CHARACTERIZATION OF ARABIDOPSIS THALIANA KINETOCHORE PROTEINS

by

SARAH RUSHING

(Under the Direction of R. Kelly Dawe)

ABSTRACT

The kinetochore is a highly conserved group of proteins involved in proper chromosome segregation by binding to the microtubules. Recent studies have identified more than 80 kinetochore components, many of which have a broad evolutionary conservation from yeast to humans. Among these are MCM21, NDC80, NUF2 and SPC25 that are predicted to be located in the core kinetochore regions and have been shown to have homologues within plants. Through mutant analysis by T-DNA and RNAi, and expression analysis using fluorescent fusion proteins, we attempted to characterize the function and localization of these proteins to further confirm their conservation in plants. We showed that SPC25 localizes to the kinetochore and is constitutively expressed throughout the tissues. It was also shown that mutation of SPC25 and NDC80 has adverse effects on the mutant plant's development compared to wildtype plants. Based on our data, we suggest that MCM21 is not a conserved feature of the plant kinetochore and SPC25 and NDC80 are most likely conserved in plants. Due to questionable data, we were unable to make any conclusions regarding the location and function of NUF2.

INDEX WORDS: Kinetochore, Centromere, *Arabidopsis thaliana*, HTR12, NDC80, SPC25, MCM21, NUF2

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DEDICATION

I dedicate this thesis to my precious fiancé, Brad. Without his love and support, this might simply never have been.

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I know I was just going to thank a few specific people, but it looks like I kept on talking as usual. Much love, Sarah

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The kinetochore is a eukaryotic specific, multi-protein structure that assembles on centromeric DNA and plays a fundamental role in accurate chromosome segregation in mitosis and meiosis. Other kinetochore functions include sister chromatid cohesion and separation, microtubule attachment, and chromosome movement. Despite poor conservation of centromeric DNA, kinetochore function has shown to be highly conserved from yeast to plants to humans (Meraldi *et al*. 2006).

Included in the kinetochore are two main regions: the inner kinetochore that is tightly associated with the centromeric DNA, and the outer kinetochore that interacts directly with microtubules. Within the inner kinetochore are "foundation" proteins that have direct involvement with the centromeres and are highly conserved. The plant homologs for these proteins have been previously described and include Centromeric Histone H3 (Talbert *et al*. 2002), Centromeric Protein-C (Dawe *et al*. 1999), and Minichromosome Instability 12 (Sato *et al*. 2005). CENH3, which is known as HTR12 in *Arabidopsis thaliana*, is a histone H3-like variant that replaces conventional H3 in the nucleosome core of centromeric chromatin. HTR12 is required for recruitment and assembly of kinetochore proteins (Talbert *et al*. 2002). CENPC binds to centromeric DNA (Du and Dawe, unpublished), is present at centromeres throughout the cell cycle, and is necessary but not sufficient for kinetochore assembly (Fukagawa *et al.* 1999). MIS12 was identified in fission yeast as an essential centromeric protein. It is part of the

kinetochore MIND complex that is required for accurate chromosome segregation (Sato *et al*. 2005).

Most investigations of centromeric proteins have dealt with yeast and mammalian systems. However, due to the discovery of CENPC homologs in *Zea mays* (Dawe *et al.* 1999) and CENH3 in *Arabidopsis* (Talbert *et al*. 2002), it has been suggested that similar centromeric proteins are being used for kinetochore assembly in plants (Sato *et al*. 2005). Strong molecular homologies for proteins outside of the foundation complex have also been detected in many organisms. As interpreted by Meraldi *et al*. 2006, we can hypothesize that there are four multi-protein complexes that form the inner core of the kinetochore: the MIND complex, the NDC80 complex, the SPC105 complex, and the COMA complex. These protein complexes are believed to be the linkers between the foundation proteins that bind directly to the centromeric DNA and the outer proteins that bind directly to the microtubules. In some models however, the NDC80 complex is shown to bind directly to the microtubules rather than the outer proteins (as proposed by Meraldi *et al*.), which have been postulated to be mostly regulatory (Ciferri *et al*. 2007).

Among the best-conserved proteins from yeast to mammals are Nuclear Division Cycle 80 (NDC80; NDC80 complex), Spindle Pole Component 25 (SPC25; NDC80 complex), Mini-Chromosome Maintenance 21 (MCM21; COMA complex), and Nuclear Filament containing (NUF2; NDC80 complex). These four proteins were chosen based on this known conservation, the availability of accession numbers from a previous study (Meraldi *et al.* 2006), and prior data revealing that NDC80 localizes to the core kinetochore in *Zea mays* (Du & Dawe, 2006). This data was an indication that the rest of the complex (SPC25 and NUF2) might also be conserved. NDC80, NUF2, and SPC25 have been studied in budding yeast, Xenopus, and humans (Janke *et al.* 2001, Wigge & Kilmartin 2001, McCleland *et al.* 2003, and Bharadwaj *et al.* 2004), and have

been shown to have sequence similarity across many different species, including plants. However, as of yet, there is no information confirming that these proteins localize to the core kinetochore in plant systems.

NDC80 and COMA Complex

Recent studies have shown that the four proteins within the NDC80 complex, NDC80, NUF2, SPC24 and SPC25, have close homologs in all eukaryotes (Janke *et al.* 2001, Wigge & Kilmartin 2001, McCleland *et al.* 2003, Bharadwaj *et al.* 2004), and that loss of function mutations in these proteins cause yeast chromosomes to detach from the spindle microtubules resulting in high rates of chromosome loss (Wigge & Kilmartin 2001). It was also noted that an inactivation of either NDC80 or NUF2 causes the other to be lost from the kinetochore and degraded. SPC25 and SPC24 however, remain in the absence of NDC80 or NUF2. This observation correlates with the position of these proteins within the NDC80 complex with respect to one another (Figure 1) (Ciferri *et al*. 2005). There are two main domains: the microtubule binding domain which includes the NDC80/NUF2 dimer, and the centromere binding domain which houses the SPC24/SPC25 dimer (Ciferri *et al.* 2007). Ciferri *et al.* also hypothesize that the SPC24/SPC25 dimer mediates the interaction of the NDC80 complex with the inner kinetochore region. Figure 1 shows an illustration of this proposed model.

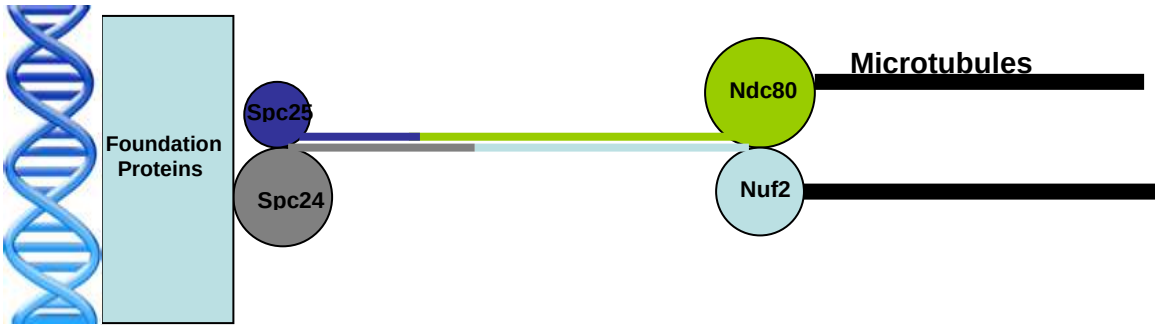

Figure 1: NDC80 complex containing an Ndc80/Nuf2 dimer that binds directly to the microtubules and a Spc25/Spc24 dimer that binds to the inner kinetochore region.

Another core complex of interest is the COMA complex which houses MCM21. Like the NDC80 complex, the COMA complex has been shown to serve as the bridge between the subunits that have direct contact to the centromeric DNA and the subunits that bind to the microtubules. Mutations in MCM21 have been shown to cause a decrease in the stability of the minichromosomes in yeast (Poddar *et al.* 1999).

In this study, the objective was to determine if these proteins were indeed part of the plant kinetochore. If so, are they constitutively expressed similar to the foundation proteins or are they peripheral in their function and not required for accurate chromosome segregation? Furthermore, do NDC80, NUF2, and SPC25 display similar characteristics to one another compared with MCM21? Finally, can we help to confirm the architectural features of Meraldi's four-complex kinetochore model in plants?

Two basic approaches were taken to characterize these proteins: expression of fusion proteins to determine their localization, and mutation analysis through T-DNA and RNAi to interpret their necessity and function.

