CLONING AND CHARACTERIZATION OF MEIOTIC GENES *REC8* AND *CDC5* AND SUBCELLULAR ANALYSIS OF KINETOCHORE ORIENTATION IN WHEAT

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ABSTRACT

Meiosis is a specialized cell division that halves chromosomes and generates haploid gametes in eukaryotes. It is a dynamic cellular process governed by a complex genetic network. Two key players of this network, *Rec8* and *Cdc5*, were cloned and analyzed using comparative genomics and subcellular immunolocalization methodologies in wheat (*Triticum turgidum* L., genome AABB). *TtRec8* and *TtCdc5* were localized to group 1 and 5 chromosomes, with two homoeoalleles in sub-genome A and B, respectively. One of the two *TtRec8* homoeoalleles, *TtRec8-A1*, contains 20 exons in a 6.5 kb-genomic DNA fragment, and the coding region encodes 608 amino acids. Two homoeoalleles of *TtCdc5* separately encode 1,081 and 1,084 amino acids. The expression profilings of *TtRec8* and *TtCdc5* were meiotic tissue dominant in LDN, and the highest levels of *TtRec8* and *TtCdc5* were at interphase through early prophase I and at pachytene stage of meiosis, separately, and then decreased as meiosis proceeded. TtRec8 protein was detected along the entire chromosomes through the early stages of prophase I. Thereafter, TtRec8 protein was mostly removed from the chromosomes. The DNA sequences and conserved domains of TtRec8 and TtCdc5 as well as their kinetics through the meiotic process in LDN were very similar as the cohesion protein Rec8 and polo-like kinase Cdc5 in models, suggesting their specific roles in meiosis.

Chromosome pairing (or synapsis) may play a role in kinetochore orientation during meiosis. Special genotypes that contained both paired (bivalents) and unpaired (univalent) chromosomes in the LDN background were constructed to determine the orientation of sister kinetochores in the univalent and bivalent chromosomes in meiosis I. Among the special genotypes included the hybrids from the crosses of the disomic LDN D-genome substitution lines LDN 1D(1B), LDN 2D(2A), LDN 2D(2B), LDN 3D(3A), LDN 4D(4B), LDN 6D(6A),

LDN 6D(6B), LDN 7D(7A), and LDN 7D(7B) with LDN, LDN 1D(1A) with rye (*Secale cereale* L., genome RR) ‘Gazelle’, LDN with *Aegilops tauschii* (genome DD) RL5286, and LDN 1D(1B) with *Ae. tauschii* RL5286. All univalents were found amphitelically orientated and all bivalents syntelically orientated at metaphase I, suggesting meiotic pairing mediates kinetochore orientation and subsequently chromosome segregation in LDN.

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CHAPTER I. GENERAL INTRODUCTION

Meiosis is an essential cell division involved in gametogenesis, which is specific for eukaryote reproduction. It is characterized by one round of DNA replication followed by two continuous rounds of nuclear divisions, resulting in formation of four daughter cells with halved chromosome number (Cnudde and Gerats, 2005). The first round of meiotic division, *i.e.* meiosis I, involves homologous chromosome pairing, recombination, and segregation. It reduces chromosomes in half and thus called reductional division. The second round of meiotic division, *i.e.* meiosis II, involves segregation of sister chromatids and is similar to mitosis. It is called equational division with an outcome of four haploid daughter cells. Matured male and female gametes developed from haploid daughter cells fertilize to form diploid or polyploid offsprings with the same chromosome number as their parents. In this way, meiosis maintains genome integrity over sexual generations. On the other hand, meiosis results in genetic variability by recombination between non-sister chromatids. Moreover, independent assortment and segregation of different pairs of homologous chromosome at anaphase I provide another mechanism for genetic variations. Therefore, not only can meiosis maintain genome integrity, but also can create genetic variability.

Deviation from normal meiosis often leads to chromosomal deletions, duplications, rearrangements, aneuploidy, and polyploidy. This is thought to be the natural driving force for gene and genome evolution, as well as polyploidization (Stadler, 1973; Bretagnolle and Thompson, 1995; Page and Hawley, 2003; Zhang, 2003; Cai and Xu, 2007).

Normal meiosis includes two successive nuclear divisions and results in reduced gametes. Failure of chromosome segregation in meiosis I or II leads to the formation of restitution nuclei with unreduced chromosomes, *i.e.* unreduced gametes. This variant meiotic cell division process is termed meiotic restitution or unreductional meiotic cell division (UMCD). There are two types of meiotic restitutions, including first division restitution (FDR) and second division restitution (SDR). They result from the failure of homologous chromosome segregation at meiosis I (FDR) and failure of sister chromatid segregation at meiosis II (SDR), respectively (Wagenaar, 1968a,b; Bretagnolle and Thompson, 1995; Xu and Joppa, 1995). Meiotic restitution or UMCD has been documented in many plants, including wheat and potato (*Solanum tuberosum* L.) (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003). Unreduced gametes provide a unique tool for breeding in polyploid crops, including potato and alfalfa (*Medicago sativa*). They were utilized to transfer the agronomically favorable gene combinations from diploid parents to the tetraploid offersprings (Veilleux, 1985). Unreduced gametes could be produced in both microsporogenesis and megasporogenesis, respectively (Ramanna and Jacobsen, 2003). The fertilization of unreduced female and male gametes results in chromosome doubling and increase of ploidy level. This has been reported as a major cytological mechanism of polyploidization in plants (Harlan and deWet, 1975; Fukuda and Sakamoto, 1992a,b; Wendel, 2000; Xu and Joppa 2000a; Lyrene *et al.*, 2003; Ramanna and Jacobsen 2003; Jauhar 2007). Furthermore, unreduced gametes have proven useful in gene introgression through intergeneric/interspecific hybridization and polyploidization, particularly in *Triticeae* (Islam and Shepherd, 1980; Balatero and Darvey, 1993; Bretagnolle and

Thompson, 1995; Ramanna and Jacobsen, 2003; Matsuoka and Nasuda, 2004; Jauhar, 2007; Shamina, 2012).

Cultivated wheats, including common and durum wheat, are the most consumed food grain around the world. They are allohexaploid and allotetraploid, respectively, and taxonomically placed in the genus *Triticum* under the tribe *Triticeae*. Common wheat originated from spontaneous hybridization between tetraploid wheat (*T. turgidum* L., genome AABB) and diploid goatgrass (*Aegilops tauschii* Coss., 2n=2x=14, genome DD), followed by chromosome doubling (Kihara, 1944; McFadden and Sears, 1946; Riley and Chapman, 1958; Dvořák *et al*., 1993; Takumi *et al*., 1993; Huang *et al*., 2002). Among the three diploid ancestors of common wheat, *T. urartu* is considered the A genome donor and *Ae. tauschii* is the D genome donor (Kihara, 1944; McFadden and Sears, 1946; Dvořák *et al*., 1993; Takumi *et al*., 1993; Petersen *et al*., 2005). *Ae. speoltoides* has been considered a possible donor of the B-genome (Riley and Chapman, 1958; Zohary and Feldman, 1962; Blake *et al*., 1999).

Haploidy-dependent UMCD has been observed in the haploids of tetraploid wheat and the hybrids between tetraploid wheat and *Aegilops* species (Xu and Dong, 1992; Xu and Joppa, 2000a,b; Cai *et al.*, 2010). This unique UMCD has been considered the major mechanism of chromosome doubling in the evolution lineage of common wheat, if not the only one (Lyrene *et al.*, 2003; Jauhar, 2007). Recently, Cai *et al.* (2010) reported that the bipolar microtubule-kinetochore attachment on the univalent chromosomes and persistence of centromeric cohesion contributed the onset of the haploidy-dependent UMCD in tetraploid wheat. However, the molecular mechanism and genetic network underlying this special UMCD remain obscure.

Kinetochore orientation and chromosome cohesion coordinately ensure proper segregation of chromosomes during meiosis (Yokobayashi *et al*., 2003). The orientation of sister kinetochores is regulated by a complex network involving multiple proteins, including meiotic cohesin Rec8, polo-like kinase Cdc5, Moa1, monopolin complex, *etc*. (Watanabe and Nurse, 1999; Clyne *et al*., 2003; Lee and Amon, 2003; Chelysheva *et al*., 2005; Yokobayashi and Watanabe, 2005; Corbett *et al*., 2010). Both cohesin Rec8 and polo-like kinase Cdc5 have been found highly conserved across many eukaryotes. In this study, the wheat homologues of *Rec8* and *Cdc5* were targeted for cloning and characterization to reveal the molecular mechanism underlying the haploidy-dependent UMCD in wheat.

Wheat has a large and complex allopolyploid genome. This has made the genome study of wheat lag behind other cereal crops, such as rice, corn, and barley. However, wheat is a great model for investigating polyploidization and related processes due to the availability of its genome ancestors, large chromosomes and spindle microtubules, and various cytogenetic stocks (Boden *et al.*, 2007; Cai and Xu, 2007; Cai *et al.*, 2010). Here, this research focused on the two major meiotic genes (*Rec8* and *Cdc5*) involved in kinetochore orientation and chromosome segregation in the tetraploid wheat 'Langdon' (LDN) that undergoes haploidy-dependent UMCD, a critical meiotic process for polyploidization in wheat. In addition, this study attempted to determine the role of meiotic pairing in kinetochore orientation and chromosome segregation in LDN.

The primary objectives of this research were:

1. to clone the *Rec8* and *Cdc5* homologues in tetraploid wheat and characterize their functions in meiosis; and

2. to determine the effect of chromosome pairing on kinetochore orientation in tetraploid wheat.

This is a foundational research for further study of UMCD in polyploidy wheat. Accomplishment of this research will enhance knowledge of meiotic cell division in wheat, especially the haploidy-dependent UMCD involved in polyplodization.

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CHAPTER II. LITERATURE REVIEW

Meiosis promotes plant genome evolution

Meiosis is a critical cell division involved in sexual reproduction of eukaryotes. It maintains genome integrity as well as creates genetic variability. It is characterized by one round of DNA replication followed by two successive rounds of nuclear divisions, giving rise to four haploid gametes (Cnudde and Gerats, 2005). The first meiotic division (meiosis I) involves homologous chromosome pairing, recombination, and segregation, in which the genetic information from both parents was reshuffled and recombined. The second meiotic division (meiosis II) resembles mitosis and generates four haploid daughter cells that eventually develop into gametes. Matured male and female gametes derived from haploid daughter cells fertilize to form progeny with the same chromosome/genome as their parents. In this way, meiosis maintains genome integrity over sexual generations. On the other hand, meiosis results in genetic variability by recombination between maternal and paternal chromosomes. Additionally, meiosis generates genetic variability through independent assortment of different homologous chromosome pairs at anaphase I.

Meiotic recombination creates genome variation

Meiotic recombination, which occurs at prophase of meiosis I, results in the exchange of genetic materials from paternal and maternal parents through a breakage-exchange event, called crossing over, between non-sister chromatids in the synapsed homologous chromosomes. Crossovers generate chiasmata that physically connect two paired homologous chromosomes together. Chiasmata are later resolved, allowing paired

homologous chromosomes to segregate at anaphase I. Recombination could occur along the entire chromosome, but recombination frequencies are not evenly distributed along the chromosomes (Zhang and Gaut, 2003; Gaut *et al*., 2007). Some chromosome regions tend to recombine more frequently than others, which are termed recombination hot and cold spots, respectively (Schnable *et al*., 1998; Faris *et al*., 2002; Cnudde and Gerats, 2005; Mezard, 2006; Gaut *et al*., 2007). It has been observed that recombination usually occurs in a higher frequency within gene-rich regions (Tanksley *et al*., 1992; Gill *et al*., 1996a,b). Also, it has been found that recombination can occur within the coding regions of a gene (intragenic recombination) as well as between genes (intergenic recombination). Moreover, homologous chromosomes can be aligned unequally because of the sequence similarity, particularly in the regions harboring repetitive DNA sequences and/or among members of gene family, resulting in unequal crossing-over. Interestingly, recombination could occur between non-homologues, such as illegitimate recombination, resulting in chromosome deletions, duplications, inversions, and translocations of the involved sequence blocks. Hence, recombination creates genome variations, and consequently leads to evolution of genes and genomes in a species.

Intragenic recombination was first reported in *Drosophila melanogaster* as early as 1940 (Oliver, 1940). When recombination occurs within the coding region of a functional gene, it is often accompanied by non-functionalization, sub-functionalization, or neo-functionalization of the involved gene. This has been found true especially in plant disease resistance genes. It has been commonly seen that plant resistance genes often co-evolve with corresponding avirulence genes in the pathogen following the gene-for-gene model (Flor, 1971). For example, tomato *Cf-9* and *9DC* genes both show resistance

against the fungal pathogen *Cladosporium fulvum*. *Cf-9* was evolved from *9DC* by intragenic recombination (Renier *et al.*, 2001). As a result, intragenic recombination is capable of creating diverged versions of alleles at a gene locus and facilitates gene evolution.

The discovery of unequal crossing over could be traced back to the 1920s in *D. melanogaster* (Sturtevant, 1925). This variant recombination is attributed to the sequence similarity among the members in a gene family or among the repetitive DNA sequences within the genome. The misalignment between the tandem repeats, commonly seen in plants, often leads to deletion and duplication of the repeats as well as the flanking sequences (Flavell *et al*., 1974; Faris *et al.*, 2002). This variant meiotic recombination has been thought important for genome evolution.

Illegitimate recombination happens between non-homologous chromosome regions under the circumstance where they share nucleotides, often in short repeats. Together with unequal crossing-over, illegitimate recombination has been considered a major mechanism to shrink the genome size expanded by retrotransposon amplification and polyploidization (Devos *et al*., 2002; Ma *et al*., 2004).

Meiosis may generate aneuploidy and polyploidy

Meiosis involves two rounds of successive nuclear divisions, *i.e.* meiosis I - reductional division and meiosis II - equational division. In meiosis I, homologous chromosomes synapse and recombine with each other at prophase and the synapsed homologues are held together by chiasmata and cohesion proteins (cohesin) until anaphase. At metaphase I, sister kinetochores for each of the synapsed homologous

chromosomes are attached by microtubules emanating from the same pole of the cell (monopolar attachment). However, the two pairs of sister kinetochores in the synapsed homologous chromosomes orient in opposite directions, generating a tension between the homologues linked by chiasmata. This tension stabilizes the pulling force created by microtubules, ensuring proper segregation of chromosomes (Yoshida *et al*., 2011). At anaphase I, chiasmata are resolved and cohesin along the chromosome arms is removed, resulting in segregation of homologous chromosomes into opposite poles. However, cohesin remains intact around centromeres, holding the sister chromatids together until anaphase II. At metaphase II, sister kinetochores are attached by microtubules from opposite poles (bipolar attachment) and the cohesin around centromeres is removed. As a result, the sister chromatids are pulled into different poles, making meiosis II equational.

A complex network composing a series of genes/proteins governs the proper chromosome segregation at each step of meiosis, such as homologous chromosomes pairing, recombination, and kinetochore orientation in both meiosis I and II (Bai *et al*., 1999; Ohi *et al*., 1999; van Heemst *et al*., 1999; Watanabe and Nurse, 1999; Tóth *et al*., 2000; Qian *et al*., 2001; Smits *et al*., 2001; Cai *et al*., 2003; Clyne *et al*., 2003; Lee and Amon, 2003; Rabitsch *et al*., 2003; Yokobayashi *et al*., 2003; Hauf and Watanabe, 2004; Hutchins *et al*., 2004; Kitajima *et al*., 2004; Marston and Amon, 2004; Zhang *et al*., 2004; Chelysheva *et al*., 2005; Watanabe, 2005; Yokobayashi and Watanabe, 2005; Golubovskaya *et al*., 2006; Nonomura *et al*., 2006; Petronczki *et al*., 2006; Zhang *et al*., 2006; Ishiguro and Watanabe, 2007; Monje-Casas *et al*., 2007; Tao *et al*., 2007; Sourirajan *et al*., 2008; Brar *et al*., 2009; Nasmyth and Haering, 2009; Iacovella *et al*., 2010; Xiong and Gerton, 2010; Gong *et al*., 2011). Loss-of-function or other changes of

involved genes/proteins often result in abnormal chromosome segregation and gametes with unbalanced chromosome numbers and eventually aneuploidy.

Environmental factors may influence homologous chromosomes paring and result in abnormal meiosis in addition to meiotic genes/proteins. For example, homologous chromosomes may occasionally fail to synapse with each other, termed asynapsis due to some unknown environmental factors. In addition, homologues can synapse with each other normally, but the connection in-between is resolved prematurely, termed desynapsis. Both asynapsis and desynapsis lead to unpaired chromosomes (univalents). The unpaired chromosomes either get lost or randomly segregate during meiosis I, subsequently producing unbalanced gametes and aneuploid progeny.

Changes in the critical genes/proteins involved in meiosis can lead to polyploidy. It is known that the balanced tension between the pulling force of microtubules from opposite poles and persistence of centromeric cohesion as well as chiasmata connection permit proper segregation of homologous chromosomes at anaphase I. Elimination of chiasmata often leads to non-disjunction of homologous chromosomes, resulting in gametes with unreduced chromosomes (Hawley, 1988; Yoshida *et al*., 2011). In fission yeast (*Schizosaccharomyces pombe*) cells without meiotic cohesin Rec8, sister kinetochores showed bipolar orientation, rather than monopolar orientation at metaphase I (Watanabe and Nurse, 1999). Since the centromeric cohesion between sister chromatids was not established during meiosis in those mutant cells, an equational segregation was observed at anaphase I, resulting in separation of sister chromatids. Chromosomes randomly segregated at anaphase II due to the lack of cohesion, subsequently leading to aneuploidy and/or polyploidy.

Meiotic restitution results in polyploidization

Normal meiotic cell division leads to haploid daughter cells with chromosome number reduced by half. However, chromosomes may fail to segregate in the first or second meiotic cell division, leading to restitution nuclei with unreduced chromosomes in the variant meiotic cell division, such as meiotic restitution. Two types of meiotic restitutions or termed unreduced meiotic cell division (UMCD), have been documented in plants, *i.e*. first division restitution (FDR) and second division restitution (SDR). The first and second division restitution result from the failure of chromosome segregation at meiosis I and II, respectively (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003; Zhang *et al*., 2010; Brownfield and Köhler, 2011; Silkova *et al*., 2011). Both FDR and SDR result in unreduced gametes, but their genetic compositions may differ from each other.

In FDR, homologous chromosomes fail to segregate at anaphase I, and instead they stay on the equatorial plate to form one unreduced nucleus after the first division. The unreduced nucleus usually undergoes normal meiosis II. As a result, FDR is featured by an equational division of chromosomes and the formation of two daughter nuclei with the same chromosome number as their mother cells. In SDR, first division is normal, leading to the separation of homologous chromosomes; however, the sister chromatids fail to segregate at anaphase II, resulting in two daughter nuclei having unreduced number of chromosomes. The daughter cells resulted from FDR and SDR differ in the chromosome complements. The FDR-derived unreduced nuclei contain an entire set of paternal as well as an entire set of maternal chromosomes, making FDR resemble mitosis. However, the SDR-derived unreduced nuclei receive a combination of paternal

and maternal chromosomes rather than a complete set of paternal and maternal chromosomes. Polyploidy has been widely observed in plants. Fertilization of unreduced gametes has been considered a major mechanism of polyploidization in nature (Wagenaar, 1968; Ramanna and Jacobsen, 2003).

Two types of polyploidy are found in plants, *i.e.* autopolyploid and allopolyploid. Autopolyploid is polyploid that contains more than two homologous chromosome sets (genomes), while allopolyploid is the polyploidy that comprises more than one heterozygous chromosome set (genome). Fertilization of unreduced gametes generated from meiotic restitution and/or other variant meiotic processes has been considered a major mechanism for the formation of autopolyploid (Singh, 2002). Allopolyploid is thought to be originated from the spontaneous crossing between genetically different species, followed by chromosome doubling of inter-specific hybrids most likely through meiotic restitution (Fukuda and Sakamoto, 1992a,b; Bretagnolle and Thompson, 1995; Xu and Joppa, 2000b; Lyrene *et al*., 2003; Silkova *et al*., 2003; Matsuoka and Nasuda, 2004; Jauhar, 2007; Silkova *et al*., 2011). Both autopolyploidization and allopolyploidization can dramatically increase the genome size as the whole genome duplication is involved.

