investigated here three T-DNA insertion alleles of AtMAD2, which affect the size of the seedling primary root. mad2-2, an allele lacking the full-length AtMAD2 transcript, has a shorter primary root in the absence of sucrose. This stunted primary root growth might be caused by the defect in cell division or in cell growth since a smaller root division zone and less number of meristematic cortex zone in the absence of sucrose. When 2% sucrose was added, the cell division defect of mad2-2 was rescued, since the meristematic cortex cell number rises to the similar level as WT, although the cell division zone size is still a slightly smaller than WT. Thus, the shorter primary root in mad2-2 with addition of 2% sucrose might be caused by the smaller cell size in the meristematic zone. These data suggest that sucrose promotes the cell division in the root, which has been suggested through up-regulation of CycD expression (Eveland and Jackson, 2011; Riou-Khamlichi et al., 2000), and that
AtMAD2 is involved in *Arabidopsis* root cell growth, since cell size in the meristematic cortex zone is smaller in *mad2-2*, loss of full-length AtMAD2. One possibility for the similar cell division rate of *mad2-2* and WT in the presence of 2% sucrose could be that some truncated AtMAD2 still function like full-length AtMAD2 to make the SAC function properly. Another possibility is the proper function of an AtMAD2-independent SAC, which has been demonstrated in *Drosophila* (Orr et al., 2007a). The phenotypes for *mad2-2* are very similar to that of *nua-4*, a null mutant of the nucleoporin NUA, suggesting that the two proteins are both required for a step in root meristem growth, the disturbance of which can be overridden by exogenous sucrose supplementation.

In contrast, the two promoter insertions *mad2-3* and *mad2-4* lead to an increased abundance of *AtMAD2* mRNA, somewhat longer primary roots and an increased meristem size. This effect is particularly revealed in the presence by exogenous sucrose, suggesting that an overabundance of AtMAD2 facilitates sucrose-based stimulation of growth (Hirano et al., 2011; Rolland et al., 2006). Consistent with these data, the 35S promoter-driven expression of a GFP-AtMAD2 fusion protein also leads to increased root length in the presence of sucrose, supporting the above notion and suggesting that GFP-AtMAD2 is functional. Together, these data suggest that AtMAD2 has a function in regulating root meristem development in *Arabidopsis*, consistent with the finding that AtMAD2 is expressed predominantly in meristematic tissues and the known role in other model systems of MAD2 as a SAC regulator that controls cell cycle progression (Caillaud et al., 2009).
Although mad2-3 and mad2-4 both have larger division zone size, their meristematic cortex cell number is similar to that in WT in the absence and in the presence of 2% sucrose, indicating that their cell division rate might be similar to WT and the cell size in the meristematic cortex zone are bigger than WT. One possibility for the similarity of cell division rate in mad2-3, mad2-4 and WT might be that SAC is still properly fulfilled in the mad2-3 and mad2-4 alleles, even AtMAD2 are over-expressed in these two mutant lines. For larger meristematic cortex cell size in mad2-3 and mad2-4 indicated by their longer division zone and similar cell number in the meristematic zone, one possibility is the involvement of AtMAD2 in cell growth, which is also suggested by the primary root growth phenotypes of mad2-2 allele.

Alternatively, the data of the division size and meristematic cortex cell number in mad2-2, mad2-3, mad2-4 and WT might mean that we don’t have data from enough seedlings to draw any conclusions, since only ten seedlings were measured from each line. To improve the accuracy of these data and the final conclusions, large data sets should be collected and analyzed.

NUA is the Arabidopsis homolog of a long coiled-coil nucleoporin named Tpr, Mtor, Mlp1, Mlp2 in mammalian systems, Drosophila and yeast (Xu et al., 2007b). In HeLa cells and yeast, interactions of Tpr and Mlp1/Mlp2, respectively, with the SAC proteins MAD1 and MAD2 have been demonstrated (Lee et al., 2008; Scott et al., 2005). In HeLa
cells, Tpr directly interacts with MAD1 and MAD2 (Lee et al., 2008). Although no physical interaction of Mlp1p and Mlp2p with yeast Mad1p and Mad2p was shown, Mad1p requires Mlp1p and Mlp2p for nuclear rim association (Scott et al., 2005). Based on our Y2H, BiFC and CoIP assays, the physical interactions among the NUA and MAD1/MAD2 vary between the different organisms investigated. In Arabidopsis, NUA interacts with AtMAD1, but not AtMAD2, while AtMAD1 interacts with AtMAD2. Thus, the physical attachment of the MAD1/MAD2 complex with the nuclear pore might be based on different affinities of the SAC proteins for nucleoporins in different organisms that lead to the same outcome: an association of MAD1/MAD2 with the NE in interphase. In Arabidopsis, NUA, AtMAD1 and AtMAD2 can be in the same complex if NUA interacts with AtMAD2 though its association with AtMAD1 as intermediate. Consistent with this hypothesis, AtMAD1 and AtMAD2 co-localize with NUA at the nuclear rim in interphase and disappear from the NE in NUA null mutants, suggesting that NUA functions as a NE scaffold for AtMAD1 and AtMAD2. It is currently not resolved whether the disappearance of AtMAD1 and AtMAD2 from the NE in NUA null mutants is also accompanied with a reduction in protein abundance or is solely a delocalization effect.