CHAPTER 2

MATERIALS AND METHODS

T-DNA Mutant Analysis

The four kinetochore mutants used in this study were T-DNA insertion alleles in the Columbia ecotype obtained from Arabidopsis Information Resource Center (TAIR) (www.arabidopsis.org) in the form of T4 seed. Accession numbers, T-DNA germplasm designations and T-DNA location within the genes are summarized in Table 1. To determine the genotype of each plant, cDNA from individuals from each line were amplified to determine the presence of the wildtype and/or insertion alleles. DNA was prepared using the DNeasy kit (Qiagen) from approximately 4 week old leaf tissue. For each mutant, specific primers were designed to amplify the wildtype allele and the insertion allele (Table 1). PCR products from flanking DNA were sequenced to determine the precise location of the T-DNA. Subsequent PCR reactions were performed to monitor the segregation of mutant and wild-type alleles and to ensure each mutant was in a genetic background displaying correct 3:1 segregation ratios. After PCR screening and propagation, T-DNA seeds were tested for kanamycin resistance by visually comparing seedlings growing on medium containing kanamycin (50 μg ml^{-1}) that appeared yellow and closed with those than remained unaffected by the antibiotic. To test the reliability of the kanamycin selection, resistant seedlings were compared with kanamycin plates containing all wildtype seedlings of the same ecotype. Once genotyped was again confirmed, homozygous mutant plants were backcrossed with wild-type Columbia plants to further reduce the likelihood of background mutations being present in the lines.

Table 1: Summary of T-DNA mutants as well as primer pair sequences used in genotyping for each wildtype and mutant allele. LBb1 is Left Border b1 which is a universal T-DNA specific primer.

Mutant	Locus name	T-DNA germplasm	T-DNA location	Wildtype allele primers	Insertion allele primers
nuf2-1	At1g61000	SALK_087432	2nd exon	Nuf2-1F: 5'-CGACTTTGTCTCCGAGCTTT 3' nuf2-1R: 5' AGCAACCTTTGCCTCACACT 3'	Nuf2-1F LBb1: 5'GCGTGGACCGCTTGCTGCAACT 3'
spc25-1	At3g54630	SALK_024260	1st exon	Spc25-1F: 5' GGTTTTGATTTAGAGCGATT 3' spc25-1R: 5' GCAAAACAATGTATGTAAGCA 3'	LBb1 Spc25-1R
ndc80-1	At3g54630	SALK_021583	promoter	Ndc80-1F: 5' TTTTGGGTTTAACAGGTGAG 3' ndc80-1R: 5' CACCGTCGTTCTTCTCTTT 3'	Ndc80-1F LBb1
mcm21-1	At5g10710	SALK_149069	1st exon	Mcm21-1F 5' CTGCTGATAGGCATGATAGGG 3' mcm21-1R 5' CACGGATGAGCAAATACTTATGA 3'	LBb1 Mcm21-1R

T-DNA Mutants: qRT-PCR Analysis

Once a homozygous line was identified, seed resulting from their self fertilization were plated on half strength Murashige and Skoog media (Murashige and Skoog, 1962). At 13 days of growth under long day conditions, approximately 100ng of fresh weight shoots were collected by weighing and used to isolate RNA using the Qiagen RNeasy plant mini kit. A total of 3 µg of total RNA was reverse transcribed using the Superscript III first-strand synthesis kit (Invitrogen) with random hexamer primers to produce cDNA. Quantitative RT-PCR was used to analyze cDNA populations using ubiquitin universal primers as the endogenous control. Real-time PCR was performed on an Applied Biosystems 7500 real-time PCR system using SYBR Green detection chemistry. Experiments are average relative quantities from at least two biological replicates. Primer sequences for each real-time reaction are listed in Table 2.

Table 2: Sequence and location of primers used for identifying T-DNA insertions and RT-PCR

Mutant	qRT-PCR Primers
nuf2-1	**Exon 1** Nuf2-1rtF1: 5' ACGAGTACCCGAGGCTCTCT 3' Nuf2-1rtR1: 5' CGAGGGCGTCGAGATAGATA 3' **Exon 2** Nuf2-1rtF2: 5' TTGGAGCAATTGGAGAAGCC 3' Nuf2-1rtR3: 5' CAAAAGTGCGCTGATGAAAA 3' **Exon 1&2** Nuf2-1rtF3: 5' AAAACCCCAACTTCCGACTT 3' Nuf2-1rtR3: 5' GGATGATTCAGGGCGTAAGA 3' **Exon 2&3** Nuf2-1rtF4: 5' TCATCAGCGCACTTTTGAAC 3' Nuf2-1rtR4: 5' ACCTTTGCCTCACACTGCTT 3'
spc25-1	**Exon 1** Spc25-1rtF1: 5' CTGGTGGAGACACAACGAAA 3' Spc25-1rtR1: 5' CTCAAGCCACTGCTCAAAGC 3' **Exon 2&3** Spc25-1rtF2: 5' AGCGGATCTGAGAGAAGCAT 3' Spc25-1rtR2: 5' GAATTATCTCCCAACAGTTGCA 3' **Exon 1&2** Spc25-1rtF3: 5' CTATGGCGTTCTGGGTCTC 3' Spc25-1rtR3: 5' TTGAAAGCGGATCTGAGAGAA 3' **Exon 3** Spc25-1rtF4: 5' GTAAAGAGGCACGGCAAATG 3' Spc25-1rtR3: 5' GAATTATCTCCCAACAGTTGCAAG 3'
ndc80-1	**Exon 1** Ndc80-1rtF1: 5' AAAAACAAAGAAATTCGAAGAGG 3' Ndc80-1rtR1: 5' TGGAACTGGATCGGGAAATA 3' Ndc80-1rtF2: 5' GCGGGAAAGAGAAGAACGAC 3' Ndc80-1rtR2: 5' GCGTCGGAGTCTCTTGAGTT 3' Ndc80-1rtF3: 5' CGTCCTTCATCAATCGGTTT 3' Ndc80-1rtR3: 5' GATCGGGAAGTTATGTGTGGA 3'

RNAi Primer Design and Two Step Subcloning

RNA interference constructs were assembled using vector pFGC5941 (www.Chromedb.org) for each of the four gene fragments. PCR primers consisted of the specific restriction sites used for cloning followed by gene specific sequence from the 5' and 3' ends for annealing (Table 3). The restriction sites flank a ChsA intron that is used as a non-specific loop that links the forward and reverse strands of the target gene (so it can form double stranded RNA). The primers were designed to yield a ~400 bp product in the 5' region of each gene. The inverted repeat was assembled directly into the binary vector by a two-step cloning process (Figure 2). In the first step, the PCR product was cleaved at the inner restriction sites, *AscI* and SwaI, and ligated to cleaved AscI and *SwaI* sites in pFGC5941. Fragments were sequenced to confirm identity before adding the second inverted segment using the outer

restriction sites, *BamHI* and *XbaI*. This produces an inverted repeat separated by the ChsA intron. The confirmed constructs were then transformed into *Agrobacterium tumefaciens* strain C58C1 and subsequently transformed into Arabidopsis using the floral dip method (Clough, 2005).