The evolution of wheat

Wheat taxonomy

Wheat is currently the most consumed food grain around the world. It taxonomically belongs to the genus *Triticum* under the tribe *Triticeae*, which comprises of about 300 species (Clayton and Renvoize, 1986). Within the genus of *Triticum*, there

are about 30 different wild and cultivated wheat species at three ploidy levels, *i.e.* diploid, tetraploid, and hexaploid (Kimber and Sears, 1987; http://www.k-state.edu/wgrc/Taxonomy/taxkas.html).

Evolutionary lineages of polyploid wheat

Genetic evidences have revealed that polyploid wheat has two evolutionary lineages. The origin of tetraploid wheat (*T. turgidum* L. 2n=4x=28, genome AABB) and hexaploid wheat (*T. aestivum* L., 2n=6x=42, genome AABBDD) comprises one lineage, and *T. timopheevii* (Zhuk.) Zhuk. (2n=4x=28, genome AAGG) and *T. zhukovskyi* Men. & Ericz. (2n=6x=42, genome A^mA^mAAGG) comprise the other. Common wheat was originated from spontaneous hybridization between cultivated tetraploid wheat (*T. turgidum* L., genome AABB) and diploid goatgrass (*Aegilops tauschii* Coss., 2n=2x=14, genome DD), followed by chromosome doubling (Kihara, 1944; McFadden and Sears, 1946; Riley and Chapman, 1958; Dvořák *et al*., 1993; Takumi *et al*., 1993; Huang *et al*., 2002; Cai and Xu, 2007; Jauhar, 2007; Cai *et al*, 2010). Among the three diploid ancestors of hexaploid wheat, *T. urartu* contributed the A genome and *Ae. tauschii* contributed the D genome (Kihara, 1944; McFadden and Sears, 1946; Dvořák *et al*., 1993; Takumi *et al*., 1993; Petersen *et al*., 2005). Though there remains some controversy, *Ae. speoltoides* is generally considered as the B-genome ancestor (Riley and Chapman, 1958; Zohary and Feldman, 1962; Blake *et al*., 1999). In the other lineage, *T. zhukovskyi* (genome A^mA^mAAGG) was evolved from amphiploidization following the hybridization between *T. timopheevii* (genome AAGG) and cultivated *T. monococcum* (2n=2x=14, genome A^mA^m).

Evolution of common wheat

Although the evolutionary lineage of common wheat described above has been widely accepted, direct evidences are needed to affirm some of the evolutionary events. A series of reproductive and genetic events are essential for the success of common wheat speciation, *i.e.* spontaneous hybridization between *T. turgidum* and *Ae. tauschii*, normal growth of fertile triploid F_1 hybrids, and genome doubling in the subsequent generations of hybrids, as well as later genetic and epigenetic changes in the common wheat genome.

The spontaneous hybridization between *T. turgidum* and *Ae. tauschii* is the prerequisite for the origin of common wheat. Although spontaneous intergeneric hybrids between *Aegilops* and *Triticum* species have been reportedly observed in the Middle Eastern regions, there has been no report about spontaneous hybrids between *T. turgidum* and *Ae. tauschii* (Van Slageren,1994). The ancestors of common wheat are reproductively isolated with each other because of the physiological and ecological barriers such as differential flowering time (Kihara *et al*., 1965; Matsuoka *et al*., 2008). How and under what conditions the spontaneous hybridization between *T. turgidum* and *Ae. tauschii* occurred remain unknown. The normal growth and reproduction of the triploid F_1 hybrids are also critical for the origin of common wheat. Apparently, spontaneous chromosome/genome doubling had taken place with the triploid hybrids, making them fertile and produce viable progeny. Genome-wide doubling could be made possible by either somatic chromosome doubling or the formation and union of unreduced gametes. Some recent studies favor the latter route with regarding to the origin of common wheat (Harlan and deWet, 1975; Fukuda and Sakamoto, 1992a,b; Xu and

Joppa, 2000a; Lyrene *et al*., 2003; Ramanna and Jacobsen, 2003; Jauhar, 2007; Cai *et al*., 2010). Common wheat combines three evolutionarily distinct genomes, *i.e.* A, B, and D. After the genome expansion, various types of genome modifications, including gene elimination, duplication, and silencing had taken place to make hexaploid wheat more productive and adaptive to different environments (Matsuoka, 2011). Moreover, in the polyploid wheat, individual genes often choose their own way to be curved in the evolutionary history, such as nonfunctionalization (deletion or pseudogenization), neofunctionalization, or subfunctionalization (Adams and Wendel, 2005; Zhang *et al*., 2011).

Gene cloning in wheat

The most commonly used strategies for cloning genes encoding for unknown products are map-based cloning and T-DNA/transposon-tagging. T-DNA/transposon can be randomly inserted into the genome and used as a "landmark" to facilitate gene cloning. However, this approach in wheat is not as efficient as that in other species, such as tobacco, *Arabidopsis*, maize, rice, and aspen (Dinesh-Kumar *et al*., 1995; Krysan *et al*., 1999; Brutnell, 2002; Jeong *et al*., 2002; Fladung *et al*., 2004). This is due primarily to the lack of an efficient T-DNA/transposon-tagging system in wheat, and high levels of noncoding repetitive DNA sequences within the wheat genome (Smith and Flavell, 1975; Faris *et al*., 2002). Map-based gene cloning also encounters various challenges in wheat, including large genome size, allopolyploidy, great portion of non-coding repetitive DNA sequences, and low level of recombination events in gene-poor regions. However, significant progress has been made on the cloning and characterization of the genes for important agronomic traits that can be visually phenotyped in wheat. It includes free-

threshing habit gene *Q*, vernalization response gene *VRN1*, powdery mildew resistance gene *Pm3b*, host-selective toxins sensitive gene *Tsn1*, frost tolerance gene *Fr-A^{m}2*, leaf rust resistance genes *Lr1* and *Lr21*, *etc*. (Faris *et al*., 2003; Huang *et al*., 2003; Yan *et al*., 2003; Yahiaoui *et al*., 2004; Lu *et al*., 2006; Cloutier *et al*., 2007; Knox *et al*., 2008).

Currently, map-based cloning has been the most popular strategy of gene cloning in wheat, especially for the genes with visually scorable phenotypes. However, it remains a challenge to clone a gene that conditions a functional or structural protein without a distinct and easily scored phenotype, such as the genes/proteins invoved in cell cycles, using the map-based cloning strategy. Rapid advances in the genome studies of models and wheat-related species, such as rice, *Brachypodium distachyon*, *Arabidopsis thaliana*, maize, and barley, have generated various genomics tools and resources useful for wheat genome studies. Comparative studies of the model and wheat genomes have opened another entry point for gene cloning from the large and complex allopolyploid genome of wheat. The comparative genomic analysis approach has been used to clone and characterize the genes/proteins in the genetic network of cell cycle in plants, such as wheat homologous chromosome pairing gene *Ph2*, meiosis regulator gene *WM5*, mitosis checkpoint gene *MAD2*, regulator genes for synaptonemal complex (SC) assembly and synapsis *ASY1*, SC assembler gene *ZYP1*, structural maintenance of chromosome (SMC) family member *Rad50*, and *RAD51* for homologous recombination in meiosis (Sutton *et al*., 2003; Kimbara *et al*., 2004; Dong *et al*., 2005; Boden *et al*., 2007; Khoo *et al*., 2008; Pérez *et al*., 2011; Khoo *et al*., 2012).

Unreductional meiotic cell division

Unreductional meiotic cell division and wheat evolution

Meiosis is a specialized cell division that produces daughter cells with chromosomes reduced in half, and eventually leads to the formation of haploid gametes. Deviation from normal meiosis due to the genetic, epigenetic, and/or environmental factors often leads to daughter cells or gametes with variant chromosome numbers and/or compositions. One of the meiotic variants, unreductional meiotic cell division (UMCD), has been found in plant species, including wheat, potato, *etc*. (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003; Fawcett and Van de Peer, 2010). UMCD often results in the formation of unreduced gametes (2n), and subsequently polyploidization. It appears to be a naturally widespread phenomenon in angiosperm (Harlan and deWet, 1975; Fukuda and Sakamoto, 1992a,b; Wendel, 2000; Xu and Joppa, 2000b; Ramanna and Jacobsen, 2003). Furthermore, UMCD has proven to be particularly important for plant improvement through its use in intergeneric/interspecific hybrids and polyploids, especially in tribe *Triticeae* (Islam and Shepherd, 1980; Balatero and Darvey, 1993; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003; Matsuoka and Nasuda, 2004; Jauhar, 2007; Shamina, 2012).

As described earlier, there are two types of UMCD, also known as meiotic restitution, *i.e.* first division restitution (FDR) and second division restitution (SDR). In FDR, all chromosomes stay on the equatorial plate to form a restitution nucleus instead of segregating at anaphase I as seen in normal meiosis. The resulting restitution nucleus has the same chromosome number as the mother cell at the end of meiosis I and undergoes a normal second division. SDR is characterized by a normal first meiotic division and

failure of sister chromatid segregation in the second division, resulting in two nuclei with unreduced chromosomes. Both FDR and SDR usually take place simultaneously during micro- and mega-sporogenesis, leading to the formation of unreduced male and female gametes, respectively (Ramanna and Jacobsen, 2003).

Haploidy-dependent UMCD has been observed in tetraploid wheat (*T. turgidum* L., 2n=4x=28, genome AABB). It happens only under the haploid condition. Meiosis goes normal under the disomic condition (Xu and Dong, 1992; Xu and Joppa, 2000a,b; Cai *et al*., 2010). This unique haploidy-dependent UMCD has been considered a primary mechanism, if not only one, of chromosome doubling in the origin of common wheat (*T. aestivum* L., 2n=6x=42, genome AABBDD) from the interspecific hybridization between tetraploid wheat (*T. turgidum*) and *Ae. tauschii* (2n=2x=14, genome DD) (Fukuda and Sakamoto, 1992a,b; Xu and Joppa, 2000b; Lyrene *et al*., 2003; Jauhar, 2007).

Cytological and molecular mechanisms of UMCD

Tetraploid wheat 'Langdon' (LDN) undergoes normal meiosis; however, its haploid and hybrid with *Ae. tauschii* and rye show UMCD (Jauhar *et al*., 2000; Xu and Joppa, 2000b; Zhang *et al.*, 2008; Cai *et al*., 2010). It has been widely accepted that proper kinetochore orientation and chromosome cohesion coordinately orchestrate chromosome segregation during meiosis (Yokobayashi *et al*., 2003). Normally, sister kinetochores orient syntelically and homologous chromosomes segregate at meiosis I. Recently, Cai *et al*. (2010) observed that LDN (disomic condition) underwent normal meiosis with syntelic orientation of sister kinetochores at meiosis I and amphitelic orientation at meiosis II, while the LDN haploid and interspecific hybrid of LDN with *Ae. tauschii* had sister kinetochores oriented amphitelically at both meiosis I and II.

Apparently, the amphitelic orientation of sister kinetochores and persistence of the centromeric cohesion between sister chromatids at meiosis I contributed to the onset of this haploidy-dependent UMCD in LDN (Cai *et al*., 2010).

What makes sister kinetochores orient differently at meiosis I in LDN and LDN haploid? Kinetochore orientation and chromosome cohesion are reportedly regulated by a complex network involving multiple genes/proteins, including cohesin Rec8 (Chelysheva *et al*., 2005; Ishiguro and Watanabe, 2007), polo-like kinase Cdc5 (Sharon *et al*., 1990; Herrmann *et al*., 1998; Pahlavan *et al*., 2000; Chase *et al*., 2000; Clyne *et al*., 2003; Lee and Amon, 2003), cohesin protector Sgo (Watanabe, 2005), monopolin-complex and Moa1 (Watanabe and Nurse, 1999; Tóth *et al*., 2000; Hauf and Watanabe, 2004; Chelysheva *et al*., 2005; Yokobayashi and Watanabe, 2005; Zhang *et al*., 2006; Ishiguro and Watanabe, 2007). The change from LDN to LDN haploid may lead to nonfunctionalization, neofunctionalization, or subfunctionalization in some of these genes/proteins responsible for chromosome cohesion and kinetochore orientation (Wendel, 2000; Adams and Wendel, 2005; Wang *et al*., 2006; Zhang *et al*., 2011). Rec8 and Cdc5 have been considered the two major players in the genetic network for kinetochore orientation and microtubule-kinetochore attachment at meiosis I. The objectives of this study were to clone the Rec8 and Cdc5 homologues in LDN and determine their role in the onset of the haploidy-dependent UMCD.

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CHAPTER III. CLONING AND CHARACTERIZATION OF THE *REC8*-LIKE GENE AND MEIOTIC COHESIN IN POLYPLOID WHEAT

Abstract

Meiosis is a specialized cell division that halves chromosomes and generates haploid gametes in eukaryotes. The meiotic cohesin Rec8 has proven to play a significant role in kinetochore orientation in addition to functioning as a cohesion protein during meiosis. Here, we report the cloning and functional analyses of the *Rec8* homologue in tetraploid wheat 'Langdon' (LDN) (*Triticum turgidum* L.), designated *TtRec8*. *TtRec8* was cloned from LDN through the comparative genomic approach. It encodes a Rec8-like cohesion protein with 608 amino acids. Two homoeoalleles of *TtRec8* were identified on the long arm of chromosome 1A and 1B in LDN and designated *TtRec8-A1* and *TtRec8-B1*, respectively. *TtRec8-A1* contains 20 exons in a 6.5 kb-genomic DNA fragment. Real-time PCR showed significantly higher levels of *TtRec8* transcripts in meiotic anthers than in roots and leaves of LDN. The expression level of *TtRec8* was highest at interphase through early prophase I of meiosis, and then decreased as meiosis proceeded. Western blotting detected a higher expression level of *TtRec8* in meiotic anthers than in leaves of LDN, and no TtRec8 was detected in roots. These results consistently indicated that *TtRec8* was expressed primarily in anthers at interphase through early prophase I as reported with *Rec8* orthologues in models. TtRec8 antibody was raised and used to localize endogenous TtRec8 protein in the meiocytes of anthers at different meiotic stages. TtRec8 protein was detected along the entire chromosomes through the early stages of prophase I. Thereafter, TtRec8 protein was mostly removed from the chromosomes. Hardly could TtRec8 protein be visualized on chromosomes after

pachytene stage. The kinetics of TtRec8 through the meiotic process in LDN was very similar as the cohesion protein Rec8 in models, suggesting its specific role in chromosome cohesion and kinetochore orientation in meiosis.

Introduction

Meiotic cell division governs the gamete formation and gene transmission over sexual reproduction in eukaryotes. It comprises two successive nuclear divisions with only one round of DNA replication, and maintains genome integrity as well as creates genetic variability. The first meiotic division (meiosis I) involves homologous chromosome paring, recombination, and segregation, while second meiotic division (meiosis II) leads to separation of sister chromatids and formation of four haploid cells. During meiosis I, homologous chromosomes synapse at prophase and sister chromatids are syntelically attached at centromere by the microtubules emanating from same pole at metaphase. The multi-subunit protein complex (cohesin complex), including Rec8, maintains the cohesion and syntelic orientation of sister kinetochores in meiosis I (Watanabe and Nurse, 1999). Sister kinetochores orient amphitelically and sister chromatids separate in meiosis II. These cohesin-involved meiotic events ensure meiosis I reductional and meiosis II equational.

Cohesins function as “glue” to connect sister chromatids and ensure proper chromosome segregation in meiosis and mitosis. The mitotic cohesin complex comprises four subunits, *i.e.* Scc1/Rad21, Scc3/Psc3, and two subunits of the ubiquitous structural maintenance of chromosomes (Smc) family (Smc1 and Smc3) (Chelysheva *et al*., 2005; Ishiguro and Watanabe, 2007; Nasmyth and Haering, 2009; Xiong and Gerton, 2010). It differs from the meiotic cohesin complex in which Scc1/Rad21 is replaced by its meiotic

counterpart Rec8 (Chelysheva *et al*., 2005; Ishiguro and Watanabe, 2007). A variety of cohesion proteins have been identified and characterized in different eukaryotes, including yeast, *Caenorhabditis elegans*, mice, *Arabidopsis*, rice, and maize, *etc*. (Birkenbihl *et al*., 1992; Molnar *et al*., 1995; Bai *et al*., 1999; Watanabe and Nurse, 1999; Dong *et al*., 2001; Pasierbek *et al*., 2001; Cai *et al*., 2003; Pasierbek *et al*., 2003; Bannister *et al*., 2004; Zhang *et al*., 2004; Chelysheva *et al*., 2005; da Costa-Nunes *et al*., 2006; Golubovskaya *et al*., 2006; Zhang *et al*., 2006; Ishiguro and Watanabe, 2007; Jiang *et al*., 2007; Tao *et al*., 2007; Gong *et al*., 2011; Lee and Hirano, 2011; Yuan *et al*., 2012). Rec8-like and Scc1-like proteins are among the most prevalent subunits of the cohesin complex in meiosis and mitosis, respectively (Chelysheva *et al*., 2005; Ishiguro and Watanabe, 2007). The meiotic cohesin is normally released from chromosomes in a two-step fashion. Initially, the endopeptidase separase removes the cohesin along the chromosome arms, but not around the centromeric region when meiosis proceeds from the prophase of meiosis I till the anaphase of meiosis II. This retains the connection between sister chromatids and ensures the segregation of two sisters into the same pole at meiosis I. At the anaphase of meiosis II, the Sgo1-mediated protection of the centromeric cohesion is broken down and cohesin within the centromeric region is removed, leading to the separation of sister chromatids (Kitajima *et al*., 2004; Watanabe, 2005).

Except for providing linkage between sister chromatids, cohesion proteins play important roles in other meiotic events, such as homologous chromosome pairing and recombination, synaptonemal complex (SC) formation, and double-stranded breaks (DSBs) repair (Klein *et al*., 1999; van Heemst *et al*., 1999; Ellermeier and Smith, 2005; Brar *et al*., 2009; Nasmyth and Haering, 2009). In addition, cohesins regulate

transcription of a series of genes, particularly meiotic genes (Peric-Hupkes and van Steensel, 2008; Merkenschlager, 2010; Dorsett, 2011; Lin *et al*., 2011a,b; Shao *et al*., 2011; Seitan and Merkenschlager, 2012; Yuan *et al*., 2012). In *Arabidopsis*, cohesin subunit SYN3 was found to regulate the transcription level of the genes for meiotic proteins essential for homologous recombination and synapsis (Yuan *et al*., 2012). Similarly, the cohesin SCC2 has proven directly to regulate meiotic gene expression in budding yeast (Lin *et al*., 2011a,b).

Kinetochore orientation and chromosome cohesion coordinately ensure proper segregation of chromosomes during meiosis (Yokobayashi *et al*., 2003). The orientation of sister kinetochores is regulated by a complex network involving multiple proteins, including meiotic cohesin Rec8, Moa1, monopolin complex, *etc*. In this network, Moa1 and monopolin complex are two distinct classes of proteins assisting Rec8 to establish monopolar orientation of kinetochores at meiosis I. Moa1 plays its role in *Schizosaccharomyces pombe* (Yokobayashi and Watanabe, 2005), while monopolin complex functions in *Saccharomyces cerevisiae* and includes Mam1, Csm2, Lrs4 and Hrr25/casein kinase 1 (Watanabe and Nurse, 1999; Hauf and Watanabe, 2004; Chelysheva *et al*., 2005; Zhang *et al*., 2006; Ishiguro and Watanabe, 2007; Corbett *et al*., 2010).

Although the conserved function of Rec8 in the centromeric cohesion at meiosis I has been widely accepted, the role of Rec8 in kinetochore orientation at meiosis I is still ambiguous. In *S. pombe*, *rec8*Δ cells showed bipolar orientation of sister kinetochores at meiosis I, suggesting Rec8 protein is required for monopolar orientation of sister kinetochores (Watanabe and Nurse, 1999; Yokobayashi *et al*., 2003; Yokobayashi and

Watanabe, 2005). However, the replacement of Rec8 by the mitotic cohesin Scc1 that is responsible for bipolar orientation of sister kinetochore in mitosis resulted in monopolar orientation of sister kinetochores at meiosis I in *S. cerevisiae* (Tóth *et al*., 2000). It suggests that other genes/proteins may be involved in the regulation of kinetochore orientation in *S. cerevisiae* in addition to Rec8, such as monopolin (Tóth *et al*., 2000; Rabitsch *et al*., 2003). When *Rec8* is deleted in *Arabidopsis*, maize and rice, sister chromatids prematurely segregated to opposite poles at anaphase I, suggesting Rec8 is required for monopolar orientation of sister kinetochores in plants (Chelysheva *et al*., 2005; Golubovskaya *et al*., 2006; Shao *et al*., 2011).