In addition to the NE localization of AtMAD1 in live and fixed Arabidopsis root tip cells, AtMAD1 also localizes to the cytoplasm in the epidermal cells of N. benthamiana, observed from the BiFC assay. One possibility for this might be that N. benthamiana is an exogenous system for Arabidopsis AtMAD1 and factors required for the NE
localization of AtMAD1 are missing in the *N. benthamiana* system. AtMAD2 was also detected different localization based on different methods. GFP-AtMAD2 showed a distribution between the nucleus and the cytoplasm in the root tip cells of *Arabidopsis*, in the epidermal cells of *N. benthamiana* and in live BY-2 cells. The nucleus and cytoplasm location of AtMAD2 is conserved through the *Arabidopsis*, *N. benthamiana*, and BY-2 cells. However, AtMAD2 is shown the NE localization in immunofluorescence assays when AtMAD2 was detected with anti-hMAD2 antibody and GFP-AtMAD2 was detected with anti-GFP antibody. The discrepancy of the AtMAD2 localization revealed by immunofluorescence microscopy and by live imaging of GFP is probably due to the technique difference between immunofluorescence microscopy and live imaging of GFP. When whole amount immunofluorescence was used to show AtMAD2 localization, the unbounded AtMAD2 in the nucleus and cytoplasm might be washed out from the cell by the MTSB buffer (Friml et al., 2003) and only the bounded AtMAD2 still stays in the NUA/AtMAD1/AtMAD2 complex at the NE. The NE bounded AtMAD2 could be probed with the anti-hMAD2 antibody and anti-GFP antibody. Alternatively, reasons currently not known might also be involved in the more AtMAD2 NE localization revealed by immunofluorescence microscopy than by live imaging of GFP.

MAD2 has been demonstrated to be a key sensor for the attachment of microtubules to kinetochores (Fang et al., 1998; Nasmyth, 2005). In live PtK1 cells, GFP-hMAD2 (GFP fused to the N-terminus of human MAD2) and GFP-XMAD2 (GFP fused at the N-terminus of frog MAD2) were distributed throughout the cell in interphase and began to
concentrate at the kinetochore during prophase and early prometaphase (Howell et al., 2000). Once cells progressed to metaphase, GFP-hMAD2 and GFP-XMAD2 signals disappeared from kinetochores remained off the kinetochores during the following anaphase and telophase (Howell et al., 2000). Consistent with the localization dynamics of GFP-hMAD2 and GFP-XMAD2, GFP-AtMAD2 was also found in the nucleus and less prominently in the cytosol in live BY-2 cells and Arabidopsis root tip cells. Along with the condensation of DNA in prophase/prometaphase, GFP-AtMAD2 began to concentrate at kinetochore-like spots but could not be found concentrated in spots once chromosomes were fully aligned in the metaphase plane. These results differ from the reported localization of AtMAD2-GFP (GFP fused to the C-terminus of the AtMAD2) in prometaphase in live BY-2 cells (Caillaud et al., 2009), where no AtMAD2-GFP concentration at kinetochores was seen during the entire cell cycle with drug treatment. It was thus proposed that the AtMAD2-dependent SAC is not activated in normal mitosis of BY-2 cells (Caillaud et al., 2009). This discrepancy is possibly due to the orientation of the GFP fusions used in the two studies. It is conceivable that only GFP-AtMAD2, but not AtMAD2-GFP can associate with the kinetochores to a level detectable by fluorescence microscopy during undisturbed cell cycle. GFP at the C-terminus of MAD2 might interfere with MAD2 function because it has been shown in mammalian cells, Xenopus and yeast that the C-terminus of MAD2 is required to bind MAD1, Tpr and Cdc20 (Chen et al., 1999; Fang et al., 1998; Lee et al., 2008). Alternatively, the discrepancy is possibly only due to the different exogenous expression levels of GFP-AtMAD2 and AtMAD2-GFP and the undetectable AtMAD2-GFP at the kinetochores in
live BY-2 cells. AtMAD2 with GFP at its C-terminus can be functional properly as AtGFP-MAD2 localizes to kinetochores in live BY-2 cells treated with the microtubule-destabilizing herbicide propyzamid (Caillaud et al., 2009).

Together, our data suggests that aspects of MAD1/MAD2 function are conserved in plants. These include the interaction of AtMAD1 and AtMAD2 with the NE in interphase, the dependence of this interaction on the nuclear pore protein NUA, and a functional role of AtMAD2 in meristematic cell division. Although plants differ in some other aspects drastically from animals and fungi, such as the acentrosomal spindle assembly (Vos et al., 2008), the SAC might be evolutionary old and functional in similar ways in all three groups of organisms (Basu et al., 1998; Howell et al., 2004).
References