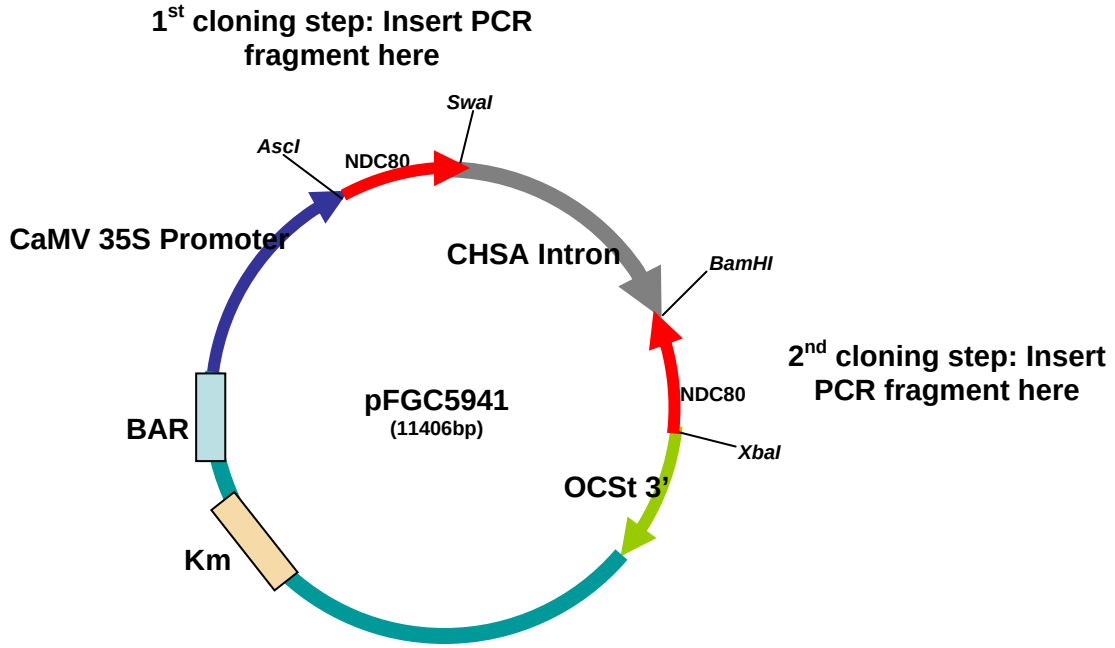

Figure 2: pFGC5941 vector and the two step subcloning process (Adapted from ChromDB). The first 400 bp of the cDNA was inserted into both orientations to produce the stem-loop structure within the CHSA Intron

Table 3: Gene specific RNAi primers. Cyan: *XbaI*, Magenta: *AscI*, Grey: *SwaI*, Red: *BamHI*

Gene	RNAi Primer pair
NUF2	NUF2RNAi-S: 5'-TACGCTTCTAGAGGCGCGCCATGTCAGCCTACGAGTACCCG-3' NUF2RNAi-A: 5'-TAGCTGGGATCCATTTAAATACCTTTGCCTCACACTGCTTT-3'
SPC25	SPC25RNAi-S: 5'-TACGCTTCTAGAGGCGCGCCATGGCGTCTCTGGGTCTCATC-3' SPC25RNAi-A: 5'-TAGCTGGGATCCATTTAAATAACGTGAAAACCGAGAGCATG-3'
NDC80	NDC80RNAi-S: 5'-TACGCTTCTAGAGGCGCGCCATGAGAGGCGGAGCCGCGGG-3' NDC80RNAi-A: 5'-TAGCTGGGATCCATTTAAATGAGGCGTATTTGGAGCTTTG-3'
MCM21	MCM21RNAi-S 5' –TACGCTTCTAGAGGCGCGCCATGGGAAAAATGATTGTTTC-3' MCM21RNAi-A 5' –TAGCTGGGATCCATTTAAATCGACTTGCTCTCAAGCACAC-3'

CFP and YFP tagging constructs

The coding region of each gene was amplified from mature flower cDNA of the Columbia ecotype using gene specific primers (Table 4). The fragments were cloned into the pENTR directional cloning vector (Gateway Technology, Invitrogen) which contains

recombination sites at the 5' and 3' ends. The gene within the entry vector was then recombined into the destination vector in the correct orientation for expression (ensured by the primer and vector design). The destination vectors used were pEarleyGate101, which contains a yellow fluorescent protein, and pEarleyGate102 that contains a cyan fluorescent protein (Earley *et al.* 2006). The recombination event between the entry and the destination vectors produced C-terminal fusions of the kinetochore proteins with YFP and CFP respectively, all under the control of the 35S promoter. All constructs used for transformation were sequence verified to ensure the correct, in-frame transcription of the gene and the fluorescent protein. Both vectors encoded a kanamycin resistance gene for bacterial selection and a BASTA herbicide resistance gene for plant selection. Transformation of the constructs occurred in Agrobacterium strain C58C1, which were subsequently transformed into wildtype Arabidopsis plants via the floral dip method (Clough, 2005). Transformants were selected on soil-grown plants by administering a 1:250 dilution of BASTA herbicide (Finale) to the first true leaves. The BASTA resistant T3 seeds were then sown on ½ Murashige and Skoog media and grown at long day conditions. On the fourth day, intact seedlings were removed from the medium for microscopic screening. A potential problem with these methods, specifically the use of the 35S promoter is that fused proteins can interfere with protein function causing abnormal or "overexpression" phenotypes within the cell.

Table 4: Gene specific primers used to amplify genes from Arabidopsis cDNA

Gene	cDNA Primer pair
NUF2	NUF2-F: 5' – CACCATGTCAGCCTACGAGTACCC -3' NUF2-R: 5' - GAGGCTTGGTAGAAACGCATCAAACGA -3'
SPC25	SPC25-F: 5' – CACCATGGCGTCTCTGGGTCTCAT -3' SPC25-R: 5' - TTTCTTACCCTTAAAGCGAGAAGAACGGC -3'
NDC80	NDC80-F: 5' – CACCATGAGAGGCGGAGCCG-3' NDC80-R: 5' - GATACCAAGGTTCTTCTTGAAGTTGGC-3'
MCM21	MCM21-F: 5' - CACCATGGGAAAAATGATTGTTTC-3' MCM21-R: 5' – GCTTGGACTAGTCTGAAATAATTG-3'

Tissue Preparation and Fluorescent Microscopy

After removal of 4 day old seedlings from ½ MS plates, the seedlings were placed on slides containing ½ MS liquid media. Cover slips were gently placed on top to prevent damaging the live tissue and sealed with nail polish. Cells were viewed using a Zeiss Axio Imager, and 3D images collected using Slidebook 4.0 software (Intelligent Imaging Innovations).

CHAPTER 3

RESULTS

Characterization of *nuf2-1*, *spc25-1*, *ndc80-1*, and *mcm21-1* insertion lines

T-DNA insertion lines were selected to identify loss-of-function mutants for the genes encoding NUF2, SPC25, NDC80, and MCM21; all of which are considered putative inner kinetochore proteins (Meraldi, 2006). Through sequencing of the T-DNA borders using T-DNA border specific primers (Table 1), the insertions were shown to be located in the 5' region of each gene (Figure 3). Upon further investigation, I found that the only publicly available insertion for NDC80 was actually in the 3'UTR of an upstream gene, *Tsa1*. It was also noted that in *spc25-1*, despite the annotated cDNA sequence on TAIR, a second start site was located in-frame downstream of the insertion (Figure 4). Quantitative realtime PCR however, failed for every primer set except for F3/R3 which showed no change in mRNA levels.

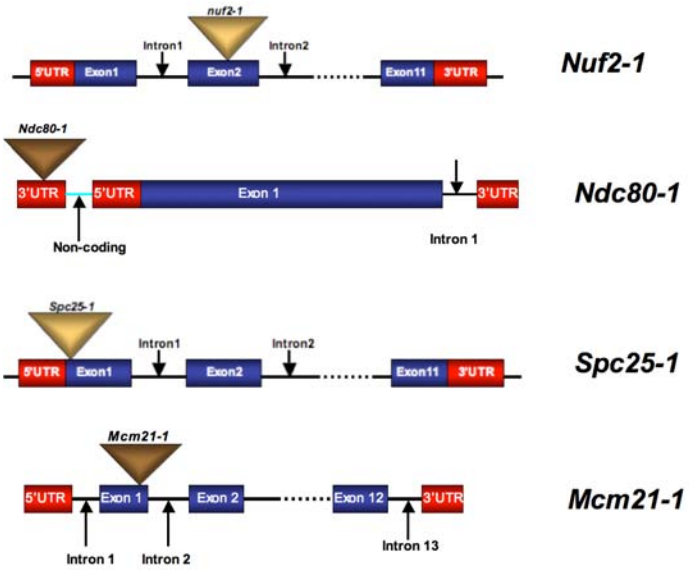

Figure 3: Location of T-DNA insertions within the four genes.