Polyploid wheat, including common and durum wheat, originated from interspecific hybridization of three diploid ancestors followed by spontaneous chromosome doubling (Kihara, 1944; Riley *et al*., 1958; Dvořák *et al*., 1993; Takumi *et al*., 1993; Huang *et al*., 2002). Both exhibit normal diploidized meiotic behavior even though they contain three and two homoeologous sub-genomes, respectively. However, unreductional meiotic cell division (UMCD) has been observed in the haploids and hybrids of durum wheat with *Ae. tauschii*, D-genome donor of common wheat (Xu and Joppa, 1995, 2000a,b; Jauhar *et al*., 2000; Zhang *et al*., 2008; Cai *et al*., 2010). This variant meiotic cell division has been considered a primary mechanism, if not only one, of chromosome doubling in the origin of common wheat from the interspecific hybridization between *T. turgidum* and *Ae. tauschii* (Lyrene *et al*., 2003; Jauhar, 2007). Recently, Cai *et al.* (2010) reported that the haploidy-dependent UMCD in durum wheat resulted from the amphitelic orientation of sister kinetochores and persistence of the centromeric cohesion between sister chromatids at meiosis I. It has been anticipated that

the meiotic cohesin Rec8, which involves in the regulation of kinetochore orientation during meiosis, might play a role in the onset of this haploidy-dependent UMCD in wheat. The objectives of the present study were to clone the *Rec8* homologue in tetraploid wheat and to perform functional analysis of this gene for further studies of this polyploidization-related meiotic cell division.

Materials and methods

Plant materials and male meiocyte collection

Durum wheat 'Langdon' (LDN) (*T. turgidum* ssp. *durum*, 2n=4x=28, genome AABB), common wheat 'Chinese Spring' (CS) (*T. aestivum* L., 2n=6x=42, genome AABBDD), a set of CS nulli-tetrasomic lines, and a series of LDN D-genome disomic substitution lines (LDN DS) were included in this study. The CS nulli-tetrasomic lines each misses one pair of homologous chromosomes, but has four homologues of another chromosome within the same homoeologous group. The LDN DS has one pair of A- or B-genome homologous chromosomes substituted by a pair of D-genome homologous chromosomes within the same homoeologous group from CS. These two sets of cytogenetic stocks were used to determine the chromosomal location of the *Rec8*-like genes in tetraploid wheat. All the wheat materials were grown in a temperature-controlled greenhouse for the sampling of male meiocytes, leaf, and root tissues in this study. Total genomic DNA of all these wheat lines was extracted from leaf tissues as described by Faris *et al*. (2000). Male meiocytes (anthers) at different meiotic stages were sampled following the procedure of Cai (1994). Anther samples were stored either in liquid nitrogen for real-time PCR and Western blotting or in 1× Buffer A (15 mM Pipes - NaOH, pH 6.8, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 0.15 mM

spermine tetra HCl, 0.05 mM spermidine, 1 mM dithiothreitol, 0.32 M sorbitol; Bass *et al*., 1997) for immunolocalization.

Production of interspecific hybrids and haploids

The LDN spikes were emasculated 2-3 days prior to anthesis, and pollinated with freshly shed pollen from *Ae. tauschii* RL5286 when the hairy stigma opened. Fourteen days after pollination, the caryopses were harvested, surface-sterilized with 20% bleach solution (200 ml bleach+800 ml dH_2O) for 5 min and 70% ethanol for 1 min. The caryopses were rinsed twice each for 1 min with sterile distilled water after each step of sterilization. Immature embryos were aseptically dissected from the caryopses and cultured on MS medium (Murashige and Skoog; 1962) at room temperature (18°C) for 4-8 weeks. Seedlings with two leaves were transplanted into pots in the greenhouse for further studies as described by Cai *et al.* (2010).

LDN haploid plants were produced by pollinating LDN plants with maize pollen and embryos were rescued following the procedure as described by Cai *et al*. (2010). Haploid plants were grown in the greenhouse to collect leaf tissues for DNA extraction.

RNA extraction and cDNA preparation

Total RNA was extracted from leaves, roots, and anthers at interphase/early prophase I, pachytene, metaphase I, metaphase II/anaphase II, tetrads, and matured pollen stages in LDN and LDN × *Ae. tauschii* hybrids using RNAqueous®-4PCR Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instruction. Prior to cDNA synthesis, total RNA was first treated with DNase I and purified with RNAqueous®-4PCR Kit (Life Technologies, Grand Island, NY, USA). After

quantification with NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and agarose gel electrophoresis, 1 µg of total RNA was used as template for first strand cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instruction.

cDNA cloning of the *Rec8*-like gene in LDN

Meiotic cohesin Rec8 has been found highly conserved among eukaryotes, particularly in grass family. The amino acid sequence of rice Rad21/Rec8-like protein Os05g0580500 (GenBank accession NP_001056426.1) was used as a query to *tblastn* the wheat tentative consensus (TC) sequence database. Wheat ESTs having high sequence homology with rice Rad21/Rec8-like protein were annotated based on the information of the *Rec8*-like genes available in models and other plants. The nucleotide sequence of the candidate EST in wheat was used to design gene specific primers using Primer3 (http://frodo.wi.mit.edu/) and primers were synthesized by the Integrated DNA Technologies (IDT, Coralville, IA, USA). The cDNAs synthesized from total RNA in the LDN anther at early prophase I/pachytene stages was used as templates for subsequent RT-PCR with the gene specific primers. 3' and 5' RACE (rapid amplification of cDNA ends) were performed to extend the cDNA sequence of the candidate gene in tetraploid wheat. The final complete cDNA sequence of the candidate gene was PCR-amplified by the primer pair GM067F (5' CACCTCCTCCTCCGACCT 3') and GM065R (5' ATTCTTTCAGCGTGGCATATCT 3') that span the start and stop codons of the gene. The RT-PCR, cloning, and RACE were performed according to Ma *et al*. (2006).

Chromosomal localization of the *Rec8*-like gene in wheat

Wheat *Rec8*-like sequences were amplified from a set of 21 CS nulli-tetrasomic lines and a series of 14 LDN D-genome disomic substitution lines (LDN DS), as well as tetraploid LDN, LDN haploid and hexaploid CS by PCR with *Rec8*-like gene specific primer pair GM008F (5' AAGACCCTCCTCAACAACA 3') and GM008R (5' CCTGACTTGACGCCTTTT 3'). 50 ng DNA was used as PCR template in a 25 µl reaction. PCR amplification was performed with the Platinum® Taq Polymerase (Invitrogen Corporation, Grand Island, NY, USA), and PCR cycles were as follows: 2 min at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minutes at 72°C, followed by 7 minutes at 72°C. After chloroform purification and ethanol precipitation, the PCR products were digested with diagnostic *Dde*I (New England Biolabs, Ipswich, MA, USA) and separated on the denaturing polyacrylamide gel, *i.e.* CAPS (cleaved amplified polymorphic sequence) technique (Konieczny and Ausubel, 1993).

Genomic DNA sequence cloning and analysis

Four cDNA fragments of the *Rec8*-like gene cloned in LDN were bulked as a probe to screen LDN BAC library as described by Huo *et al.* (2006). The positive BAC clones were further verified by PCR with the *Rec8*-like gene specific primers. The verified BAC clones were characterized by fingerprinting with *Hin*dIII and CAPS to identify the BAC clones that contain different homoeoalleles of the *Rec8*-like gene in tetraploid LDN. Subcloning was performed to delineate the homoeoallele into smaller genomic fragments for sequencing using the pWEB-TNC™ Cosmid Cloning Kit (Epicentre Biotechnologies, Madison, WI, USA). The genomic sequences of the

homoeoalleles in LDN were obtained and sequenced by the primer waking method with DNA Walking *SpeedUp*^{TM} Premix Kit II (Seegene, Inc., Gaithersburg, MD, USA).

Function prediction of the candidate gene was performed using BLASTP 2.2.26+ in NCBI nr database (www.ncbi.nlm.nih.gov). Comparative analysis of the Rec8 cohesion proteins from different species was performed using through the use of ClustalX 2.1. Bootstrap Neighbor-Joining phylogenetic tree was built with the use of ClustalX 2.1 and visualized through the use of software FigTree v1.4.0. PEST motif was predicted with the use of EPESTFIND (www.emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). Other motifs were scanned with Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The cDNA and genomic DNA sequences were aligned and analyzed for *Rec8* gene structure with Splign software at NCBI website to (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) and visualized with the use of online drawing tool fancyGENE (http://bio.ieo.eu/fancygene/).

Quantitative real-time PCR

Real-time RT PCR was conducted to quantify the relative levels of *Rec8*-like gene transcripts in leaves, roots, and anthers at interphase/early prophase I, pachytene, metaphase I, metaphase II/anaphase II, tetrads, and matured pollen stages in LDN and the hybrids between LDN and *Ae. tauschii* using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described by Chao (2008). Two pairs of *Rec8*-like gene specific primers were used, *i.e.* GM010F (5' CGCCTGGAGGATTTGG 3')/GM010R (5' TTGCTCGTTTAGTTGGTTGT 3') and LWC6 (5' ACACTAGTTCCCTCTCCACCAA 3')/LWC7 (5' CCTGGGATCATTATCTGGTTGT 3'). After dissociation test and primer validation, 18S rRNA gene was used as

endogenous control and the primer combination is GM003 (5' GAGGGACTATGGCCGTTTAGG 3')/GM004 (5' CACTTCACCGGACCATTCAATCG 3'). Two technical and three biological replications were included in these experiments. The comparative C_T method was used to determine changes in *Rec8*-like gene expression in different samples (test) relative to anthers of interphase/early prophase I stage (control) as described by Chao *et al*. (2010). Fold difference in gene expression is $2^{-\Delta CT}$, where $-\Delta C_T = C_{T,test} - C_{T,control}$.

Antibody production and affinity-purification

A 464 bp cDNA segment (named R26) of the *Rec8*-like gene in LDN was chosen, based on its low hydrophobicity and sequence uniqueness, to raise antibody against the wheat Rec8-like cohesion protein. The segment was PCR amplified from the primer pair GM026F (5' AATAGAATTCAAAATGATCCCAGGAAATGTA 3') and GM026R (5' ACATGTCGAC*TCA*CCCAGGACTTCCAGGTGTA 3'), which were modified with the addition of *Eco*RI and *Sal*I recognition sites at 5' ends (underlined), respectively, and the addition of a stop codon (italic) at 3' of *Sal*I site of GM026R. This cDNA segment was cloned into two expression plasmid vectors pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ, USA) and pMAL-c2X (New England Biolabs, Ipswich, MA, USA), respectively. After verified by sequencing, the two constructs were transformed into *E. coli* strain BL21-Star (DE3) (Invitrogen Corporation, Grand Island, NY, USA). Upon IPTG (isopropyl β-D-1-thiogalactopyranoside) induction, the fusion polypeptides, pGEX-R26 and pMAL-R26, were accumulated in insoluble pellets and resolubilised after sonication. The generation of two fusion peptides, pGEX-R26 and pMAL-R26, was conducted as described by Chao *et al.* (2007). The total proteins were separated on SDS-

PAGE gel and the candidate bands were cut out as per the estimated molecular weight. Upon the verification with protein ID test (Appendix A and B) done in Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University (Stanford, CA, USA), the polypeptide pGEX-R26 was used for the immunization and generation of the polyclonal antibody in rabbits by Affiniity BioReagents (ABR, Golden, CO, USA; now Thermo Fisher Scientific, Inc.).

In order to isolate the antibodies that can specifically recognize the Rec8-like protein in LDN, the anti-pGEX-R26 crude serum after second booster was affinity-purified as described by Chao *et al.* (2007) with minor modifications. The affinity-purified pMAL-R26 polypeptide was first coupled to AminoLink coupling resin with the use of AminoLink Plus Immobilization Kit (Pierce Biotechnology, Rockford, IL, USA) and then incubated with crude serum. After incubation, the mixture of crude serum and resin was loaded to the column, and the serum was collected and stored in -80°C for later use. The anti-Rec8 antibody was eluted with the IgG Elution Buffer (Pierce Biotechnology, Rockford, IL, USA) after washing the column with 20 column volumes of 1× PBS solution. Aliquots of anti-Rec8 antibody were made and stored in -80°C for later use, one of which was taken out for concentration determination.

Immunoprecipitation, Western blotting, and immunolocalization

Total proteins of LDN were phenol-extracted from leaves, roots, and anthers at interphase, prophase I, metaphase I/anaphase I, and metaphase II/anaphase II stages and were loaded 5 µg per lane for blotting. The protein extraction, immunoprecipitation, and Western blotting were performed following the procedures of Chao *et al*. (2007). The

anti-Rec8 antibody was diluted in a ratio of 1:500 for Western blotting according to the result from immunoprecipitation test.

Immunolocalization was conducted as described by Golubovskaya *et al*. (2006) with minor modifications to monitor the changes of endogenous Rec8 protein in LDN anthers during meiosis. The primary anti-Rec8 antibody was probed by the secondary Anti-Rabbit IgG (whole molecule)-FITC Antibody produced in goat (Sigma-Aldrich Co., St Louis, MO, USA), and chromosomes were counterstained by propidium iodide (PI). Two negative control experiments were performed to monitor the specificity of the antibodies in meiocytes. In the first negative control, the thin layer of polyacrylamide gel containing meiocytes was directly incubated with secondary antibody, while in the second one, the thin layer of polyacrylamide gel containing meiocytes was incubated with primary anti-Rec8 antibody that was preabsorbed overnight with fusion polypeptide pGEX-R26 at a molar ratio of blocking peptide to antibody of 50 to 1.

Microscopy

Confocal microscopy was conducted with Zeiss Axioplan 2 Imaging Research Microscope equipped with ApoTome confocal component (Carl Zeiss Light Microscopy, Jena, Germany). Two-dimensional (2-D) and three-dimensional (3-D) images were captured and analyzed with the use of Zeiss Axio Vision 4 software as described by Cai *et al.* (2010).

Results

Cloning and phylogenetic analysis of the *Rec8* homologue in tetraploid wheat

The meiotic cohesion protein gene *Rec8* has been found highly conserved across a variety of eukaryotes, including fungi, plants, and animals in terms of DNA and protein sequences and subcellular functions (Watanabe and Nurse, 1999; Tóth *et al*., 2000; Chelysheva *et al*., 2005; Golubovskaya *et al*., 2006; Shao *et al*., 2011). Rice (*Oryza sativa*) has been a model for monocot plants, including wheat and barley (Kellogg, 2001; Gaut, 2002; Salse *et al*., 2008). In this study, the amino acid sequence of Rad21/Rec8-like protein Os05g0580500 (GenBank accession NP_001056426.1) in rice Japonica group was used as query to search against common wheat TC database (previously at www.tigr.org; and now at http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat), which runs *wu-blast* 2.0. An 808-bp EST from common wheat (GenBank accession BQ744508) was found to have highest sequence similarity (80% identity; E-value: 1.3E-101) and annotated as a cohesin-like protein gene after *tblastn* search. According to the nucleotide sequences of this wheat EST and rice *Rad21-4* gene, wheat cohesin-like gene specific primer combinations were designed in the conserved region. RT-PCR was performed on the cDNAs from LDN anther at early prophase I/pachytene stages when the *Rec8*-like genes were highly expressed. The amplicons from the RT-PCR were sequenced and analyzed, and then used to design gene-specific primers for the next round of 3' and 5' RACE. The draft cDNA sequences of the candidate gene were obtained in LDN after several rounds of RACE reactions. Since LDN is an allotetraploid with two homoeologous sub-genomes, *i.e.* A and B, it generally contains two homoeoalleles with high sequence similarities on each of two

homoeologous chromosomes (Murai *et al*., 1999; Huang *et al*., 2002; Kimbara *et al*., 2004; Zhang *et al*., 2011; Brenchley *et al*., 2012). Thus, errors could occur when assembling the cDNA segments from the RACE reactions. To avoid the assembling errors, two 5' and 3' gene-specific primers from the final round of RACE, which spanned the start and stop codons, were used to amplify full-length cDNA sequences of the gene. Only one full-length cDNA copy, instead of two, was obtained from these amplifications. It was 1,824 bp long and encoded 608 amino acids with a predicted molecular weight of 67.6 kDa.

The predicted protein encoded by the candidate gene was compared to the cohesin orthologues from other eukaryotes, including sister chromatid cohesion 1 protein 1-like protein (Scc1-like) in *Brachypodium distachyon* (GeneID: 100824802), Rad21/Rec8-like protein in rice (GeneID: 4339720), absence of first division 1 (Afd1) protein in maize (GeneID: 732730), sister chromatid cohesion 1 protein 1 (Syn1) in *Arabidopsis thaliana* (GeneID: 830432), and Rad21 from *Drosophila melanogaster* (GeneID: 113590) and *Xenopus laevis* (GeneID: 399129). The comparative analysis revealed high levels of amino acid sequence similarity (Table 3.1). In addition, the predicted protein encoded by the candidate gene contains two conserved domains of Rad21/Rec8 cohesin, *i.e.* pfam04825 at N-terminus and pfam04824 at C-terminus (Figure 3.1). Further analysis showed that this protein has a serine-rich region conserved among the Rec8 cohesins in plants (Figure 3.1). The serine-rich region is essentially important for the cohesin to interact with other proteins in the network. Furthermore, there are two potential proteolytic cleavage sites (PEST motifs) characterized as signals for rapid protein degradation in this predicted protein (Figure 3.1). All these results support the identity of

this candidate gene as a *Rec8* homologue in tetraploid wheat (*T. turgidum*), designated *TtRec8*.

Table 3.1. Comparative analysis of the predicted protein of the *Rec8*-like gene in wheat and cohesion proteins from other eukaryotic species

Species	Cohesin orthologue	GeneID	Genbank Accession	Similarity
B. distachyon	Scc1-like	100824802	XP_003567819.1	80%
O. sativa	Rad21	4339720	NP_001056426.1	68%
Z. mays	Afd1	732730	NP_001105829.1	67%
A. thaliana	Syn1	830432	NP_196168.1	42%
D. melanogaster	Rad21	113590	AAD33593.1	40%
X. laevis	Rad21	399129	AAH97558.1	36%

Bootstrap Neighbor-Joining phylogenetic tree (bootstrap value= 1,000) showed that TtRec8 was genetically closest to BdScc1L, followed by OsRad21 and ZmAfd1, which all belong to the grass family. Dicotyledon cohesin AtSyn1 was relatively further as compared to grass cohesin counterparts; while Rad21 proteins in animals, DmRad21 and XlRad21, were genetically furthest (Figure 3.2).