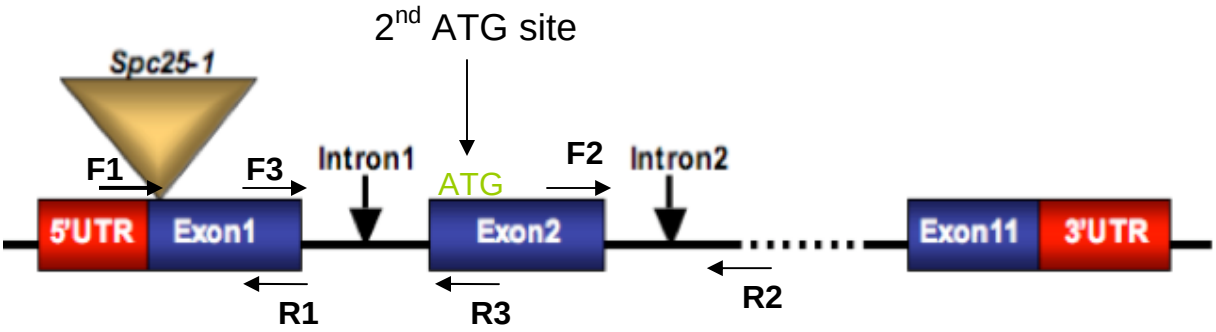

Figure 4: *Spc25-1* T-DNA insertion. The first start site in exon 1 was deleted by the T-DNA. A 2nd start site in- frame and downstream of the insertion in exon 2 was discovered. The deletion caused by the T-DNA insertion had no effect on the plant's phenotype or levels of mRNA expression. Basepairs #39-344 of the genomic DNA were deleted by the T-DNA. F and R designate primer pair locations.

Quantitative real-time PCR results showed no significant change in mRNA levels for *nuf2-1* primer sets F1/R1 and F4/R4. However, for primer sets F2/R2 and F3/R3, all PCR attempts failed (Figure 5). Quantitative real-time PCR results for *mcm21-1* were never completed based on the multiple failures of the previous experiments.

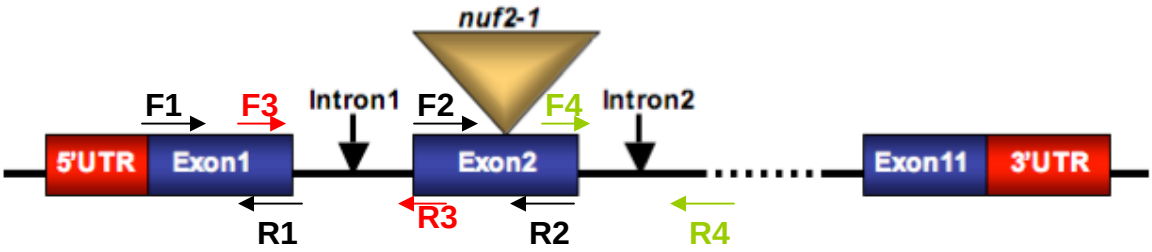

Figure 5: *Nuf2-1* T-DNA insertion located within exon 2 at basepair #563. There was no sequence deleted as a result of this insertion based on PCR of the T-DNA borders. Primer pairs are color coded for easier visualization.

Quantitative real-time PCR results for *Ndc80-1* showed a 50-60% reduction in transcript levels when compared with wildtype (Figure 6A). Figure 7A shows the comparison of wildtype and *ndc80-1* Ct values. Notice that it takes approximately eight more cycles for the *ndc80-1*

amplification to be detected than wildtype, indicating that there is ~2^8 increase in Ndc80 expression in the wildtype compared with the mutant.

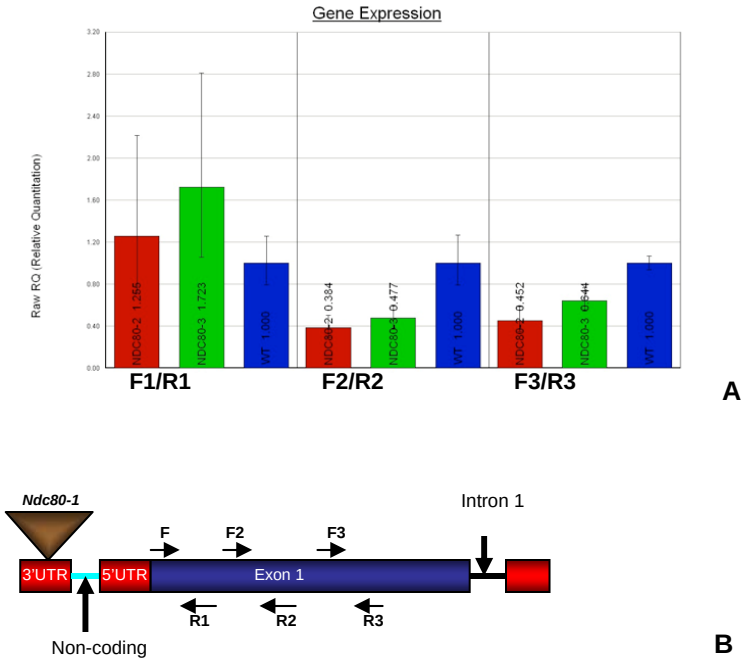

Figure 6: A) Relative quantity of NDC80 transcript in wildtype (blue) and ndc80-1 plants (red and green) as detected by real-time RT-PCR. There is approximately a 50-60% reduction in transcript level in the mutants compared with the wildtype. B) Diagram of the location of the T-DNA insertion in the 3' of TSAI. The real time primer locations are also shown.

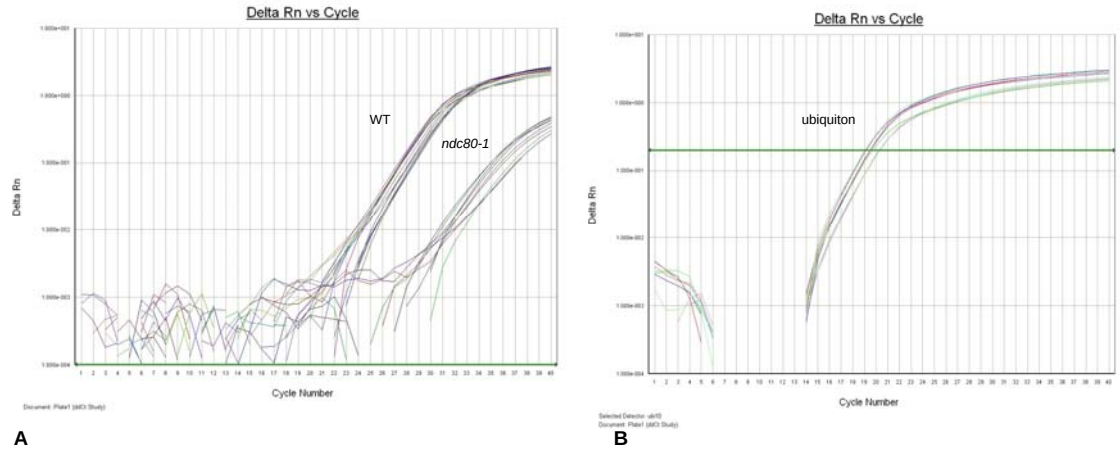

Figure 7: A) Real-time RT-PCR results show that wildtype expression of NDC80 expresses 2^8 times more mRNA that *ndc80*-1. B) Curve of the housekeeping gene ubiquiton that was used as a control. The *Rn* value represents the normalized reported fluorescence emission as the reporter dye is released, and the base line, shown in green is the point at which all reactions are linear.

Correlating with the decrease in mRNA levels, a subtle phenotype was observed in *ndc80-1* mutants whereby plants were prematurely senescing in the mature stages of growth. Normally at approximately 8-9 weeks of growth wildtype plants will begin senescing and losing pigment. However, *Ndc80-1* plants reached this mature stage at 1-2 weeks prior to wildtype (Figure 8).

Figure 8: The phenotype of premature senescence is shown in the ndc80-1 mutant on the right. Wildtype is shown on the left. All plants are at 9 weeks of growth.

The initiation of leaf senescence depends upon several factors. The age of the leaf and the reproductive phase of the plant, external factors such as nutrient deficiency, pathogenic attack, drought, light limitation, and temperature can all induce premature senescence (Smart, 1994). To date, there has not been any correlation between the decline of NDC80 expression and the acceleration of senescence in Arabidopsis. Due to these results and the lack of visible phenotypes relating kinetochore defects to any of the mutations, it is difficult to draw conclusions from these data.

RNAi Mutant Analysis

As an alternative approach to T-DNA mutagenesis, we used RNAi to establish a link between gene identity and gene function of NDC80, SPC25, NUF2 and MCM21. Since RNAi results in a knockdown (not a knockout) of gene expression, this allows us to observe the effects of a partial loss of function with phenotypes ranging from mild to severe. Because the core kinetochore proteins have been shown to be essential, (Choo, 1997), a full knockout would probably be lost in the gametophyte generation. Previous studies from our lab have shown that RNAi is an effective tool in determining *Mis12* function in *Zea mays* (Xuexian Li, unpublished). We speculated that this method might be useful for detecting more subtle, transmissable defects. In addition, the fact that RNAi is genetically dominant (faster to work with) made it a reasonable and rapid alternative for studying the chosen genes.

As soon as the RNAi lines were transformed and placed under Basta selection, it was apparent which lines were experiencing deficiencies as a result of the decrease in expression of the targeted genes. In wild type plants, transformation rates are typically at 1% (one transformant for every 100 seed harvested from Agrobacterium treated plants) (Clough and Bent, 1998). This rate was observed in our RNAi mutant line *Mcm21*-RNAi, and is used here as a control of sorts (since I did not perform a simultaneous empty vector control). To test transformation frequency, the number of plants produced by *Mcm21*-RNAi lines was compared to the other mutant lines. Approximately 40mg or 2000 seeds were planted for each line. After spraying with Basta, the *Mcm21* RNAi lines produced 22 resistant plants, *Ndc80* RNAi lines retained only 2 resistant plants and *Spc25* RNAi lines only retained one plant. Unfortunately, NUF2 was never transformed into the construct due to time constraints and cloning difficulties. The prediction had been that *Nuf2* RNAi lines would produce a similar phenotypic effect as

Ndc80-RNAi due to its relative location within the NDC80 complex and similar proposed role of binding directly to the microtubules.

Distinguishing the mutants with mild knockdown phenotypes from those with moderate to severe phenotypes was fairly straightforward. From germination to maturity, the phenotype of *Mcm21*-RNAi was identical to that of wildtype. Quantitative real time PCR results revealed that the mRNA expression levels of MCM21 compared with that of wildtype were also indistinguishable.

Following BASTA screening, the two *Ndc80* RNAi plants and one *Spc25* RNAi plant showed a dwarfed phenotype that was considerably stunted in growth compared to wildtype and *Mcm21* RNAi. At approximately five weeks following sowing, *Ndc80* RNAi plants had only produced 4 rosette leaves, each measuring at less than a centimeter in length, while the *Mcm21* RNAi lines had reached their adult height and produced 14 mature rosettes. The inflorescence of *Mcm21* RNAi was at 12 inches in height and of the flower buds produced, greater than 50% had opened (Figure 9A).

Spc25 RNAi expressed developmental deficiencies similar to that of *Ndc80* RNAi in the first four weeks after sowing. Such defects included small rosettes at 1-2cm long and diameters remaining at approximately 20% of their mature size. In comparison, at four weeks, rosette growth should be nearing completion in wildtype Columbia plants. However, within the next 2-3 weeks, *Spc25* RNAi developed rapidly at almost twice the rate as previously observed and produced flower buds in conjunction with *Mcm21* RNAi and wildtype. It is possible that a somatic clone emerged with weaker RNAi phenotype.

Meanwhile, the two *Ndc80* RNAi plants remained at ~20% of their mature rosette diameter at 6 weeks of growth and had many other obvious developmental defects that were

persistent well into maturity. Such defects included shortened siliques that reached only half the length of wildtype siliques, standard looking siliques that contained fewer or missing seeds, and delayed silique development by up to two weeks. Amongst these defects were most notably the presence of only a single inflorescences compared with wildtype which produced up to five inflorescences throughout its development. Another obvious defect was the occurrence of multiple siliques (usually between 4-8), developing at a single flower bud (Figure 9B) as opposed to wildtype which produces one silique per flower. Quantitative rtPCR was performed on 3 week old *Ndc80* and *Spc25* RNAi mutant leaf tissue. However, due to time constraints, our results remain inconclusive. Our prediction for the results is that there will be a noticeable reduction in mRNA levels for both *Ndc80* RNAi and *Spc25*RNAi compared to the wildtype. These predictions are based on the moderate phenotypes observed in the mutants as well as past *Mis12* RNAi expression data (Xeuxian Li, unpublished).

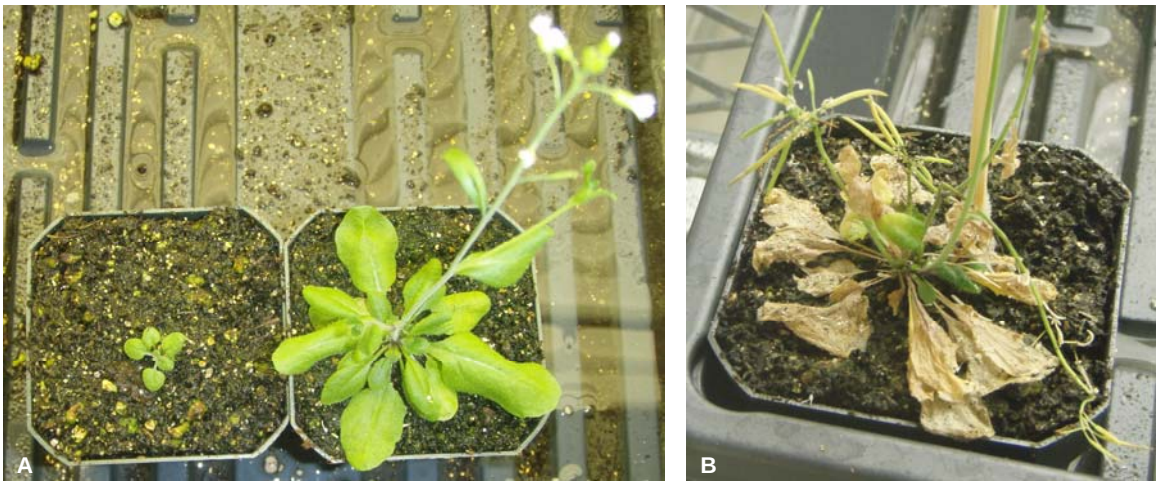

Figure 9: A) NDC80:RNAi on the left compared with MCM21:RNAi on the right. Both plants are shown at 6 weeks after germination. B) A mature NDC80:RNAi plant showing mutant phenotypes

CFP and YFP Tagging Results

It has been shown in previous studies that constitutively expressing YFP tagged CENH3 in *Arabidopsis* results in the incorporation of the tagged CENH3 into centromeric regions of living tissues (Talbert *et al*. 2002). In this study a similar approach was used to interpret the localization of NDC80, SPC25, MCM21 and NUF2. The live cell system allowed us to investigate at what point during the cell cycle the proteins were incorporated into kinetochores. During these procedures we found that it was important to work quickly to prevent oxygen depletion. Oxygen depletion within living tissue results in cell death if the seedlings are submerged in water for more than 2-3 hours.

With the live tissue in place, fluorescent microscopy was used to analyze 4-day old root tips and shoot apical meristems in order to best visualize dividing cells. Root tips and shoot apical meristems are tissues that are accessible to live imaging without dissection. 3D images were taken at every 5 microns to get a more accurate depiction of chromosomal positioning. One problem we encountered with visualizing fluorescent proteins was their propensity to quickly bleach when exposed to UV light. When taking 3D pictures of many levels along the Z axis, the exposure over time can cause a significant loss of signal. This problem was resolved by reducing the total exposure time to less than 400ms by adjusting the exposure/image and the number of z sections collected.

The localization results among the four genes were diverse in their overall pattern of fluorescence and the intensity of the signal. When examining tagged SPC25 most living root tip nuclei displayed up to 10 kinetochore "spots," (2n=10) when fused to either fluorescent protein (Figure 10A). However, there was no evidence that NUF2 and MCM21 were localizing to the nuclear region when fused to either fluorescent protein. Compared to wildtype, the roots of

NDC80-YFP and MCM21-YFP exhibited an increase in fluorescence activity confirming the presence of the construct within the transformant, however there was no indication that the fluorescence was localizing to any specific part of the cell. One explanation is that MCM21 and NUF2 are not present in the kinetochore during interphase and are recruited at the onset of mitosis, while SPC25 is constitutively expressed throughout the cell cycle. This is consistent with data showing that maize NDC80 (which is located in the same complex as SPC25) is also a constitutive feature of the central kinetochore (Du & Dawe 2006). We predict that if NDC80 and SPC25 are both constitutively expressed, NUF2 should have the same expression patterns due to its similar position within the NDC80 complex. That being said, more experimental data is required to examine this question further.