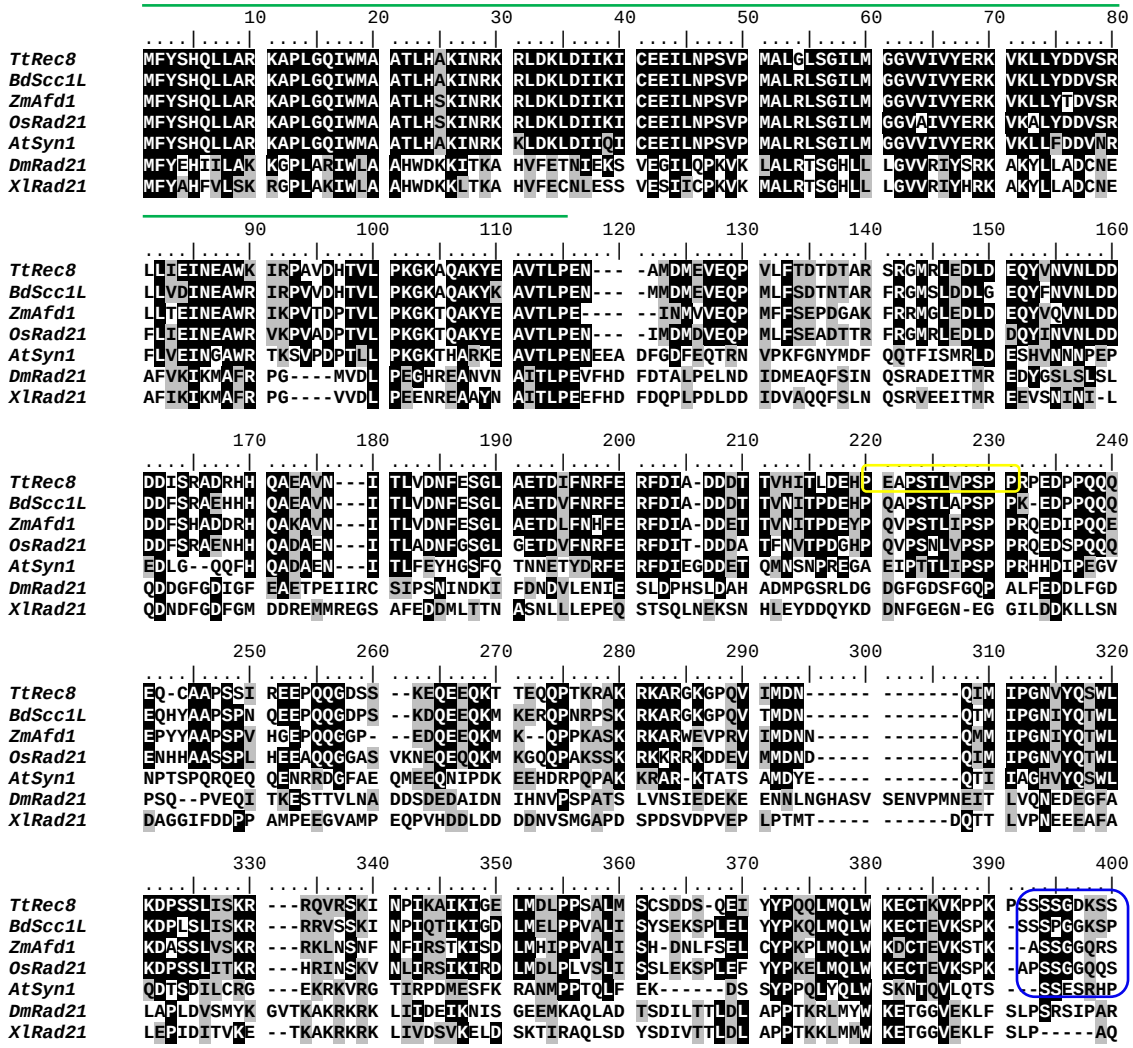

Figure 3.1. Alignment of multiple eukaryotic cohesion proteins. Amino acid sequences of TtRec8 is aligned with *Brachypodium distachyon* sister chromatid cohesion 1 protein 1-like protein BdScc1L (GeneID: 100824802), maize absence of first division1 ZmAfd1 (GeneID: 732730), rice Rad21/Rec8-like protein OsRad21 (GeneID: 4339720), *Arabidopsis thaliana* sister chromatid cohesion 1 protein 1 AtSyn1 (GeneID: 830432), *Drosophila melanogaster* Rad21 (GeneID: 113590), and *Xenopus laevis* Rad21 (GeneID: 399129) by ClustalW. Black, grey, and white backgrounds indicate the levels of conservation of amino acids. Green and red bold lines above the sequence indicate the conserved domains pfam04825 (N-terminus) and pfam04824 (C-terminus), respectively. Blue boxes highlight the serine-rich regions conserved among plant cohesin proteins. Yellow boxes highlight the potential PEST motifs.

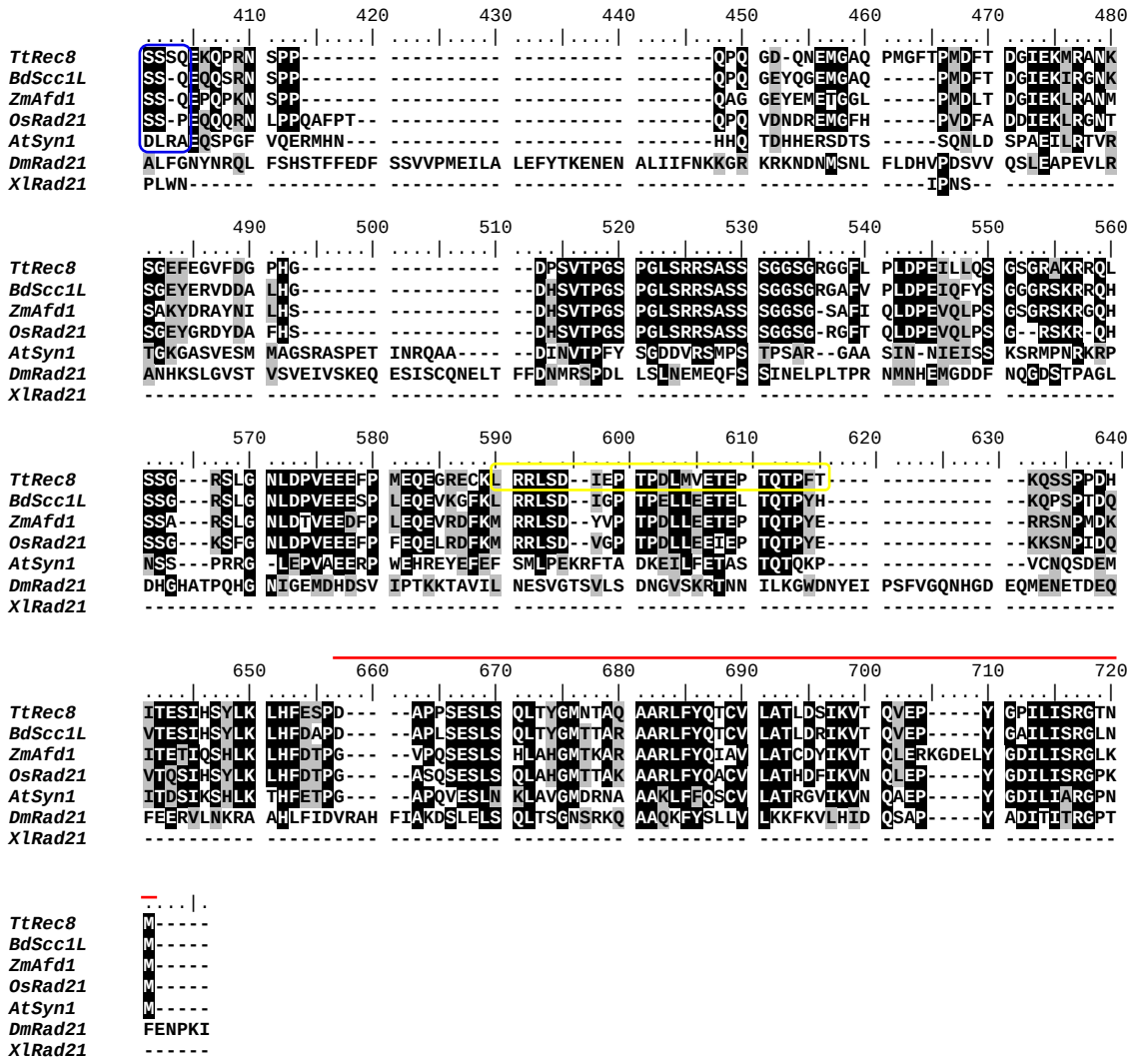

Figure 3.1. (continued) Alignment of multiple eukaryotic cohesion proteins.

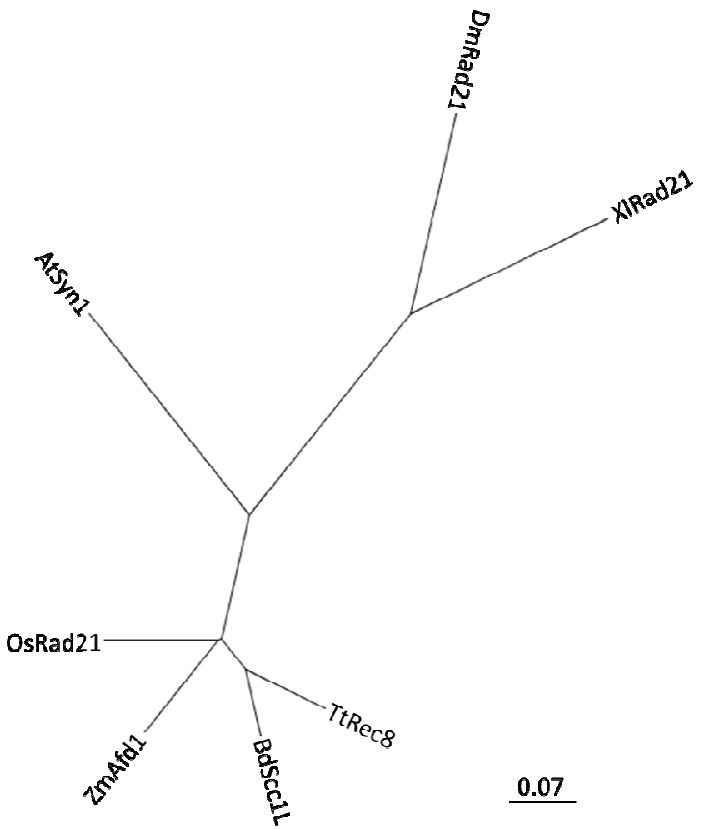

Figure 3.2. Phylogenetic tree of Rec8 orthologues in tetraploid wheat and other eukaryotic species. Phylogenetic tree was built from the amino acid sequences of TtRec8, BdScc1L (GeneID: 100824802), ZmAfd1 (GeneID: 732730), OsRad21 (GeneID: 4339720), AtSyn1 (GeneID: 830432), DmRad21 (GeneID: 113590), and XlRad21 (GeneID: 399129) with Bootstrap Neighbor-Joining method.

Subcellular localization of TtRec8 protein

The polyclonal antibody against TtRec8 raised in rabbits was used to detect endogenous TtRec8 protein in the male meiocyte nuclei at different meiotic stages (Figure 3.3). TtRec8 was found to be associated with the entire chromosomes from early leptotene through pachytene stage at meiosis I (Figure 3.3, a1-a3 and b1-b3). After pachytene stage, TtRec8 was scarcely visualized on the chromosomes, and most of the

TtRec8 protein was removed or degraded from the chromosomes (Figure 3.3, c1-j3). TtRec8 protein was not detected in the somatic cells of anthers that undergo mitosis from interphase to anaphase, indicating TtRec8 is meiosis-specific (Appendix C). In the first negative control where meiocytes were incubated directly with the secondary antibody, no signal could be detected, indicating the endogenous TtRec8 proteins did not react with the secondary antibody. In the second negative control where the primary anti-Rec8 antibody was incubated with fusion polypeptide pGEX-R26 overnight, no signal was detected, indicating the paratope on the anti-Rec8 antibody has specifically recognized and completely combined with the epitope on R26 portion of the fusion polypeptide. Both negative control experiments showed that the anti-Rec8 antibody and Rec8 are mutually specific. The kinetics of TtRec8 through the meiotic process in LDN was very similar as the cohesion protein Rec8 in yeast and other models. All these results further confirm that TtRec8 is the Rec8 homologue in tetraploid wheat.

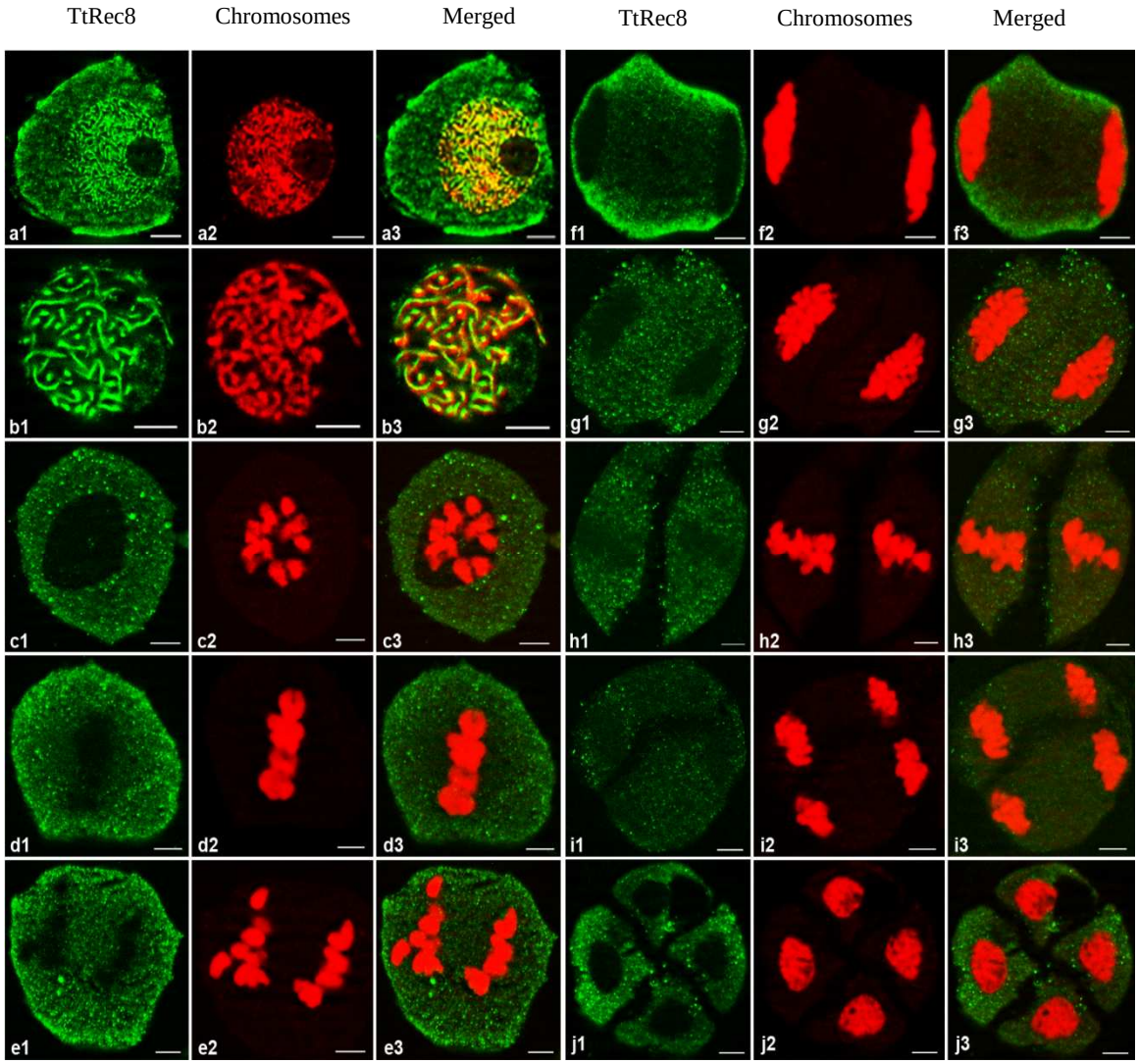

Figure 3.3. Fluorescent immunolocalization of TtRec8 protein (green) on the meiotic chromosomes (red) in LDN. a1-a3: leptotene; b1-b3: pachytene; c1-c3: diakinesis; d1-d3: metaphase I; e1-e3: anaphase I; f1-f3: telophase I; g1-g3: prophase II; h1-h3: metaphase II; i1-i3: anaphase II; and j1-j3: tetrads. Scale bar: 5 µm.

Expression analysis of *TtRec8*

In LDN, significantly higher levels of *TtRec8* transcripts were detected in anthers at early prophase of meiosis I than in roots and leaves by real-time PCR with two *TtRec8*-specific primer pairs. The transcription level of *TtRec8* was highest at interphase through

early prophase I of meiosis. After that, transcription constantly declined to a level of 11-17% at the end of meiosis, *i.e*. tetrad stage (Figure 3.4). The relative transcription levels of *TtRec8* in roots and leaves were only about 4.8-7.9% and 0.015-0.020% of that in the anthers at interphase/early prophase I stages, respectively. The expression profile of *TtRec8* revealed by real-time PCR was similar to the *Rec8*-like genes in models, further confirming the identity of *TtRec8* as a *Rec8* homologue in tetraploid wheat.

TtRec8 exhibited similar expression patterns in both LDN and LDN × *Ae. tauschii* hybrid (Figure 3.4). However, the *TtRec8* transcript level in the LDN × *Ae. tauschii* hybrid was only about 40% of that in LDN at early prophase I (Figure 3.4).

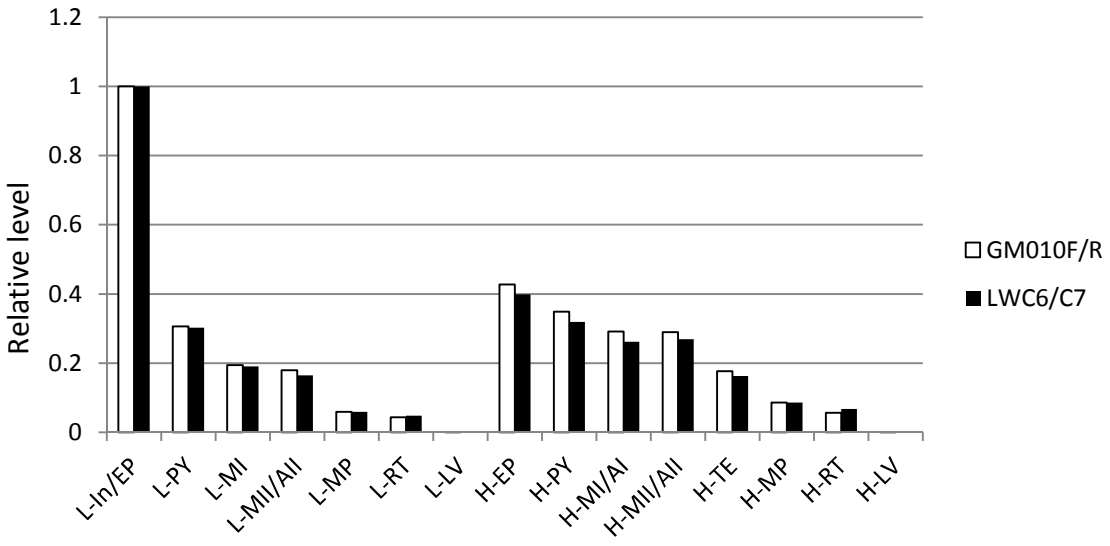

Figure 3.4. Relative transcript levels of *TtRec8* in roots, leaves, and anthers at different meiotic stages in LDN and LDN × *Ae. tauschii* hybrid. Data from two primer pairs LWC6/LWC7 (LWC6/C7) and GM010F/GM010R (GM010F/R) were plotted in the filled and open bars, respectively. In/EP: Interphase/Early Prophase I; PY: Pachytene; MI: Metaphase I; MII/AII: Metaphase II/Anaphase II; TE: Tetrads; MP: Matured Pollen; RT: Roots; and LV: Leaves. The prefix "L-" refers to the samples collected from LDN, and "H-" to the samples collected from the LDN × *Ae. tauschii* hybrid.

Immunoprecipitation was performed to verify the specificity of the anti-TtRec8 antibody. After anti-Rec8 antibody was incubated with the total protein extract from anthers undergoing meiosis, a protein with a molecular weight of a little over 60 kDa was immunoprecipitated. This molecular weight matched with the predicted molecular weight 67.6 kDa of TtRec8 (Figure 3.5, top). In addition, this protein was not present in the supernatant after immunoprecipitation. Also, no precipitation was observed when anti-TtRec8 antibody was not included into the protein extract for incubation (Figure 3.5, top). This confirmed the specificity of the antibody for TtRec8 protein in tetraploid wheat.

Western blotting with the TtRec8 antibody detected the highest level of TtRec8 protein in the anthers collected primarily at prophase I. Also fair amounts of TtRec8 were detected in the anther samples primarily at other meiotic stages. A relatively low level of TtRec8 was detected in leaves, but not in roots (Figure 3.5, bottom). There were some unspecific bindings of TtRec8 antibody with other proteins in root samples, forming a heavy band with a lower molecular weight than TtRec8 (Figure 3.5, bottom).