Localization of NDC80 fused to a yellow fluorescent protein

There were two types of localization patterns found in NDC80-YFP lines. The first and most obvious pattern was the excess of fluorescent spots within all tissues of the plant. The spots were greater in number than the expected 10 (2n=10), sometimes exceeding 30 spots per nucleus. It is predicted that these spots are not indicative of the kinetochore, but more likely a surge in the expression of YFP creating what appears to be an enhanced NDC80 response. Additionally, the over abundance of spots occasionally appeared to be randomly partitioned throughout the root tip, and not localizing specifically to nuclear regions (Figure 10D). In approximately 10% of the NDC80 plants, a second phenotype was noticed displaying similar kinetochore localization patterns as those seen in SPC25-YFP and HTR12-YFP. However, NDC80-YFP differed in that its 10 spots appeared to localize outside of the nuclear region (Figure 10C). One possible explanation for this discrepancy is that the previous results were also an artifact of

overexpression and that normally the NDC80 protein localizes to the kinetochores within this nuclear region.

Currently, in an effort to address these abnormal phenotypes, the 35S promoter was replaced with an endogenous NDC80 promoter. To achieve this, an OCS terminator, NDC80-YFP, and 1kb of sequence upstream of NDC80 were cloned separately into an empty pCAMBIA vector and expressed in Arabidopsis (www.cambia.org). This work is currently in progress and we predict that NDC80-YFP under the control of its endogenous promoter will display localization patterns similar to that of SPC25-YFP.

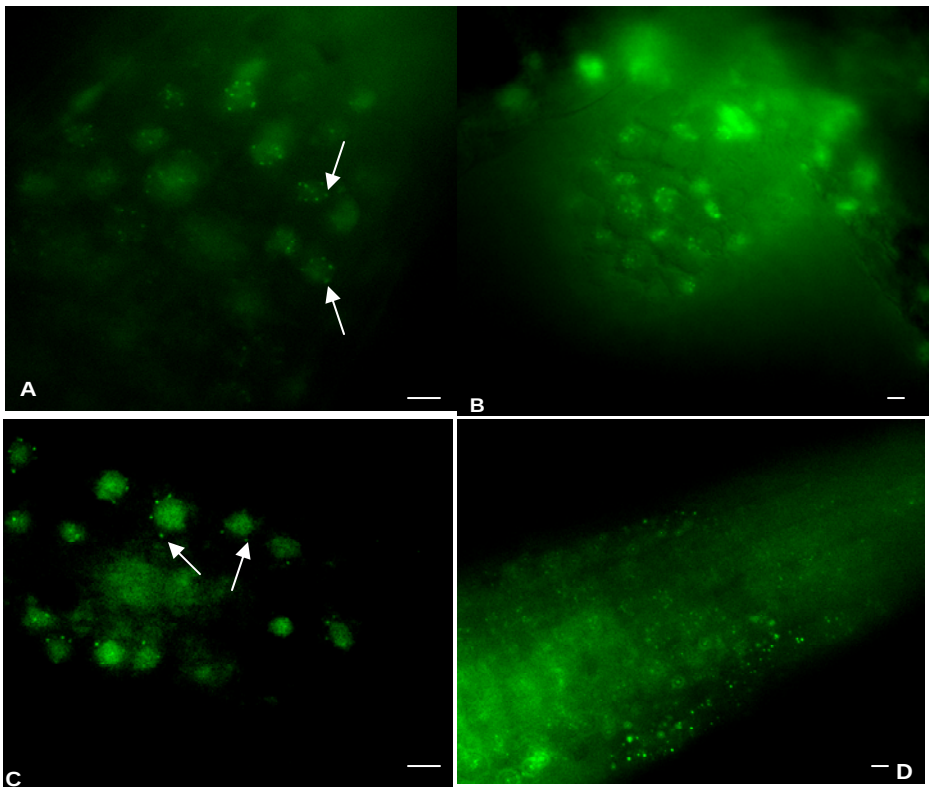

Figure 10: A) SPC25 root tip at 63X magnification. Arrows point to the localization of SPC25-YFP within the nucleus B) Positive control, HTR12 shoot apical meristem at 40X. HTR12-YFP spots are similar to SPC25-25 showing that they both localize to the same general region C) NDC80 root tip at 63X. Arrow shows localization of NDC80-YFP which appears to localize outside of the nuclear region D) NDC80 root tip at 40X displaying an overexpression phenotype where the spots localize to all regions rather than just the nuclear area. Size bars = 2 µm

CHAPTER 4

DISCUSSION AND CONCLUSIONS

T-DNA and RNAi Mutants

Earlier I described the results associated with T-DNA insertions in putative kinetochore proteins in *Arabidopsis thaliana*. Of the four mutations analyzed, *mcm21-1, nuf2-1, ndc80-1*, and *spc25-1*, only *ndc80-1* produced a visible mutant phenotype and change in RNA expression levels. We were unable to find any research previously reported, linking premature senescence with defects in chromosome segregation. This lead us to believe that as a result of the location of the T-DNA with respect to NDC80 (the 3' end of the previous gene), this phenotype is not related to a reduction of NDC80 expression, but more likely a reduction in the upstream gene, *Tsa1*. Further investigation revealed that TSA1 encodes a tryptophan synthase gene. Tryptophan synthase is involved in auxin sythesis and regulates such plant processes as cell division, root initiation, flowering, fruit ripening, and most notably, plant senescence (Normanly *et al*. 1993). Due to these findings and the lack of visible phenotypes relating kinetochore defects in any of the mutations, it is difficult to draw conclusions from these data. One hypothesis is that because the genes are presumed to be essential, very few viable mutations were recovered during the TDNA mutagenesis, and those that were had little or no effect on the phenotype. These four genes are single copy in *Arabidopsis thaliana*, eliminating the likelihood of genetic redundancy. Because of what has already been established about the location and the complex that encompasses NUF2, NDC80, and SPC25, we cannot assume that because of a lack of phenotype and lack of change in mRNA expression levels that these genes are of little

importance in regards to the kinetochore's structure and function. More reverse genetic investigation will be necessary to make any further conclusions.

The silencing of genes can also be achieved using DNA-vector systems expressing short, hairpin RNAs, much like the vectors we used in our RNAi study. There are many reasons for using RNAi as a means of silencing gene expression. With RNAi, the gene product is commonly knocked down anywhere from 20%-90% (Li C.X. *et al*. 2006), which doesn't fully eliminate it like a knockout would do. However, due to our limited time for thorough testing, we were unable to assay for heritability or RNA expression to confirm that the mutant phenotypes were directly linked to a decrease in kinetochore gene expression.

If conclusions were based solely on the mutant phenotypes collected, we would conclude that a decrease in gene expression in *Spc25* and *Ndc80* would cause adverse effects to the plant system. This makes sense based on their similar location within the NDC80 complex.

The mutant analysis of *Mcm21* has left many questions unanswered. It did not display any changes in expression when mutated with T-DNA or RNAi. This could very well be attributed to the inability of both methods to properly cause mutations or a different portion of the gene with more unique sequence could be inserted into the RNAi construct making it more efficient in silencing. It could also be that *Mcm21* is simply unnecessary for proper chromosome segregation. Again, RNAi results for *Nuf2* would tie up many loose ends in this study because it would help to validate the results we obtained for *Mcm21* by increasing the reliability of our assays. An obvious *Nuf2* mutant phenotype would support the notion that it is also an integral component to the NDC80 complex, as well as correlating with the results of *Ndc80* and *Spc25*. Then we could tentatively conclude that *Mcm21*, with a transformation efficiency and phenotype

directly comparable to wildtype, is probably not an integral component of the core kinetochore. However, at this point our data are inconclusive.

Spc25-YFP localizes to the kinetochore

The NDC80 complex is well conserved throughout evolution, suggestive of its critical role in binding to the microtubules and crucial location within the kinetochore (Cheeseman & Desai, 2008). Because of this, one would predict that all of the proteins within this complex would localize to the nucleus when tagged with a fluorescent protein and observed *in vivo*. Based on our studies, we found that SPC25-YFP also localizes to the kinetochore as previously seen in HTR12-YFP. It is found localizing in all tissues of the plant leading us to believe that it is also constitutively expressed however this was never confirmed by PCR. The YFP signal in SPC25-YFP is noticeably dim compared with that of HTR12-YFP. This could be a result of natural variation amongst expression levels of the transgenes or a considerable difference in the amount of the protein present at each kinetochore.

The expression patterns of NDC80-YFP as described in the results section lead us to believe that NDC80 does localize to the kinetochore despite the overexpression phenotype witnessed. This is based on prior studies showing that it is constitutively localized to the kinetochore in maize (Du & Dawe, 2006), and that it is located in a complex that has been highly conserved throughout evolution. We conclude that the localization phenotype observed is an artifact of overexpression, and with the completion of the native promoter studies, this hypothesis can be confirmed.