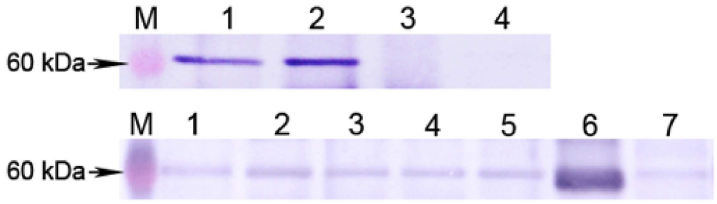

Figure 3.5. Immunoprecipitation and Western blotting of the endogenous TtRec8 in LDN. Immunoprecipitation and Western blotting assays are shown at top and bottom, respectively. Top: M-protein size marker; 1-total protein extracted from anthers undergoing meiosis; 2-proteins that are immunoprecipitated by anti-TtRec8 antibody; 3-negative control without anti-TtRec8 antibody in the reaction; and 4-supernatant from the immunoprecipitation experiment. Bottom: M-protein size marker; 1-interphase; 2-prophase I; 3-metaphase I/anaphase I; 4-metaphase II/anaphase II; 5-matured pollen; 6-roots; 7- leaves.

Chromosomal localization and genomic sequences of *TtRec8*

Screening of the LDN BAC library identified six BAC clones that contain part or full-length genomic DNA sequence of *TtRec8* (Appendix D, A-B). Fingerprinting with *Hin*dIII and CAPS analysis categorized these BAC clones into two groups (Appendix D, C-G). Likely, these two groups of BAC clones each harbored a different homoeoallele of *TtRec8*. Two homoeoalleles of *TtRec8* were identified in LDN and assigned to chromosome 1A and 1B, respectively, using CS nulli-tetrasomic and LDN DS lines (Figure 3.6). They were designated *TtRec8-A1* and *TtRec8-B1*, respectively. Several PCR products amplified from the BACs that contain *TtRec8-A1* and *TtRec8-B1*, respectively, by *TtRec8*-specific primers were sequenced and compared for DNA sequence similarity. High levels of DNA sequence similarities were found in the investigated regions of these two homoeoalles (Appendix E). In addition, chromosome 1D of CS was found to contain another homoeoallele of *Rec8* gene (Figure 3.6). The rice *Rec8*-like gene *OsRad21-4* (GenBank accession NP_001056426.1) and *Brachypodium* gene encoding sister chromatid cohesion 1 protein 1-like protein (GenBank accession XP_003567819.1) were assigned to the long arm of chromosome 5 and chromosome 2, respectively. Both chromosomes are collinear with wheat chromosomes in the homoeologous group 1, *i.e.* 1A, 1B, and 1D (Zhang *et al*., 2006; Kumar *et al*., 2012).

A 40 kb genomic DNA fragment containing *TtRec8-A1* was subcloned into a cosmid vector for the ease of sequencing. A 6.5 kb DNA segment harboring *TtRec8-A1* was completely sequenced. Alignment of the genomic DNA sequence with the full-length cDNA sequence of *TtRec8* indicates that *TtRec8-A1* contains 20 exons and 19 introns (Figure 3.7). The largest exon has 268 bp and the smallest 20 bp in length with an

average of 102 bp/exon. The largest intron has 1,491 bp (between exon 6 and 7) and the smallest is 71 bp (between exon 15 and 16) in length with an average of 234 bp/intron (Figure 3.7). Interestingly, *TtRec8* and the rice *Rec8*-like gene *OsRad21-4* share extremely high similarities in the number, size, and distribution pattern of exons/introns despite of slight difference in length of the genomic DNA sequences. This result indicates the *Rec8* gene is rather phylogenetically conserved between rice and wheat (Figure 3.7). Also, the genomic DNA sequence of *TtRec8-A1* showed high homology with a CS genomic DNA segment assigned to the long arm of chromosome 1A (IWGSC chr1AL v2 ab k71 contigs; http://urgi.versailles.inra.fr/) (99.8% nucleotide sequence similarity in 5,996 bp comparable region), suggesting the location of *TtRec8* on the long arm of the group 1 chromosomes. Cloning and analysis of the complete *TtRec8-B1* genomic sequence is underway in LDN.

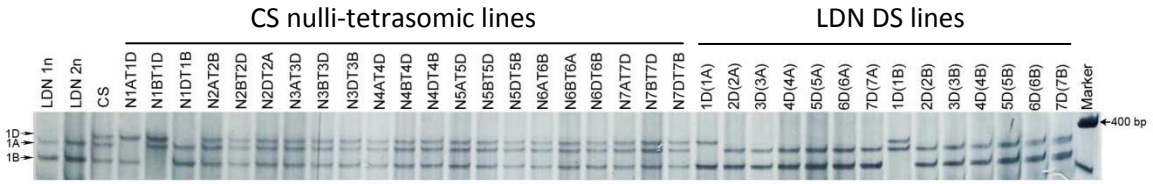

Figure 3.6. Chromosomal localization of *TtRec8* using *TtRec8*-specific primers. The three arrows at left point to the DNA fragments amplified from the homoeoalleles of the *Rec8* homologue in hexaploid and tetraploid wheat on chromosome 1D, 1A and 1B, respectively. Two fragments were amplified in LDN 2n and LDN 1n (haploid), indicating two homoeoalleles of *TtRec8* in tetraploid wheat LDN, *i.e. TtRec8-A1* and *TtRec8-B1*, respectively.

The genomic sequence of *TtRec8-A1* was used as a query to blast the genomic sequences of CS (URGI database, http://urgi.versailles.inra.fr) and *Ae. tauschii* (NCBI

database, www.ncbi.nlm.nih.gov). Comparative analysis of the three *Rec8* homoeoalleles in CS (*TaRec8-A1*, *TaRec8-B1*, and *TaRec8-D1*) revealed over 94% similarities in their genomic sequences, *i.e. TaRec8-A1* vs. *TaRec8-B1* – 94.1%, *TaRec8-B1* vs. *TaRec8-D1* – 94.2%, and *TaRec8-A1* vs. *TaRec8-D1* – 95.3%. In addition, *TaRec8-D1* showed 98.7% genomic sequence similarities with the *Rec8* homoeoallele in *Ae. tauschii* (GenBank accession AOCO010021311.1). Higher levels of similarities would be expected in the coding regions than intronic regions of these homoeoalleles.

Figure 3.7. Comparison of gene structures of wheat *TtRec8-A1* and rice *OsRad21-4*. *OsRad21-4*: GenBank accession NP_001056426.1. Left: Gene structure of *TtRec8-A1*. Right: Gene structure of rice *OsRad21-4.* Green and red wide boxes highlight the N-terminal and C-terminal conserved domains for Rad21/Rec8 cohesin, *i.e.* pfam04825 at N-terminus and pfam04824 at C-terminus. 5' and 3' UTR (untranslated regions) are highlighted with purple narrow boxes at both ends. Black lines in *TtRec8-A1* gene show the relative position of primer pair GM008F and GM008R to the introns and exons along the *TtRec8-A1* gene. Bar scale: 500 bp in length.

Discussions

Both common and durum wheat are allopolyploids with three (*i.e.* A, B, and D) and two (*i.e.* A and B) homoeologous sub-genomes, respectively. Generally, there are three homoeoalleles at a gene or marker locus in common wheat and two in durum wheat (Murai *et al*., 1999; Huang *et al*., 2002; Kimbara *et al*., 2004; Zhang *et al*., 2011; Brenchley *et al*., 2012). The recent whole genome sequence analysis of common wheat suggests that homoeoalleles of probably about one-third of genes in the common wheat genome share high levels of similarity in nucleotide sequences (Brenchley *et al*., 2012). For instance, *MAD2* (*mitotic arrest deficient 2*), a gene involved in spindle checkpoint control, shares 99.2-99.7% nucleotide similarity in the coding regions among the three homoeoalleles *wMAD2-A1*, *wMAD2-B1*, and *wMAD2-D1* of common wheat (Kimbara *et al*., 2004). The three homoeoloalleles of *Waxy* gene, encoding for granule-bound starch synthase, in common wheat share 95.6-96.3% similarity in their coding regions (Murai *et al*., 1999).

In this study, two homoeoalleles of *TtRec8, i.e. TtRec8-A1* and *TtRec8-B1*, were identified and cloned in tetraploid wheat LDN (Figure 3.6). However, only one cDNA clone of *TtRec8* was recovered from the LDN anthers at the meiotic interphase/early prophase I stages where *TtRec8* genes are highly expressed. This could be attributed to high levels of sequence similarities between these two homoeoalleles in coding regions or low expression level of one of these two homoeoalleles.

Comparative analyses of the *Rec8* homoeoalleles in wheat and *Ae. tauschii* suggest that *TtRec8-A1* and *TtRec8-B1* likely share high levels of sequence similarities, especially in coding regions (cDNAs) (Appendix E). In addition, the preliminary

genomic sequence data indicate that both *TtRec8-A1* and *TtRec8-B1* contain the primer sequences that were used to amplify the full-length cDNA of *TtRec8*, *i.e.* GM067F (5' CACCTCCTCCTCCGACCT 3') and GM065R (5' ATTCTTTCAGCGTGGCATATCT 3'). Therefore, it is likely that both *TtRec8-A1* and *TtRec8-B1* share the same coding sequence. This will be further verified by analyzing the full-length genomic DNA sequences of *TtRec8-A1* and *TtRec8-B1* and the full-length cDNA sequence.

Another possibility for recovering only one cDNA clone for *TtRec8* might be due to the low abundance of the transcript for one of the two homoeoalleles in LDN. If this was the case, the homoeoallele with the underrepresentated transcript most likely would be *TtRec8-B1* based on the comparative sequence analyses with CS homologues. Further studies are underway to characterize the expression of these two homoeoalleles using LDN D-genome disomic substitution lines 1D(1A) and 1D(1B). A better understanding will be expected for *TtRec8* after the full-length genomic sequences of the both homoeoalleles are obtained and their expression profiles are determined in LDN.

Rec8 is a highly conserved meiotic cohesin in eukaryotes, including yeast, plants, animals, and humans. In addition, Rec8 has been found essential to ensure syntelic orientation of sister kinetochores and reductional division of chromosomes at meiosis I (Molnar *et al.*, 1995; Watanabe and Nurse, 1999; Chelysheva *et al.*, 2005; Zhang *et al.*, 2006). As a meiotic cohesin, Rec8 is synthesized and incorporated into replicating chromosomes at as early as S phase when DNA replicates. It functions like “glue” to hold the newly synthesized sister chromatids together along the entire replicated chromosome. The highest levels of *Rec8* transcripts and Rec8 cohesion proteins have been detected from S phase through early prophase of meiosis I over the entire meiotic process in

models. After that, cohesion protein on both arms is removed, but cohesin in the centromeric region persists to hold sister chromatids together till sister chromatids segregate at anaphase of meiosis II (Klein *et al.*, 1999; Waizenegger *et al.*, 2000; Cai *et al.*, 2003). However, slight variation on the kinetics of the Rec8 cohesin was observed over the meiotic stages in different species. The Rec8 homologue in *Arabidopsis* was detected on the meiotic chromosomes at interphase and later in the centromeric region till metaphase I (Cai *et al*., 2003; Chelysheva *et al.*, 2005). In maize, the Rec8 cohesin is not detectable after prometphase I (Pawlowski, personal communication). Rice cohesin OsREC8 labeling could be detected along the entire length of meiotic chromosomes from interphase till metaphase I (Shao *et al*., 2011).

In this study, TtRec8 cohesion protein was detected along the entire chromosomes at early prophase I, such as leptotene and pachytene. After pachytene and prior to anaphase II, chromosomes became more condensed and shorter; and TtRec8 protein was hardly visualized on the chromosomes, including the centromeric regions. Most likely, TtRec8 cohesin was still retained around the centromere then, but not detectable using the immunolocalization procedure in this study because of large chromosomes in wheat as well as chromosome condensation and covering of TtRec8 protein by condensed chromatin in the centromeric region.

Yeast has much smaller chromosomes than plants, such as *Arabidopsis*, maize, rice, and wheat. The fission yeast Rec8 appeared in the centromere and the surrounding chromosome arms in the premeiotic S phase and the centromeric Rec8 remained detectable during meiosis I and disappeared at anaphase II (Watanabe and Nurse, 1999). The budding yeast Rec8 cohesion protein, Rec8p, was detectable on the chromosomes at

early prophase I till anaphase I around the centromeric region (Klein *et al*., 1999). However, Rec8 in the centromeric region was hardly visualized at later meiotic stages in the plant species with large chromosomes, such as maize, rice, and wheat. This is particularly true in wheat that has largest chromosomes among *Arabidopsis*, maize, and rice. Seemingly, chromosome size is negatively correlated with the visibility of the cohesion protein on chromosomes.

The real-time PCR results indicated that the transcript level of *TtRec8* in the meiotic anthers constantly decreased from interphase/early prophase I through the end of meiosis, *i.e.* tetrad stage. Apparently, variation of the *TtRec8* transcript levels over the different meiotic stages consistently supports the role of TtRec8 as meiotic cohesin in tetraploid wheat. Minimal levels of *TtRec8* transcripts were detected in roots, but not in leaves, suggesting minimal expression of this meiosis-specific cohesin gene in roots.

Western blotting detected the highest TtRec8 level primarily in the anthers at prophase I stage. After that, TtRec8 protein level declined. However, fair amounts of TtRec8 were still detected at later meiotic stages. This probably resulted primarily from the use of unsynchronized anther/meiocyte samples for protein extraction. In other words, the samples used for protein extraction very likely contained anthers and meiocytes at different meiotic stages, rather than all at the same meiotic stage as expected. Obviously, it is critical for meiotic studies to sample meiocytes synchronized at a particular meiotic stage targeted. Real-time PCR encountered the same sampling problem as Western blotting, but to a less extent because it involved a rather small anther/meiocyte sample for RNA extraction comparing to Western blotting. Both real-time PCR and Western blotting detected low levels of *TtRec8* transcript and TtRec8 protein in somatic tissues, indicating

minimal expression of *TtRec8* in non-meiotic tissues. Similar results were reported for *Rec8* in other species (Watanabe and Nurse, 1999; Cai *et al.*, 2003; Zhang *et al.*, 2006).

Clearly, TtRec8 functioned as a meiotic cohesin in tetraploid wheat. The other functions TtRec8 may have in tetraploid wheat LDN are under determination, including its role in kinetochore orientation and haploidy-dependent UMCD. Significant lower level of *TtRec8* transcript was observed in the anthers at early prophase I of the LDN × *Ae. tauschii* hybrid (haploid) than LDN (tetraploid). This might suggest the possible involvement of TtRec8 in the onset of haploidy-dependent UMCD in LDN. Further studies are needed to completely reveal the functions of TtRec8 under both haploid and disomic conditions.

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CHAPTER IV. PRELIMINARY STUDIES ON THE PUTATIVE POLO-LIKE KINASE GENE *CDC5* IN TETRAPLOID WHEAT

Abstract

A complex genetic network involving multiple genes conditions meiotic cell division. Within this network, polo-like kinase Cdc5 has proven to promote meiosis in an orderly progress. It is required to ensure monopolar attachment of sister kinetochores at meiosis I and is involved in the formation of chiasmata as well as stepwise removal of cohesin in meiosis. The objectives of this study were to perform initial studies towards cloning the *Cdc5* homologue in tetraploid wheat 'Langdon' (LDN) (*T. turgidum* L.), designated *TtCdc5*. Two homoeoalleles of the putative *TtCdc5*, encoding for 1,081 and 1,084 amino acids respectively, were cloned from LDN through the comparative genomic analysis. They were localized to chromosome 5A and 5B, respectively. Real-time PCR showed significantly higher levels of the putative *TtCdc5* transcripts in meiotic anthers than in roots and leaves of LDN. The transcript level of the putative *TtCdc5* was highest at pachytene stage of meiosis I, and then decreased as meiosis proceeded. Moreover, a higher expression level of *TtCdc5* was observed in meiotic anthers than in somatic tissues of LDN. These results indicated that the putative *TtCdc5* expressed primarily in anthers at pachytene stage of meiosis I as reported with *Cdc5* orthologues in models. The sequences and conserved domains of *TtCdc5* as well as its kinetics through the meiotic process in LDN were very similar to the polo-like kinase Cdc5 in models. Additional functional analyses are needed to verify the identity of this gene as a Cdc5 homologue in tetraploid wheat.

Introduction

Meiotic cell division involves one round of DNA replication and two continuous nuclear dividions and leads to four haploid daughter cells with reduced chromosomes. The first round of cell division of meiosis, *i.e.* meiosis I, differs from meiosis II and mitosis in chromosome behavior and genetic outcome (Miyazaki and Orr-Weaver, 1994; Roeder, 1997; Pagliarini, 2000; Mitchison and Salmon, 2001; Hirano, 2002; Gerton and Hawley, 2005; Harrison *et al*., 2010). At meiosis I, each pair of sister kinetochores in the paired homologous chromosomes (bivalent) orient syntelically (*i.e.* monopolar orientation), but the two pairs of sister kinetochores in a bivalent are attached by spindle microtubules emanating from opposite poles in the mother cell. This drives paired homologous chromosomes to segregate, while sister chromatids still connect to each other at meiosis I. At meiosis II, sister kinetochores orient amphitelically and are attached by spindle microtubules emanating from opposite poles (*i.e.* bipolar orientation). This kinetochore-microtubule attachment makes sister chromoatids divide and migrate toward opposite poles, leading to a mitosis-like cell division at meiosis II.

Polo-like kinase (Plk), Cdc5, is a curial regulator of mitosis in yeast (Toczyski *et al*., 1997; Glover *et al*., 1998; Shirayama *et al*., 1998; Alexandru *et al*., 2001). Also, Cdc5 has been found to play a significant role in meiosis (Clyne *et al*., 2003; Lee and Amon, 2003).

Polo was first identified in *Drosophila* to be a regulator for correct structure and function of centrosome (Sunkel *et al*., 1988). Polo-like kinases are a conserved subfamily of serine/threonine protein kinases that play central roles in cell cycle and proliferation. Only single copy of Plks (Polo, Cdc5, and Plo1) was found in *Drosophila melanogaster*,

S. cerevisiae, and *S. pombe*, respectively (Sunkel *et al*., 1988; Kitada *et al*., 1993; Ohkura *et al*., 1995). In higher mammals, four Plks apparently show different spatial distributions and execute differential functions. Three of them, Plk1, Plk2 (also called Snk), and Plk3 (also called Prk or Fnk), are closely related with one another, in the viewpoints of both sequence similarity and function conservation (Simmons *et al*., 1992; Clay *et al*., 1993; Lake *et al*., 1993; Hamanaka *et al*., 1994; Holtrich *et al*., 1994; Donohue *et al*., 1995; Li *et al*., 1996; Liby *et al*., 2001). The fourth member of Plks, Plk4 (also called Sak), is distantly related to the other three (Fode *et al*., 1994).

Plks are found functionally conserved at several checkpoints during mitosis in models, such as entry and exit of mitosis, spindle pole assembly, and cytokinesis (Golsteyn *et al*., 1995; Donaldson *et al*., 2001; Park *et al*., 2010). First, Plk1 activates the phosphatase Cdc25 that functions as a positive regulator for Cdc2-Cyclin B. Cdc2-Cyclin B is a major cyclin that facilitates the G_2-M transition in the cell cycle (Ohi *et al*., 1999; Smits *et al*., 2001; Qian *et al*., 2001; Hutchins *et al*., 2004). Second, Plks regulate mitosis checkpoints in partially activating the anaphase-promoting complex (APC), which is an E3 ubiquitin protein ligase facilitating the degradation of anaphase inhibitor. This anaphase inhibitor is also called securin, *i.e.* Pds1 in budding yeast and Cut2 in fission yeast, because of its function in controlling separation of two sister chromatids (Nasmyth *et al*., 2000). At anaphase in budding yeast, destruction of securin Pds1 releases the Esp1 protease, which cleaves Scc1, a subunit of cohesin complex, and leads to the separation of sister chromatids. Similarly, in fission yeast, degradation of securin Cut2 results in the release of Cut1, an Esp1 homologue that can remove cohesin Scc1 proteins. Esp1 and Cut1 are thus termed as separin. Moreover, Plks can function in more than one pathway

to assist removal of cohesion proteins at the beginning of anaphase. Beside the pathway above, Plk Cdc5 can phosphate the cohesin Scc1 that in turn increases the susceptibility of Scc1 to Esp1 action in budding yeast. Third, Plk Cdc5 plays an essential role in the Cdc FEAR (Cdc Fourteen Early Anaphase Release) network, in which it induces the release of phosphatase Cdc14 from nucleolus at early anaphase. The released activated Cdc14 inactivates a series of mitotic kinases, resulting in the exit of mitosis and ultimately cytokinesis (Geymonat *et al*., 2002; Stegmeier *et al*., 2002).

Polo-like kinases have been found conservatively essential in meiosis in addition to their function in mitosis. During meiosis, the mitotic cohesin Scc1 is fully replaced by its meiotic counterpart Rec8 (Sharon *et al*., 1990; Herrmann *et al*., 1998; Chase *et al*., 2000; Pahlavan *et al*., 2000). Meiotic cohesin Rec8 shows distinct resistance to Esp1 around centromeres at meiosis I from mitotic cohesin Scc1. The difference between these two versions of cohesion proteins has been thought vital in the segregation of homologous chromosomes at meiosis I. Cohesion protein Rec8 is removed from meiotic chromosomes in a two-step fashion, leading to the segregation of homologous chromosomes at meiosis I and separation of sister chromatids at meiosis II, respectively. Prior to anaphase I, Rec8 is cleaved along the chromosomal arms by separase Esp1 with the same mechanism as Scc1 cleavage in mitosis, while remaining intact around the centromeric regions. This permits the recombined homologous chromosomes to segregate to opposite poles of the mother cell at meiosis I. The remained cohesin Rec8 around centromeric regions is further removed prior to anaphase II, resulting in the separation of sister chromatids. It is experimentally shown that Rec8 displays different resistance pattern to degradation as Scc1. During the meiosis of budding yeast, cohesin Rec8 is

degraded in the two-step fashion as described above, while when cohesin Rec8 is replaced by Scc1, it was completely degraded along the entire chromosome length at meiosis I (Tóth *et al*., 2000).

The Plk Cdc5 is required for monopolar attachment of sister kinetochores in meiosis I. A meiosis-specific kinetochore protein, Mam1, was characterized as an important suppressor of bipolar attachment of sister kinetochore in budding yeast (Tóth *et al*., 2000). It has been found that phosphorylated Mam1 and released Lrs4 in the presence of Cdc5 localized the Mam1-Lrs4 monopolin complex in the kinetochore region, leading to monopolar attachment of sister kinetochores (Rabitsch *et al*., 2003). Lee and Amon (2003) observed that Cdc5 was required for monopolar attachment in meiosis I. They also found that the Cdc5-depleted cells in which Rec8 was replaced by Scc1 (*pRec8-Scc1*) went through an equational rather than reductional cell division at meiosis I. In the positive control set (*Cdc5+ pRec8-Scc1*) where Cdc5 was present, the mother cells underwent reductional division at meiosis I.

Cdc5 acts in a consistent manner for the removal of cohesion protein Rec8, as compared with its role in Scc1 degradation. On one hand, Cdc5 phosphorylates Rec8, which in turn enhances the susceptibility of Rec8 to Esp1 cleavage. On the other hand, Cdc5 elevates the activation of anaphase-promoting complex (APC), which will further degrade the securin Pds1. The released form of separase Esp1 is thus activated to cleave Rec8 proteins along the chromosome arms prior to anaphase I. However, Rec8 remains intact around centromeric regions, due to its specific resistance to cleavage prior to anaphase II. As the cohesion is removed, the paired homologous chromosomes are pulled

apart by the spindle microtubules emanating from opposite poles (Clyne *et al*., 2003; Lee and Amon, 2003).

In addition, Cdc5 is required for the formation of chiasmata, which are the physical links between paired and recombined homologous chromosomes (Clyne *et al*., 2003). It has been suggested that Cdc5 participates in the regulation of late pachytene events, other than monopolin deposition and localization. At pachytene stage, recombination intermediates containing double Holliday junctions (dHJ) are reportedly resolved into crossovers (COs) that hold paired homologous chromosomes together. However, dHJ-containing intermediates failed to be resolved in Cdc5-depleted cells (*pScc1-Cdc5*) and the CO formation decreased to 1/7 of the level in wild-type cells (Clyne *et al*., 2003). Rec8 has been considered a putative substrate during the maturation of recombination products.

Watanabe (2003) summarized the function of Plk Cdc5 at meiosis I in budding yeast (Figure 4.1a). As shown in Figure 4.1b, when Cdc5 is depleted during meiosis, removal of the cohesin Rec8 lessens along the chromosome arms and sister kinetochores are attached by spindle microtubules emanating from opposite poles, resulting in meiotic arrest at anaphase I.

Furthermore, the Polo-like kinase Cdc5 promotes the exit from pachytene stage in budding yeast (Sourirajan *et al*., 2008). During prophase I, the transition from pachytene to diplotene stage involves a number of important events, including the resolution of recombination intermediates into COs, synaptonemal complex (SC) disassembly, and kinetochore positioning for monopolar orientation of sister chromatids, *etc*. The failure of

these events often results in chromosome nondisjunction, and eventually the generation of aneuploids and polyploids. To minimize errors in these meiotic events, multiple intrinsic checkpoint systems have been identified to monitor every step to proceed in an orderly way and prevent the exit from pachytene until these steps are completed (Roeder *et al*., 2000). The transcription factor Ndt80 has been found to be the central target of the checkpoint systems, which activates the expression of more than 200 genes in meiosis (Xu *et al.*, 1995; Chu and Herskowitz, 1998; Chu *et al*., 1998; Tung *et al*., 2000). In normal meiosis, Ndt80 is phosphorylated in a wide scope; however, it is either hypophosphorylated or less abundant in cells that are arrested at pachytene stage and the target genes under its regulation are not expressed (Tung *et al*., 2000). Polo-like kinase Cdc5 has been considered a major target for Ndt80 regulation. Sourirajan *et al*. (2008) concluded that Cdc5 was the only member in the Ndt80 regulation process required for the exit of pachytene. Also, they found that induced expression of *Cdc5* in *ndt80Δ* cells efficiently promoted the resolution of recombination intermediates into COs and SC disassembly which confirmed the conclusion of Clyne's group (2003) (Clyne *et al*., 2003; Sourirajan *et al*., 2008; Iacovella *et al*., 2010). In addition, Cdc5 possesses other functions during meiosis, such as involving in the adaption of DNA damage, and maintaining the spindle integrity (Sourirajan *et al*., 2008; Iacovella *et al*., 2010).

Polo-like kinase Cdc5 is not only an irreplaceable regulator in meiosis and mitosis, but also plays an essential role in mRNA splicing (Burns *et al*., 1999; Tsai *et al*., 1999) and as a crucial component of spliceosome (McDonald *et al*., 1999; Ajuh *et al*., 2000). Polo-like kinase conservatively contains a putative DNA-binding domain (DBD), which shares most similarities to those contained within Myb-related proteins. Myb-like

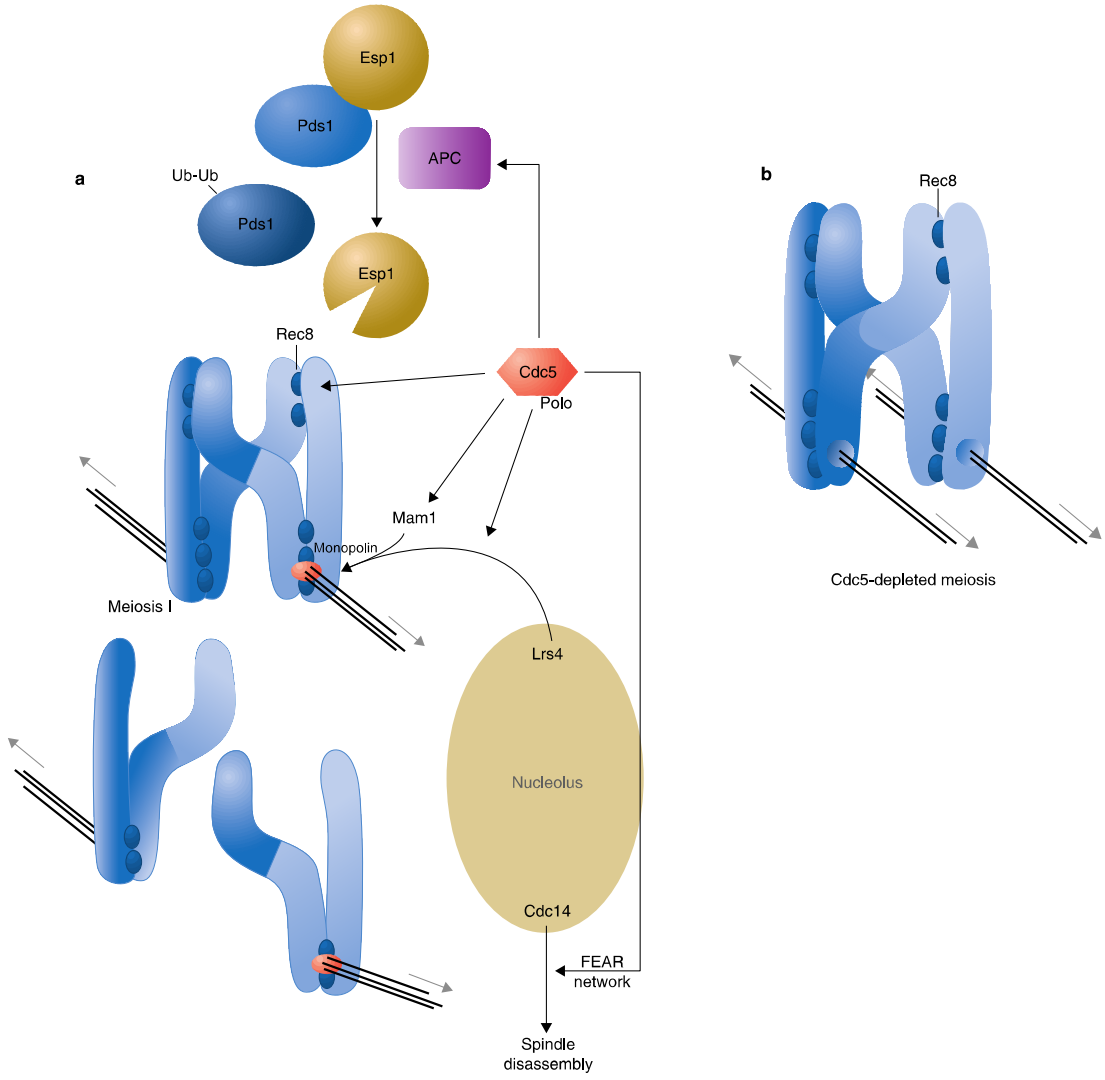

Figure 4.1. Roles of the Cdc5 polo-like kinase during meiosis I in budding yeast. With reference to Watanabe, 2003.

Cdc5 is the only putative transcription factor involved in the G_2-M transition in fission yeast (Ohi *et al.*, 1998). Although the essential functions of Myb-related Cdc5 have been well characterized in yeast and animals, its role in plants remains obscure. The first Cdc5-like homologue in multicellular organisms was cloned from *Arabidopsis* and characterized as involving in the expression regulation of a set of genes necessary for the

progression through G_2 phase (Hirayama and Shinozaki, 1996). Lin *et al*. (2007) used RNA interference (RNAi) and virus-induced gene silencing (VIGS) methodologies to study the function of *Cdc5* in *Arabidopsis* and found that *AtCdc5* is essential for G_2-M transition and programmed cell death (PCD). Currently, there is no report about how Cdc5 plays the tango in polylploid species like wheat. The objective of this study was to perform initial cloning and fuctional analyses toward a better understanding of the *Cdc5* homologue in tetraploid wheat (*T. turgidum* L.), designated *TtCdc5*.

Materials and methods

Plant materials and male meiocyte collection

Durum wheat 'Langdon' (LDN) (*T. turgidum* ssp. *durum*, 2n=4x=28, genome AABB), common wheat 'Chinese Spring' (CS) (*T. aestivum* L., 2n=6x=42, genome AABBDD), a set of CS nulli-tetrasomic lines, and a series of LDN D-genome disomic substitution lines (LDN DS) were included in this study. The CS nulli-tetrasomic lines each misses one pair of homologous chromosomes but has four homologues of another chromosome within the same homoeologous group. The LDN DS has one pair of A- or B-genome homologous chromosomes substituted by a pair of D-genome homologous chromosomes from CS within the same homoeologous group from CS. All the wheat materials were grown in a temperature-controlled greenhouse room for the sampling of male meiocytes, leaf, and root tissues in this study. Total genomic DNA of all these wheat lines was extracted from leaf tissues as described by Faris *et al*. (2000). The CS nulli-tetrasomic lines and LDN DSs were used to determine the chromosomal location of the *Cdc5*-like genes in tetraploid wheat. Male meiocytes (anthers) at different meiotic

stages were sampled following the procedure of Cai (1994). Anther samples were stored in liquid nitrogen for real-time PCR and Western blotting analyses.

Production of LDN haploids

Langdon haploid plants were produced by pollinating LDN plants with maize pollen and embryos were rescued following the procedure as described by Cai *et al*. (2010). Haploid plants were grown in the greenhouse to collect leaf tissues for DNA extraction.

RNA extraction and cDNA preparation

Total RNA was extracted from leaves, roots and anthers of LDN at interphase/early prophase I, pachytene, metaphase I, metaphase II/anaphase II, tetrads, and matured pollen stages using RNAqueous®-4PCR Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Prior to cDNA synthesis, total RNA was first treated with DNase I and purified with RNAqueous®-4PCR Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. After quantification with NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and agarose gel, 1 µg of total RNA were used as templates for first strand cDNA synthesis with the use of SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions.

cDNA cloning of the *Cdc5*-like gene in LDN

Cdc5 is reported to be conserved among eukaryotes, especially among plants. Yeast Cdc5 homologue CDC5p (GenBank accession NP_013714.1) was chosen to search

for the homologues in plants. After search in NCBI HomolGene database (www.ncbi.nlm.nih.gov/homolgene), the mRNA sequences of *Cdc5* gene from rice and *Arabidopsis* (GenBank accession NP_001059056 and NM_100849.2, respectively) were selected as query to *tblastn* the wheat tentative consensus (TC) sequence database. Wheat ESTs having high sequence homology with rice and *Arabidopsis Cdc5* were annotated based on the information of the *Cdc5*-like genes available in models and other plants. The nucleotide sequence of the candidate EST in wheat was used to design gene specific primers using Primer3 (http://frodo.wi.mit.edu/), which were then synthesized in Integrated DNA Technologies (IDT, Coralville, IA, USA). The cDNAs synthesized from total RNA in the LDN anther at early prophase I/pachytene stages was used as templates for subsequent RT-PCR with the gene specific primers. 3’ and 5’ RACE (rapid amplification of cDNA ends) were performed to extend the cDNA sequence of the candidate gene in tetraploid wheat. The final complete cDNA sequence of the candidate gene was PCR amplified by the primer pair LWD1 (5' CAAGGGAGCGGGCAAGAT 3') and GM014 (5' CCCGCAGATGAGGTATG 3') that spanned the start and stop codons of the gene. The RT-PCR, cloning, and RACE were performed according to Ma *et al*. (2006).

Sequence alignment and phylogenetic tree construction

The annotation of putative Cdc5 orthorlogue in tetraploid wheat was performed by the use of BLASTP 2.2.26+ in NCBI nr database (www.ncbi.nlm.nih.gov). The functional domains were searched against the conserved domain database embedded in NCBI website (http://www.ncbi.nlm.nih.gov/cdd). The multi-alignment and bootstrap Neighbor-Joining phylogenetic tree of amino acid sequences of Cdc5 proteins from

different species were analyzed through the use of ClustalX 2.1, and the phylogenetic tree was visualized with software FigTree v1.4.0. The leucine-rich nuclear export signal (NES) was predicted with the software NetNES 1.1 (http://www.cbs.dtu.dk/services/NetNES/).

Quantitative real-time PCR

Real-time RT PCR was conducted on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to quantify the relative levels of *Cdc5*-like gene transcript in leaves, roots, and anthers at interphase/early prophase I, pachytene, metaphase I, metaphase II/anaphase II, tetrads, and matured pollen stages in LDN as described by Chao (2008). One pair of *Cdc5*-like gene specific primers were used, *i.e.* GM018F (5' GAAATCGTGCTGCTGAG 3') and GM018R (5' GTCAATGGCTCCCTCA 3'). After dissociation test and primer validation, 18S rRNA gene was used as endogenous control and the primer pair is GM003 (5' GAGGGACTATGGCCGTTTAGG 3') and GM004 (5' CACTTCACCGGACCATTCAATCG 3'). Two technical and three biological replications were performed. The comparative C_T method was used to determine changes in *Cdc5*-like gene expression in different samples (test) relative to anthers of interphase/early prophase I stage (control) as described by Chao *et al*. (2010). Fold difference in gene expression is $2^{-\Delta CT}$, where $-\Delta C_T = C_{T,test} - C_{T,control}$.

Chromosomal localization

Wheat *Cdc5*-like gene sequences were amplified from a set of 21 CS nulli-tetrasomic lines and a series of 14 LDN D-genome disomic substitution lines (LDN DS), as well as tetraploid LDN, LDN haploid, and hexaploid CS by PCR with *Cdc5*-like gene

specific primer pair GM017F (5' AACAACTTGAGGAGCACA 3') and GM017R (5' GTCAGGCAATGGAGGA 3'). 50 ng DNA was used as PCR template in a 25 µl reaction system. PCR amplification was performed with the Platinum® Taq Polymerase (Invitrogen Corporation, Grand Island, NY, USA), and PCR cycles were as follows: 2 min at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, followed by 7 minutes extension at 72°C. Upon PCR amplification, the products were separated on the denaturing polyacrylamide gel and scanned for analysis.

Antibody production and affinity-purification

A 675 bp cDNA segment (named C31) of the *Cdc5*-like gene was chosen, based on its low hydrophobicity and sequence uniqueness, to raise antibody against the wheat Cdc5-like protein. The segment was PCR amplified from the primer pair GM031F (5' AATAGAATTCATGGCTGGTGCCTATCGT 3') and GM031R (5' ACATGTCGAC*TCA*CTTGTCAATGGCTCCCTCA 3'), which were modified with the addition of *Eco*RI and *Sal*I recognition sites at 5' ends (underlined), respectively, and the addition of a stop codon (italic) at 3' of *Sal*I site of GM031R. After purification, this cDNA segment was cloned into two expression plasmid vectors pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ, USA) and pMAL-c2X (New England Biolabs, Ipswich, MA, USA), respectively. After verification by sequencing, the two constructs were transformed into *E. coli* strain BL21-Star (DE3) (Invitrogen Corporation, Grand Island, NY, USA). Upon IPTG (isopropyl β-D-1-thiogalactopyranoside) induction, the fusion polypeptides, pGEX-C31 and pMAL-C31, were accumulated in insoluble pellets and resolubilised after sonication. The generation of two fusion peptides, pGEX-C31 and pMAL-C31, was conducted as described by Chao *et al.* (2007). The total proteins were

separated on SDS-PAGE gel and the candidate bands were cut out as per the estimated molecular weight. Upon the verification with protein ID test (Appendix F and G) done in Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University (Stanford, CA, USA), the polypeptide pGEX-C31 was used for the immunization and generation of the polyclonal antibody in rabbits by Affiniity BioReagents (ABR, Golden, CO, USA; now Thermo Fisher Scientific, Inc.).

In order to isolate the antibodies that can specifically recognize Cdc5-like protein in LDN, the anti-pGEX-C31 crude serum after second booster was affinity-purified as described by Chao *et al.* (2007) with minor modifications. The affinity-purified pMAL-C31 polypeptide was first coupled to AminoLink coupling resin with the use of AminoLink Plus Immobilization Kit (Pierce Biotechnology, Rockford, IL, USA) and then incubated with crude serum. After incubation, the mixture of crude serum and resin was loaded to the column, and the serum was collected and stored in -80°C for later use. The anti-Cdc5 antibody was eluted with the IgG Elution Buffer (Pierce Biotechnology, Rockford, IL, USA) after washing the column with 20 column volumes of 1× PBS solution. Aliquots of anti-Cdc5 antibody were made and stored in -80°C for later use, one of which was taken out for concentration determination.

Immunoprecipitation and Western blotting

Total proteins of LDN were phenol-extracted from leaves, roots, and anthers at interphase, prophase I, metaphase I/anaphase I, and metaphase II/anaphase II stages and were loaded 5 µg per lane for blotting. The protein extraction, immunoprecipitation and Western blotting procedures were performed following the procedure described by Chao

et al. (2007). The anti-Cdc5 antibody was diluted in a ratio of 1:2,000 for Western blotting upon verification of endogenous TtCdc5 concentration gradient test.