Based on the aforementioned results we predicted that NUF2 would also localize to the nucleus due to its coupled orientation with the NDC80 protein. However, of the 9 YFP lines and 13 CFP lines observed, there was no sign of localization visible in any of the interphase nuclei.

To be sure of the transgene's presence, the NUF2 cDNA was confirmed via PCR screen prior to transformation and the vector's presence was confirmed within Arabidopsis based on its acquired Basta resistance. This indicated that NUF2 might only be present during non-interphase stages. However, if NDC80 is confirmed to be constitutively expressed, then this prediction will seem unlikely due to their close relationship within the complex.

Finally, our conclusion of MCM21 localization, based on the preliminary T-DNA, RNAi, YFP, and CFP studies, reveals that MCM21 is not an important feature of the core kinetochore. *Mcm21* mutants showed no change in mRNA levels in either mutant investigations, and when fused to YFP and CFP separately, it showed no visible localization to the kinetochore. These results, as well as its location within the COMA complex (which has only been confirmed as an essential component of the core kinetochore in fungi) lead us to believe that its function is potentially different in plants than in other species.

Summary of Results

Table 5: Summary of experimental results

Gene	RNA expression in T-DNA lines	T-DNA mutant phenotypes	RNA expression in RNAi lines	RNAi mutant phenotypes	YFP overexpression	CFP overexpression
SPC25	No change in mRNA levels compared to WT	At 13 days, homozygous seedlings had 54% less fresh weight than WT	Data inconclusive	Mild developmental phenotype compared to WT	Similar expression pattern to HTR12	Currently, no data
NDC80	40% reduction in mRNA levels compared to WT	Mutants initiate senescence two weeks prior to WT	Data inconclusive	Severe developmental phenotypes	Excess of fluorescent spots	No fluorescent activity
NUF2	No change in mRNA levels compared to WT	At 13 days, homozygous seedlings had 65% less fresh weight than WT	Currently, no data	Currently, no data	No visible fluorescence localizing to the kinetochore	No visible fluorescence localizing to the kinetochore
MCM21	No change in mRNA levels compared to WT	No apparent phenotype	No change in mRNA levels compared to WT	No apparent phenotype	No visible fluorescence localizing to the kinetochore	No visible fluorescence localizing to the kinetochore

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APPENDIX A

HTR12-YFP EXPRESSION CYTOLOGY

Introduction

The centromere specific histone variant CENH3 is an integral component in chromosome segregation and the formation of the kinetochore. Designated HTR12 in *Arabidopsis thaliana*, it was the first centromeric histone to be discovered in plants and was shown to be a centromere identifying protein (Talbert *et al*. 2002). Talbert *et al.* also demonstrated that HTR12 localizes to both mitotic and meiotic cells and is present constitutively on chromosomes.

The conservation of CENH3 across all eukaryotes suggests its critical role as a component of kinetochore assembly. It has been found in a number of different organisms including *D. melanogaster* (Henikoff *et al.* 2000), *S. pombe* (Takahashi *et al*. 2000), *S. cerevisiae* (Meluh *et al.* 1998), *C. elegans* (Buckwitz *et al*. 1999) and is among the most conserved kinetochore proteins (Henikoff *et al.* 2000). CENH3 has also been shown to be present at all native centromeres but notably absent at mutated or inactivated centromeres (Tyler-Smith *et al*. 1999). These reasons amongst many others have attributed to the firmly established notion that CENH3 is the best candidate for maintaining centromeres.

Although CENH3 has already been shown to be constitutively expressed in *Arabidopsis thaliana,* additional insight is needed to determine the timing and mechanism of CENH3 and its connection with the rest of the kinetochore during mitosis. One method of interpreting gene function is through intentional overexpression of the gene. Increasing the strength of the promoter can cause dramatic increases in the amount of mRNA and protein present, which can

lead to the induction of abnormal phenotypes. For example, the overexpression of CENH3 in Drosophila causes mislocalization to chromosome arm regions and promotes formation of ectopic kinetochores (Heun *et al.* 2006). These results lead us to try a similar experiment in Arabidopsis CENH3 which is designated HTR12 (Talbert *et al*. 2002). This experiment was started by my colleague Chris Topp. My contribution was to analyze the lines in vivo.

In this study, HTR12 was fused to a yellow fluorescent protein and overexpressed to visualize *in vivo* the effects of this mislocalization on the cell cycle (Chris Topp, unpublished). It had been previously shown that HTR12 is loaded to the centromere mainly during G2 phase (Lermontova *et al.* 2006) and that in yeast, its restriction to the kinetochore is regulated by proteolysis (Collins *et al.* 2005). However it still remains unclear as to the role of HTR12 after its initial identification of the centromere and recruitment of the other kinetochore proteins.

MATERIALS AND METHODS

Generation of HTR12-YFP fusion constructs and transgenic seed lines

HTR12 constructs were created previously by Chris Topp using the same protocol as described in Earley *et al*. 2006. pEarleyGate vector 101 was used as the destination vector and adds a C-terminal fusion of HTR12 in-frame with a yellow fluorescent protein. Primers used for amplification were a gene specific forward: 5’ AAATTGT GAAGCAGATTCGA 3’ and a gene specific reverse primer: 5’ CAACGAAAAGCAG ATAGAA 3’. The transgenic seed lines were created following the methods previously reported.

Tissue Preparation and Fluorescent Microscopy

After removal of 4 day old seedlings from ½ MS plates, the seedlings were placed on slides containing ½ MS liquid media, fully intact. Cover slips were gently placed on top to prevent damaging the live tissue and sealed with nail polish. Cells were viewed using a Zeiss Axio Imager, and all images were collected using Slidebook 4.0 software (Intelligent Imaging Innovations). 4D time-lapse images were collected at 5 minute increments for a maximum of 2 hours and an exposure time of 100ms. This short exposure time was integral in maintaining the level of fluorescence and minimizing bleaching. Z axis images were taken at every 5microns throughout the width of the root.

RESULTS AND DISCUSSION

Arabidopsis thaliana plants constitutively expressing YFP tagged HTR12 were analyzed for the incorporation of CENH3 at the predicted kinetochore location. Compared with maize, Arabidopsis' chromosomes are very small and difficult to distinguish from the uniform kinethochore spots seen during interphase. This made it challenging to differentiate HTR12 signal at the kinetochore versus YFP staining at other locations on the chromosomes. As was previously shown in Lermontova *et al.* (2006), constitutively expressing CENH3-YFP localizes with centromeric regions in most nuclei during mitosis and is displayed as 10 signal foci. In our lines the CENH3-YFP kinetochores displayed a similar pattern of localization within interphase nuclei (Figure 1). The main objective then, was to capture a non-interphase cell in order to follow the movement of HTR12 throughout the different stages of mitosis. This experiment would be an extension of the work done by Lermontova *et al*., as well as taking a closer look at the exact localization of HTR12-YFP and its behavior under the 35S promoter.

During mitosis, plant cells spend over 90% of the cell cycle within the interphase stage, and the other 10% is divided amongst prophase, metaphase, anaphase and telophase. Once telophase has come to an end, cytokinesis is initiated followed by a return to interphase. Due to the limited time that the cell is in the process of dividing, the challenge became finding the tissues that would include the highest proportion of dividing cells. All of the dividing cells that were discovered were in the root tips and shoot apical meristems of 4 day old seedlings.

During the non-interphase stages, we observed that the spots became much brighter and more elongated in shape. This was initially thought to be the natural condensing of the chromatin at prophase. However, that would mean that YFP would no longer be staining just the kinetochore region as it had been in interphase, but the entire chromosome. It was suggested that this phenotype could be an effect of overexpression. The effects of overexpression on proteins *in vivo*, although beneficial for observing localization of naturally low expressing proteins, can cause a wide array of localization phenotypes that would otherwise not be inherently occurring (Peel *et al.* 2007). These observations led to the hypothesis that the increase of the YFP signal was not actually staining the chromatin, but staining an over-abundance of deposited CENH3 at numerous different locations on the chromosome.

During the regular cell cycle, it is known that CENH3 is loaded onto the centromere at the late stages of G2 (Lermontova *et al*. 2006). However, the mechanism for targeting CENH3 to the centromere is still unknown. Under endogenous expression, CENH3 is visible within the nucleus throughout all of interphase. Even during DNA synthesis, when the DNA is replicated, the total amount of CENH3 remains constant (Lermontova *et al*. 2007). This results in the dilution of CENH3 levels within the nucleus. To counteract this dilution, additional CENH3 is added in the late stages of G2, right before the sister kinetochores separate and mitosis is

initiated. After mitosis, the nuclei divide forming two daughter nuclei that both display the same CENH3-interphase pattern as their parent.