Microscopy

Phase/Fluorescent microscope Olympus CX41RF (Olympus Optical Co. Ltd., Tokyo, Japan) was used to determine the meiotic stages of meiocytes in each anther.

Results

Cloning and characterization of *Cdc5* homologues in tetraploid wheat

The polo-like kinase gene *Cdc5* has been found highly conserved across a variety of eukaryotes, including fungi, plants, and animals in terms of DNA and protein sequences and subcellular functions (Hirayama and Shinozaki, 1996; Clyne *et al*., 2003; Lee and Amon, 2003; Lacovella *et al*., 2010). Since yeast Cdc5 homologue CDC5p (Genbank accession NP_013714.1) functions to promote chiasma formation and sister cosegregation, it is chosen to search for homologues in plant models (Clyne *et al*., 2003). Rice (*Oryza sativa*) and *Arabidopsis* have long been used as models for plants (Rédei, 1992; Fink, 1998; Kellogg, 2001; Gaut, 2002; Salse *et al*., 2008; Koornneef and Meinke, 2010). In this study, the mRNA sequences of *Cdc5* genes in rice (Genbank accession NM_001059056.2) and *Arabidopsis* (Genbank accession NM_100849.2) were used as queries to search against common wheat TC database (previously at www.tigr.org; and now at http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat), which runs *wu-blast* 2.0. A 962-bp EST from common wheat (TC257703; currently split into TC392389) was found to have the highest sequence similarity with both queries (85% identity and E-value 3.9E-154 with rice *Cdc5*; 69% identity and E-value 32.7E-85 with

Arabidopsis Cdc5) and annotated as a Cdc5 protein gene after *tblastn* search. According to the nucleotide sequences of this wheat EST, wheat *Cdc5*-like gene specific primer combinations were designed in the conserved region. RT-PCR was performed on the cDNAs from LDN anthers at early prophase I/pachytene stages when the *Cdc5*-like genes were highly expressed. The amplicons from the RT-PCR were sequenced and analyzed, and then used to design gene-specific primers for the next round of 3' and 5' RACE. The draft cDNA sequences of the candidate gene were obtained in LDN after several rounds of RACE reactions. Since LDN is an allotetraploid with two homoeologous sub-genomes, *i.e.* A and B, it generally contains two homoeoalleles with high sequence similarities on each of two homoeologous chromosomes (Murai *et al*., 1999; Huang *et al*., 2002; Kimbara *et al*., 2004; Zhang *et al*., 2011; Brenchley *et al*., 2012). Thus, errors could occur when assembling the cDNA segments from the RACE reactions. To avoid the assembling errors, two 5' and 3' gene-specific primers of the final round of RACE (LWD1: 5' CAAGGGAGCGGGCAAGAT 3' and GM014: 5' CCCGCAGATGAGGTATG 3'), which spanned the start and stop codons, were used to amplify full-length cDNA sequences of the gene. Two cDNA clones were obtained from the amplicons. They were 3,243 and 3,252 bp long and encode for 1,081 and 1,084 amino acids, respectively. They have a predicted molecular weight of 120.74 and 121.05 kDa, respectively. In addition, the two homoeoalleles of the putative *Cdc5* genes shared significantly high similarity. At the nucleotide level, there were only 75 (3%) single nucleotide differences between the two homoeoalleles. Fifty-three of them are purine-purine or pyramidine-pyramidine differences and 22 are purine-pyramidine or pyramidine-purine differences. Moreover, there were three indels in these two

homoeoalleles, one of which was 9-bp indel and the other two were 3-bp indels. The amino acids of the proteins encoded by these two homoeoalleles showed 97% similarity.

The predicted protein encoded by the candidate gene was compared to Cdc5 orthologues from other eukaryotes, *e.g.* Cdc5-like protein from *Brachypodium distachyon*, *Zea mays*, *Glycine max*, *A. thaliana*, *Xenopus laevi*, *Danio rerio*, and *Meleagris gallopavo*. The comparative analysis revealed high levels of amino acid sequence similarity (Table 4.1).

Table 4.1. Amino acid similarities of the predicted protein of *Cdc5*-like gene in tetraploid wheat with Cdc5 proteins in other eukaryotic species

Species	GeneID	Genbank Accession	Similarity
B. distachyon	100830760	XP_003576174.1	94%
Z. mays	N/A	AAL59389.1	90%
G. max	100790369	XP_003536137.1	73%
A. thaliana	837506	NP_172448.1	71%
D. rerio	394059	NP_957378.2	49%
M. gallopavo	100549354	XP_003204698.1	49%
X. laevis	443636	NP_001131045.1	48%

The deduced polypeptides of the putative *TtCdc5* homoeoalleles had two specific adjacent domains located at the N terminus, *i.e.* 'SWI3, ADA2, N-CoR and TFIIIB (SANT)' DNA-binding domain and SANT/myb-like DNA-binding domain of CDC5-like protein repeat II (Figure 4.2), suggesting their function in the binding of transcription

factors. A threonine-proline-rich region was found at the position of 345-452 in both proteins encoded by the candidate homoeoalleles of *TtCdc5* (Figure 4.2). Some of these Thr-Pro di-amino acid sequences were followed by Arg/Lys or Xaa-Arg/Lys residues, suggesting the consensus sequences of the target for protein serine-threonine kinases. The phosphorylation status of these threonine residues might be related to the activity of Cdc5. The amino acids at the positions 811 and 813 were predicted to participate in the leucine-rich nuclear export signal (NES) (Figure 4.2 and 4.3).

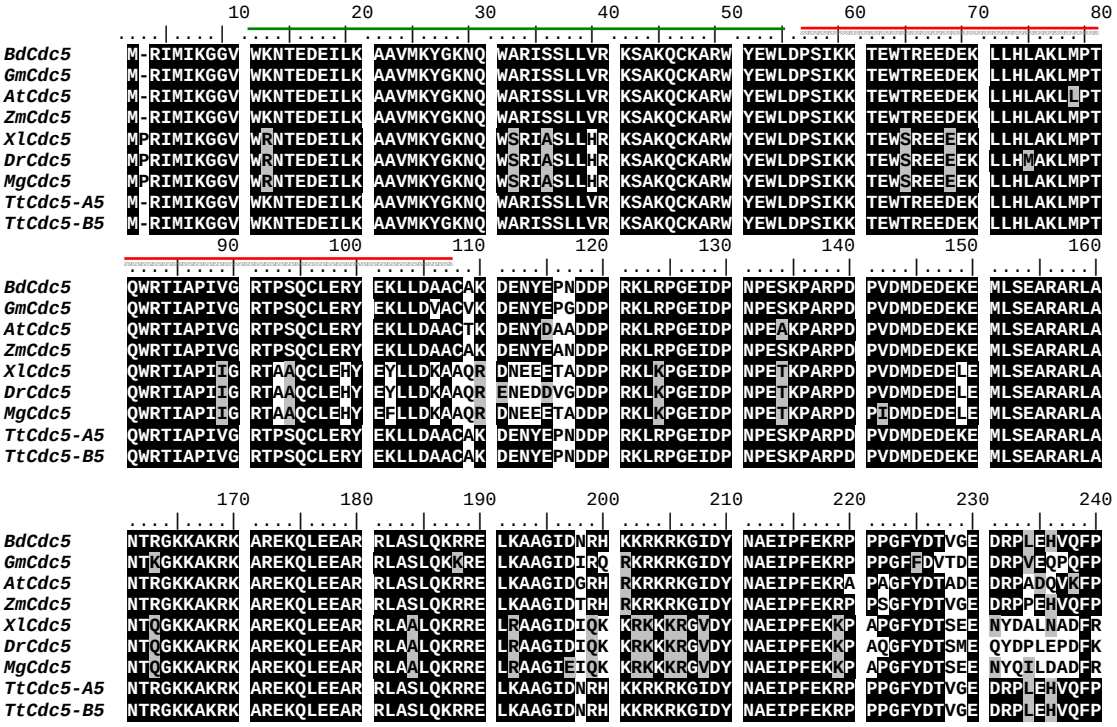

Figure 4.2. Alignment of putative Cdc5 orthologues in tetraploid wheat with other eukaryotic Cdc5 proteins. Deduced amino acid sequences of two homoeoalleles of *TtCdc5*-like genes are aligned with Cdc5 orthologue from *Brachypodium distachyon* (GeneID: 100830760), maize (GenBank accession AAL59389.1), Glycin max (GeneID: 100790369), *Arabidopsis thaliana* (GeneID: 837506), *Xenopus laevis* (GeneID: 443636), *Danio rerio* (GeneID: 394059), and *Meleagris gallopavo* (GeneID: 100549354) by ClustalW. Black, grey, and white backgrounds indicate the levels of conservation of amino acids. Green and red bold lines above the sequence indicate the N-terminal conserved domains of SANT DNA-binding domain and SANT/myb-like DNA-binding domain, respectively. Threonine-proline rich region in the middle is highlighted with pentagrams. The leucine-rich nuclear export signals (NES) in position 811 and 813 are highlighted with diamonds.

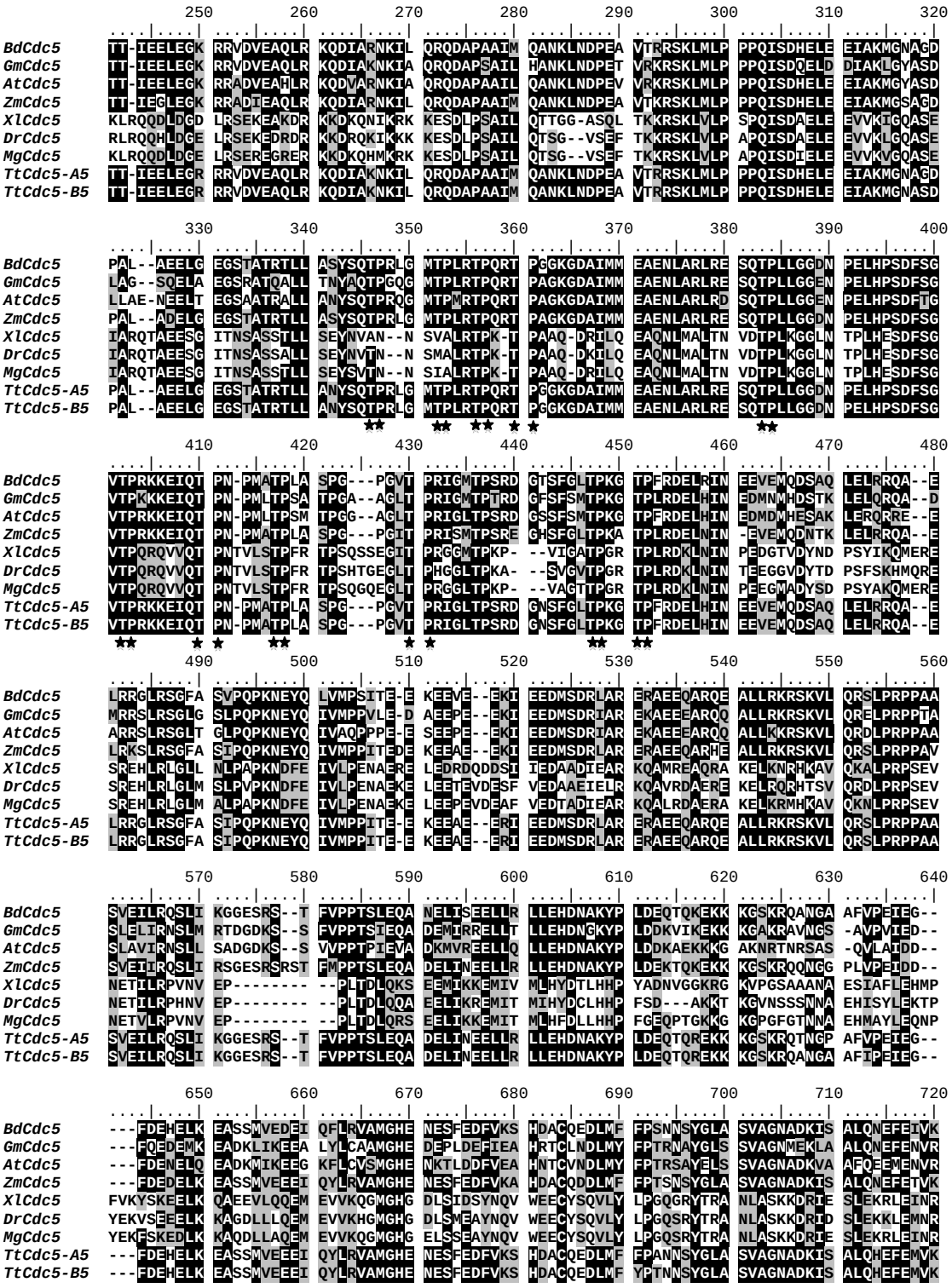

Figure 4.2. (continued) Alignment of putative Cdc5 orthologues in tetraploid wheat with other eukaryotic Cdc5 proteins.

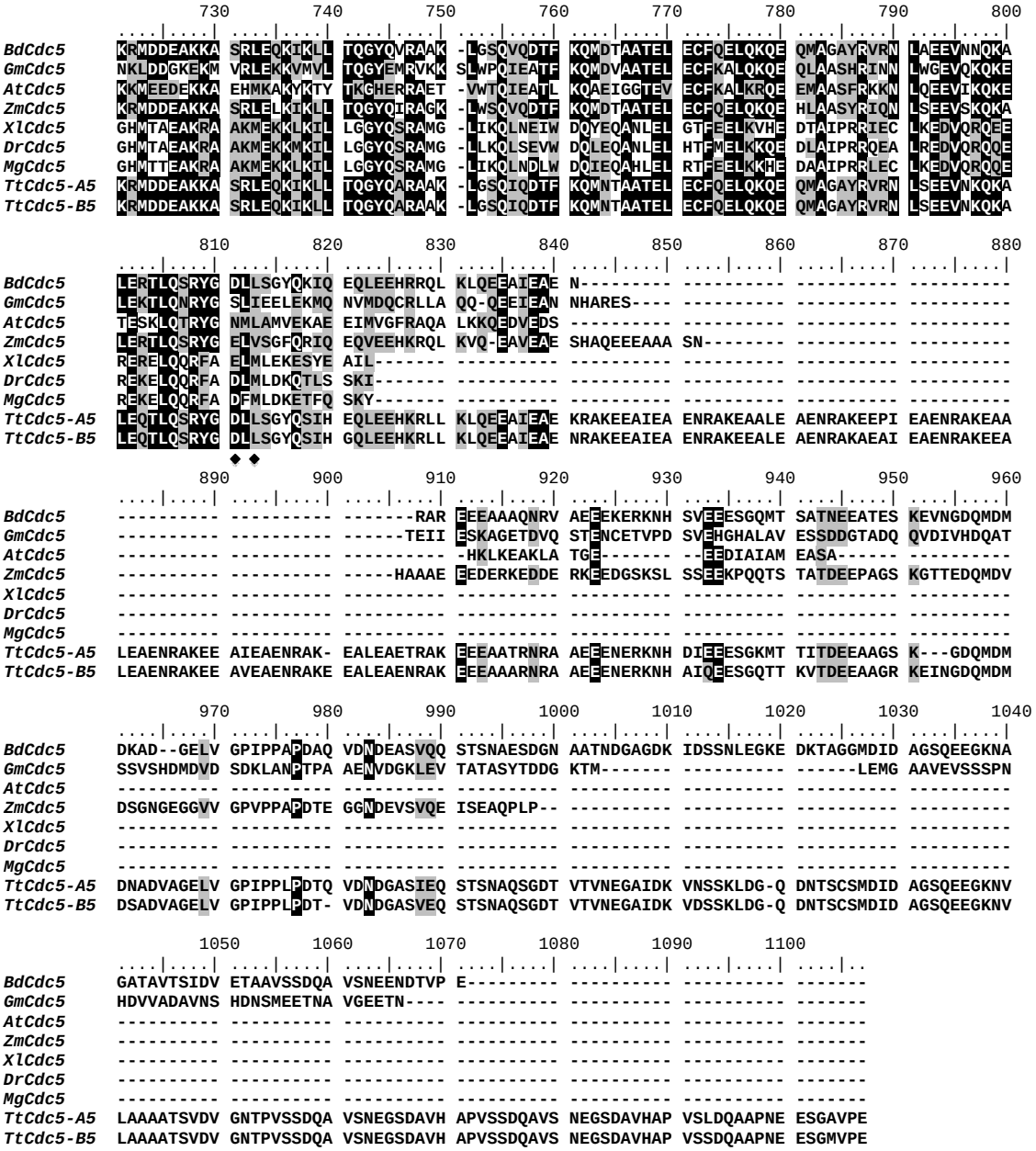

Figure 4.2. (continued) Alignment of putative Cdc5 orthologues in tetraploid wheat with other eukaryotic Cdc5 proteins.

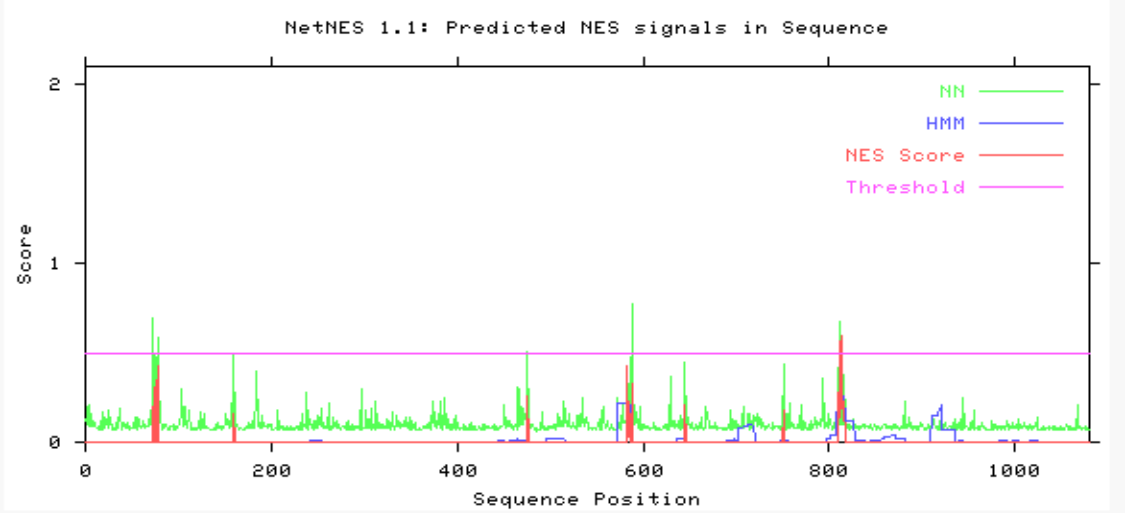

Figure 4.3. The leucine-rich nuclear export signal (NES) prediction of putative TtCdc5 protein. The NES scores of amino acid 811 and 813 (red peaks) of putative TtCdc5 are over the threshold (purple horizontal baseline) and predicted to be the potential NES.

Expression analysis of the putative *TtCdc5*

Significantly higher levels of putative *TtCdc5* transcripts were consistently detected by real-time PCR in anthers at early meiotic stages than in roots and leaves in three biological replicates. The expression level of putative *TtCdc5* reached highest at pachytene stage, and gradually decreased after that as meiosis proceeded. At tetrad stage, the transcripts of putative *TtCdc5* dropped to 37% of the highest level at pachytene stage (Figure 4.4). The relative transcript levels of putative *TtCdc5* in the somatic tissues, *i.e.* roots and leaves, were only about 37% and 1% of that in anthers at pachytene stages, respectively.

Immunoprecipitation was performed to verify the specificity of the anti-TtCdc5 antibody. After anti-TtCdc5 antibody was incubated with the protein extract from anthers undergoing meiosis, a protein with a molecular weight close to the predicted proteins (~120 kDa) was not immunoprecipitated. Also, similar results were obtained in the

anthers at different meiotic stages by Western blotting. These results suggested that the anti-Cdc5 antibody probably was not specific enough for the TtCdc5 protein and it might interact with other cellular proteins.