During CENH3 overexpression however, it appears that CENH3 loading is altered to accommodate the drastic increase in levels of CENH3 caused by the 35s promoter. We propose that during overexpression of CENH3, the loading mechanism at G2 becomes unregulated, resulting in the deposition of CENH3 to normally non-centromeric chromosome arm regions. This hypothesis correlates with the *Drosophila melanogaster* discovery that overexpression of CENH3 causes the formation of ectopic kinetochores in euchromatic regions throughout the chromosomes (Heun *et al*. 2006). After mitosis is initiated, overabundance of CENH3-YFP signal results in the appearance of whole chromosome staining which continues through all stages of mitosis. Immediately following mitosis, the newly divided cells return to their regular interphase configuration (Figure 2). Figure 1 shows this occurrence in live cells as the HTR12-YFP signal appears to lessen after cytokinesis and returns to its interphase kinetochore arrangement.

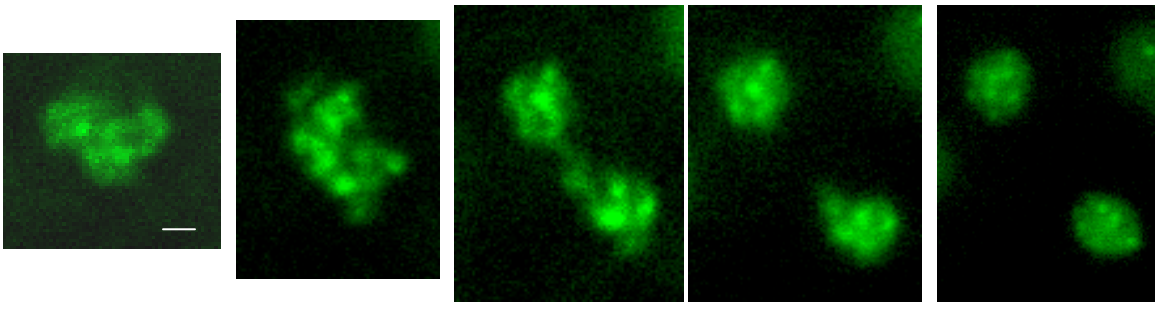

Figure 1: Phases of HTR12-YFP mitosis. Images taken at 5 minute increments. The staining appears much brighter and covers more area until the cells completely divide and return to interphase. Size bar = 2µm

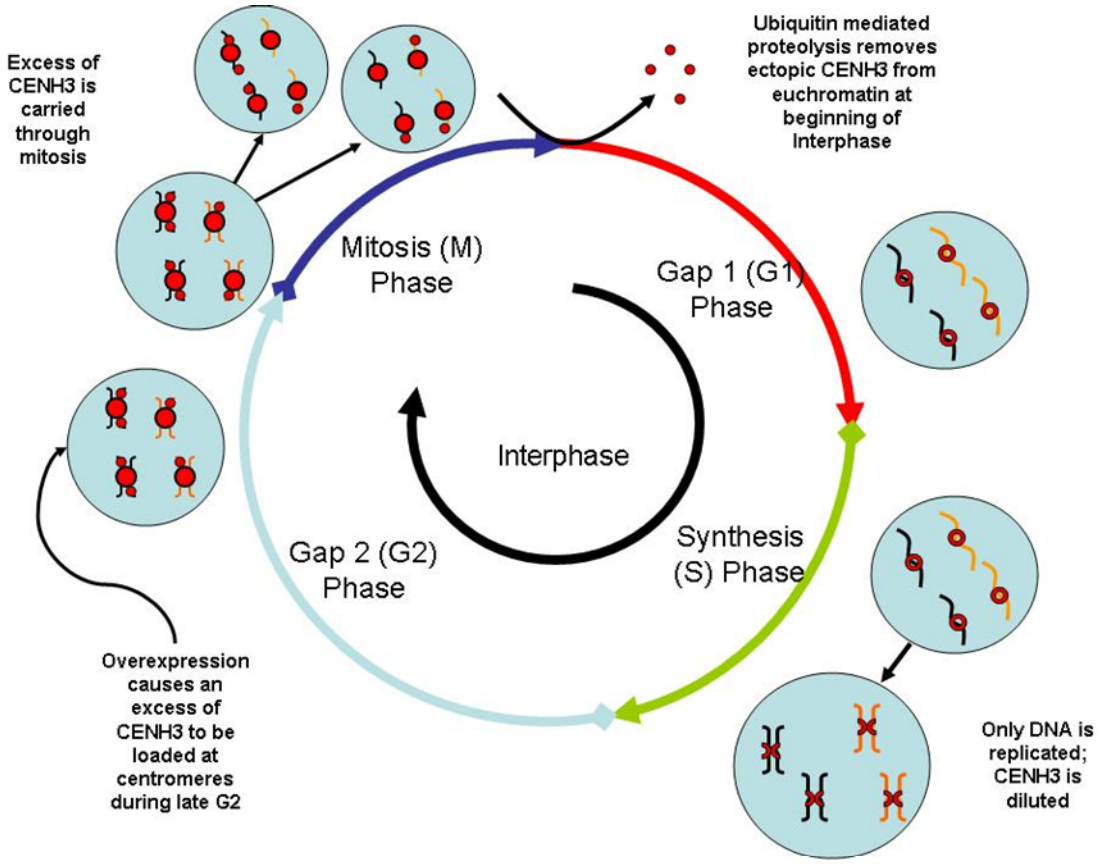

Figure 2: Model showing the effects of overexpression of CENH3 on the chromosomes throughout the cell cycle. CENH3 is added in excess during late G2. This causes mislocalization of CENH3-YFP throughout the chromosome arm to continue through mitosis. At the end of mitosis, the excess CENH3 is removed returning the cell to its original interphase state.

The question then becomes: how is the excess CENH3 removed from the noncentromeric regions following mitosis? It has been shown in Drosophila (Moreno *et al.* 2006) and budding yeast (Collins, K.A. *et al.* 2004), that CENH3 is regulated by an ubitquitin-mediated proteolysis. This is thought to restrict CENH3 to the kinetochore by releasing a protease upon CENH3 localization in euchromatic regions.

Another phenotype observed in overexpressed CENH3-YFP lines was the appearance of lagging chromosomes at the onset of anaphase (Figure 2). Lagging chromosomes are caused by

the delay and failure of sister chromatid separation (Kurihara *et al.* 2006) and this delay can be clearly seen in telophase (Figure 3).

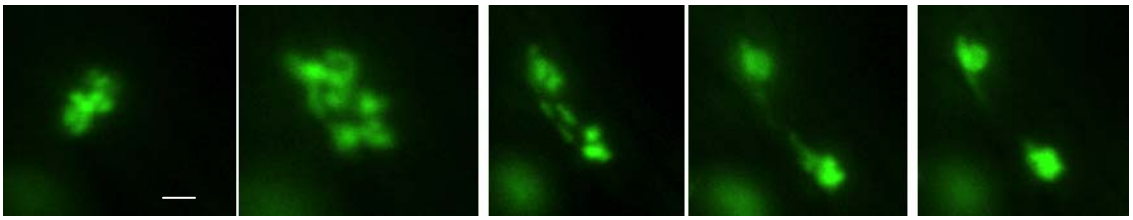

Figure 3: Phases of HTR12-YFP mitosis. Images taken at 5 minute increments. Lagging chromosomes can be seen at the onset of anaphase and eventually catch up with the dividing cells. Size bar = 2µm.

We suggest that the delay is caused by CENH3 overexpression, whereby the excess CENH3 at non-centromeric regions causes new kinetochore protein recruitment and eventually new centromere formation. These neocentromeres can cause defects in chromosome segregation creating aneuploidy (Pidoux *et al.* 2000); however in these particular lines the lagging chromosomes eventually catch up to the main chromosome mass resulting in normal cytokinesis.

In conclusion, despite not being observed by Lermontova *et al* 2006 (who may have selected for lines with lower levels of expression), the overexpression of HTR12-YFP appears to lead to ectopic CENH3 placement and lagging chromosomes at anaphase. Since the lagging chromosomes were shown to realign with the rest of the chromosome mass after nuclear division, further studies will be needed to determine whether this phenomenon has any adverse effects on the plant itself.