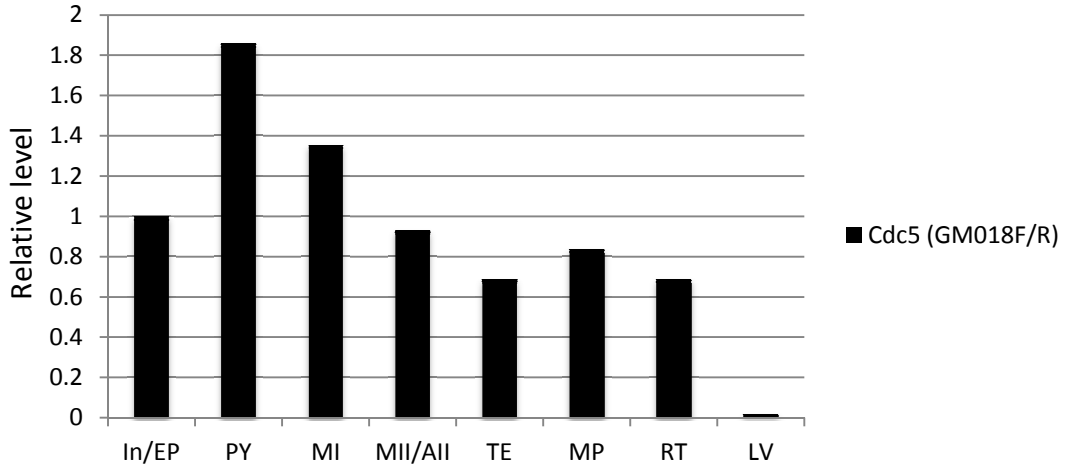

Figure 4.4. Relative transcript levels of *TtCdc5* in roots, leaves, and anthers at different meiotic stages in LDN. Data from one primer pair GM018F/GM018R were plotted in the filled bars. In/EP: Interphase/Early Prophase I; PY: Pachytene; MI: Metaphase I; MII/AII: Metaphase II/Anaphase II; TE: Tetrads; MP: Matured Pollen; RT: Roots; and LV: Leaves.

Phylogenetic analysis of Cdc5-like protein in tetraploid wheat

Bootstrap Neighbor-Joining phylogenetic tree (bootstrap value =1,000) showed the predicted proteins of the two putative *TtCdc5* homoeoalleles were genetically closest related to each other, followed by the monocots counterparts *B. distachyon* and maize *Cdc5*. The Cdc5 proteins in dicotyledon *A. thaliana* and *G. max* were a little genetically further related with the putative TtCdc5 as compared to the monocots Cdc5. The Cdc5 proteins from animals showed the furthest phylogenetic distances from the putative

TtCdc5, *i.e.* Cdc5 in *D. rerio* DrCdc5, Cdc5 in *M. gallopavo* MgCdc5 and Cdc5 in *X. laevi* XlCdc5 (Figure 4.5).

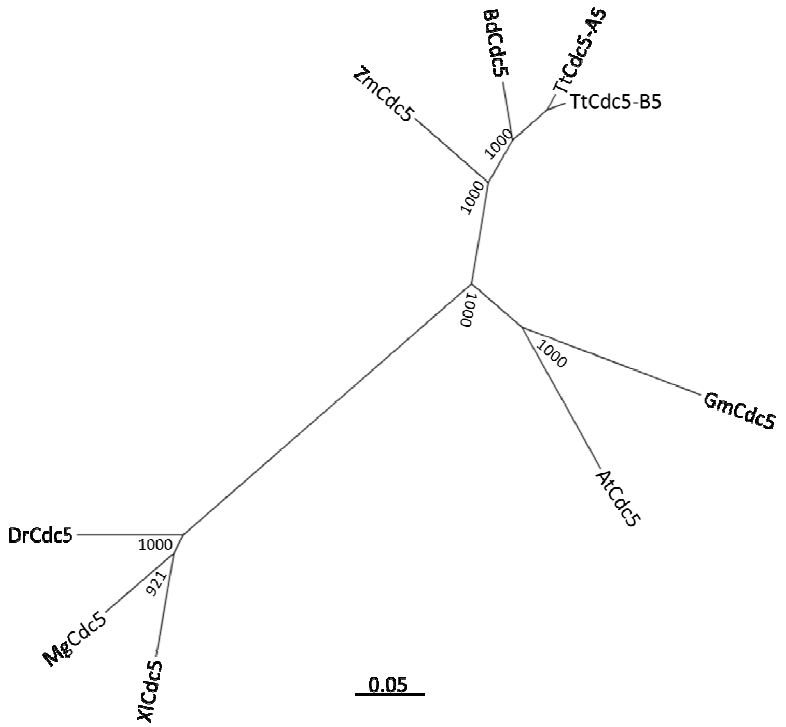

Figure 4.5. Phylogenetic tree of the putative Cdc5 orthologues in tetraploid wheat and other eukaryotic species. Phylogenetic tree was built from the amino acid sequences of the putative Cdc5 orthologues of tetraploid wheat, Cdc5 orthologues from *B. distachyon*, *Z. mays*, *G. max*, *D. rerio*, *M. gallopavo*, and *X. laevi* with Bootstrap Neighbor-Joining method.

Chromosomal localization

Two homoeoalleles of the putative *TtCdc5* were identified in LDN and assigned to chromosome 5A and 5B, respectively, using CS nulli-tetrasomic and LDN DS lines (Figure 4.6). They were designated as *TtCdc5-A5* and *TtCdc5-B5*, respectively. In addition, chromosome 5D of CS was found to contain another homoeoallele of the putative *Cdc5* (Figure 4.6). The *B. distachyon Cdc5* gene was assigned to chromosome 4

that is collinear with wheat chromosomes in the homoeologous group 5 (Kumar *et al*., 2012).

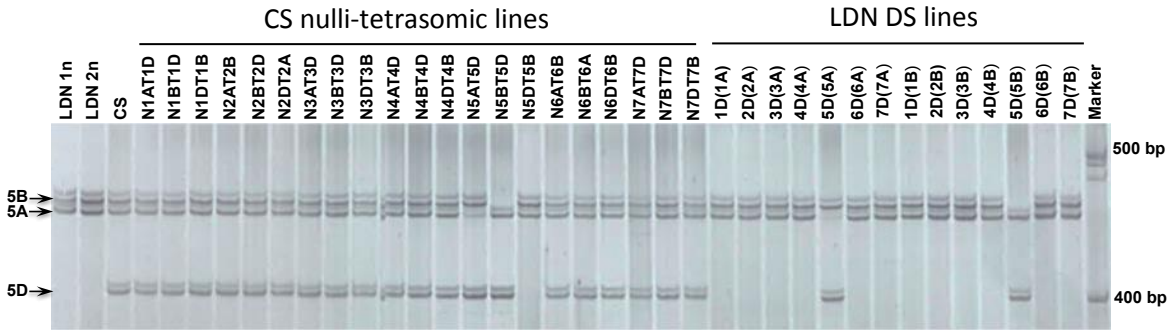

Figure 4.6. Chromosomal localization of the putative *TtCdc5* using PCR with gene specific primers. The three arrows at left point to the DNA fragments amplified from the homoeoalleles of the putative *Cdc5* homologue in hexaploid and tetraploid wheat on chromosome 5B, 5A and 5D, respectively. Two fragments were amplified in LDN 2n and LDN 1n (haploid), indicating two homoeoalleles of the putative *TtCdc5* in tetraploid wheat LDN, *i.e. TtCdc5-A5* and *TtCdc5-B5*, respectively.

Discussions

This research took the advantage of the genomic resources about the *Cdc5*-like genes in models to perform initial studies toward cloning the *Cdc5* homologues in tetraploid wheat. This comparative genomic analysis-based gene cloning strategy has proven useful to clone and characterize the genes that condition a functional or structural protein without a distinct and easily scored phenotype, such as some of the regulatory genes/proteins invoved in cell cycles (Sutton *et al.*, 2003; Kimbara *et al*., 2004; Dong *et al*., 2005; Boden *et al*., 2007; Khoo *et al*., 2008; Pérez *et al*., 2011; Khoo *et al*., 2012). The rapidly expanding genomic information and resource in model species, particularly plant models, provided a variety of genomic and genetic tools for wheat genome studies.

In the effort of *Cdc5*-like gene cloning in wheat, the *Cdc5* gene information was first retrieved from yeast, which has proven to promote chiasma formation and sister chromatids cosegregation (Clyne *et al.*, 2003). Since proper chromosome segregation and chiasmata formation are very conserved meiotic events at meiosis I, it was obviously reasonable to believe that wheat should contain a *Cdc5*-like gene with similar functions as that in yeast. Direct *blast* search of the *Cdc5*-like gene in the wheat TC database did not identify any ESTs with low E-values and annotated as *Cdc5*-like gene using the yeast *Cdc5* gene as query. However, the yeast *Cdc5* gene identified homologues in rice and *Arabidopsis*, two models in plants. The *Cdc5* homologues in rice and *Arabidopsis* were then used to blast the wheat EST pool and one wheat EST was identified as a candidate of the *Cdc5* homologue in wheat. Evidently, this comparative genomic approach worked well for cloning the genes like *Cdc5* that do not have a distinct and easily-scored phenotype from a large and complex genome.

The comparative analysis of the amino acid sequences of the putative TtCdc5 and Cdc5 proteins in other plant and animal species revealed high similarities, *i.e.* 48-94%. Especially, the predicted protein of the putative TtCdc5 showed extremely high homology (90-94%) with the Cdc5 protein in *B. distachyon* and maize. In addition, the expression profiling by real-time PCR indicated the putative *TtCdc5* predominantly expressed in meiotic tissues (anthers), rather than somatic tissues (roots and leaves). The transcription level of the putative *TtCdc5* peaked at pachytene stage, and then declined when meiosis proceeded. The kinetics of this putative *TtCdc5* gene in meiosis were in accordance with those of the *Cdc5* gene in yeast (Clyne *et al*., 2003).

In this study, two homoeoalleles of the putative *TtCdc5* gene were localized on chromosome 5A and 5B in LDN, respectively. The *B. distachyon Cdc5* gene is assigned to chromosome 4 that is collinear with wheat chromosomes in the homoeologous group 5 (Kumar *et al*., 2012). Apparently, the *Cdc5*-like gene is highly conserved in wheat and *B. distachyon*, a monocot model closely related to wheat.

As a Myb-related kinase, the function of Cdc5 in cell cycle has well characterized in yeasts and animals. However, its role in the complex network of mitosis and meiosis remains obscure in plants. Lin *et al*. (2007a) utilized VIGS technique to knockout *CDC5* in *Arabidopsis* and they found the *AtCDC5* VIGS plants, in which *AtCDC5* was specifically silenced, displayed accelerated cell death. In another study, Lin *et al*. (2007b) knocked down *AtCDC5* with RNAi technique and they found G_2/M transition was affected in the *AtCDC5*-RNAi plants, and that endoreduplication was increased. RNAi or VIGS induced *cdc5* individuals in plants often show partial suppression of phenotypes, which is different from that of yeasts, because of the complexity of plant genomes. The case would be even more complicated in polyploid wheat. Actually we have tried to knock out or knock down the candidate *TtCdc5* genes in wheat using VIGS technique. Seemingly, the viral infection negatively influenced the spike development and entire reproductive growth. Thus, informative results have not been obtained from those experiments.

The results of sequence conservation, expression profiling, conserved domains, collinearity with models, and phylogenetic relationships all concertedly suggested the identity of the candidate gene as *TtCdc5* homologue in tetraploid wheat. However, significant further studies, such as complementation test (transformation) and yeast two-

hybrid assay, are needed to completely reveal the function and precisely determine the identity of this gene.

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CHAPTER V. KINETOCHORE ORIENTATION OF PAIRED AND UNPAIRED MEIOTIC CHROMOSOMES IN TETRAPLOID WHEAT

Abstract

Sister kinetochores orient syntelically and are attached by the microtubules emanating from the same pole (*i.e.* monopolar attachment) at meiosis I. However, the two pairs of sister kinetochores in the paired homologous chromosomes (bivalents) orient amphitelically and are attached by the microtubules emanating from opposite poles, enabling paired homologous chromosomes to segregate at meiosis I. It has been reported that sister kinetochores orient differently in the tetraploid wheat Langdon (LDN) and its haploid at meiosis I. Homologous chromosomes in tetraploid LDN generally paired as bivalents, while chromosomes in LDN haploid appeared as univalents (unpaired chromosomes) at meiosis I. The objective of this study was to determine whether chromosome pairing plays a role in kinetochore orientation. Crosses involving LDN, LDN D-genome substitution lines (LDN DS), *Aegilops tauschii*, and rye were made to construct special cytogenetic stocks that undergo meiosis with both paired (bivalents) and unpaired (univalent) chromosomes. A total of 12 hybrids that formed both bivalents and univalent involving different chromosomes were obtained from the crosses of LDN 1D(1B) × LDN, LDN 2D(2A) × LDN, LDN 2D(2B) × LDN, LDN 3D(3A) × LDN, LDN 4D(4B) × LDN, LDN 6D(6A) × LDN, LDN 6D(6B) × LDN, LDN 7D(7A) × LDN, LDN 7D(7B) × LDN, LDN 1D(1A) × rye 'Gazelle', LDN × *Ae. tauschii* RL5286 and LDN 1D(1B) × *Ae. tauschii* RL5286. Sister kinetochores of almost all paired homologous chromosomes in a bivalent oriented syntelically (monopolar attachment), while sister kinetochores of unpaired chromosomes (*i.e.* univalents) mostly orientated amphitelically

(bipolar attachment) at meiosis I. Apparently, chromosome pairing or synapsis conditions kinetochore orientation and chromosome segregation at meiosis I in the tetraploid wheat LDN.

Introduction

Meiosis is characterized by two successive rounds of nuclear divisions with only one round of DNA replication, leading to the formation of gametes with half chromosomes of somatic cells. The first meiotic division (meiosis I) involves homologous chromosome recognition, pairing, recombination, and finally segregation, which reduces chromosome number in half (reductional division). The second meiotic division (meiosis II) involves segregation of sister chromatids and leads to four haploid daughter cells (equational division), which resembles mitosis (Kleckner, 1996).

Deviation from normal meiosis leads to chromosome variation in the gametes, and subsequently the offspring involving the gametes, such as aneuploids and polyploids. One of the variant meiotic cell divisions, termed unreductional meiotic cell division (UMCD) or meiotic restitution, has been observed in a number of plant species, including wheat and potato (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003; Fawcett and Van de Peer, 2010). Unreductional meiotic cell division results in unreduced gametes (2n), due to the failure of chromosome segregation at either meiosis I or meiosis II. Fertilization of unreduced gametes increases the ploidy level and leads to polyploidization. It is reportedly a widespread evolutionary event in angiosperm (Harlan and deWet, 1975; Fukuda and Sakamoto, 1992a,b; Wendel, 2000; Xu and Joppa, 2000b; Ramanna and Jacobsen, 2003). Furthermore, UMCD has been used in plant improvement and synthesis of new species, particularly in tribe *Triticeae* (Islam and

Shepherd, 1980; Balatero and Darvey, 1993; Bretagnolle and Thompson, 1995; Ramanna and Jacobsen, 2003; Matsuoka and Nasuda, 2004; Jauhar, 2007; Shamina, 2012). The tetraploid wheat LDN undergoes normal meiosis. However, the LDN haploid and hybrids of LDN with *Ae. tauschii* and rye (*Secale cereale* L., 2n=14, genome RR) were found to undergo UMCD or called meiotic restitution (Xu and Dong, 1992; Xu and Joppa, 2000a,b; Cai *et al*., 2010). This unique haploidy-dependent variant meiotic cell division has been considered a primary mechanism, if not only one, of chromosome doubling in the origin of common wheat (*T. aestivum* L., 2n=6x=42, genome AABBDD) from the interspecific hybridization between tetraploid wheat (*T. turgidum*) and *Ae. tauschii* (2n=2x=14, genome DD) (Fukuda and Sakamoto, 1992a,b; Xu and Joppa, 2000b; Lyrene *et al*., 2003; Jauhar, 2007). Cai *et al*. (2010) reported that sister kinetochores of the paired chromosomes (bivalents) in LDN oriented syntelically (monopolar microtubule-kinetochore attachment), while sister kinetochores of the unpaired chromosomes (univalents) in the LDN haploid and hybrids of LDN with *Ae. tauschii* mostly oriented amphitelically (bipolar microtubule-kinetochore attachment). The amphitelically-oriented chromosomes (univalents) may undergo an equational division (separation of sister chromatids) at meiosis I if cohesion does not persist between sister chromatids; or they may fail to divide if cohesion is maintained between sisters. Also, they may randomly segregate to either pole because the pulling force from one pole is overwhelmed by the other (Watanabe and Nurse, 1999; Yokobayashi *et al*., 2003; Parra *et al*., 2004; Chelysheva *et al*., 2005). Direct visualization of the chromosome and spindle behavior during meiosis suggested that the amphitelic orientation of sister kinetochores and persistence of cohesion between sister chromatids at meiosis I contributed to the onset of

UMCD in the LDN haploid and hybrids of LDN with *Ae. tauschii* (Cai *et al*., 2010). Why did sister kinetochores of the chromosomes orient differently under disomic and haploid conditions? This study attempted to determine whether chromosome pairing play a role in kinetochore orientation in LDN.

Materials and methods

Plant materials

The plant materials involved in this study include durum wheat 'Langdon' (LDN) (*T. turgidum* ssp. *durum* L., 2n=4x=28, genome AABB), LDN D-genome disomic substitution lines (LDN DS), cultivated rye 'Gazelle' (*S. cereal* L., 2n=2x=14, genome RR), *Ae. tauschii* RL5286 and sweat corn cultivar 'Early Sunglow'. They were grown in a temperature-controlled greenhouse room for crossing and sampling of meiotic anthers. The LDN DS has one pair of A- or B-genome homologous chromosomes substituted by a pair of D-genome homologous chromosomes withtin the same homoeologous group from hexaploid wheat 'Chinese Spring' (CS). They were used as female parents in the crosses with LDN, 'Gazelle' rye, and RL5286.

Production of hybrids between LDN DS and LDN

The LDN DS spikes were emasculated 2-3 days prior to anthesis, and pollinated with freshly shed pollen from LDN when the hairy stigma opened. Seeds of hybrids were collected upon maturation.

Production of interspecific hybrids and haploid

About 2-3 days before anthesis, LDN, LDN 1D(1A), and LDN 1D(1B) spikes were emasculated, and pollinated with freshly shed pollen from rye 'Gazelle', *Ae.*

tauschii RL5286 and maize 'Early Sunglow' when the hairy stigma opened. The maize-pollinated wheat spikes were sprayed with 2,4-D solution (213.05 mg/L 2,4-dichlorophenoxyacetic acid, 80 µl/L Tween 80, and 50 mg/L GA_3; pH 10.36) 24 h after pollination. 2,4-D was not applied to the *Ae. tauschii*- and rye-pollinated wheat spikes. Fourteen days after pollination, the caryopses were harvested and surface-sterilized with 20% bleach solution (200 ml bleach+800 ml dH_2O) for 5 min and 70% ethanol for 1 min. The caryopses were rinsed twice for 1 min with sterile double distilled water after each step of sterilization. Immature embryos were aseptically dissected from the caryopses and cultured on MS medium (Murashige and Skoog, 1962) at room temperature (18°C) in dark. Upon germination, the seedlings were moved to 16 h photoperiod growth room (18°C) for 4-8 weeks. Seedlings with two leaves were transplanted into pots in the greenhouse for further studies as described by Cai *et al.* (2010).

Sampling of meiotic anthers

One of the three anthers from each of the florets along the spike of the hybrid plants was dissected and stained with 1% acetocarmine for observation of meiotic stages. The meiotic stages of meiocytes in anthers were determined based on chromosome morphology, behavior, and other cellular features in the meiocytes as described by Xu and Joppa (1995). Upon determination, the two remaining anthers within the corresponding floret were kept in 8% (m/v) paraformaldehyde solution and fixed for 2 h at room temperature for immunofluorescent analysis of meiotic microtubules and chromosomes.

Immunofluorescent analysis of meiotic microtubules and chromosomes

Indirect immunofluorescence was performed as described previously (Chan and Cande, 1998) with minor modifications. After collection and fixation, the meiocytes extruded from anthers were embedded in an agarose block and treated with 1.5% β-glucuronidase at room temperature for 10 min to partially digest the cell walls. Then the agarose block was first incubated in the 1× PBS-diluted (1:60 dilution) mouse monoclonal antibody against α-tubulin (Invitrogen Corporation, Grand Island, NY, USA) and then in 1× PBS-diluted (1:40) FITC-conjugated goat-anti-mouse antibody (Sigma-Aldrich Co., St Louis, MO, USA) each for at least 12 h at room temperature. Prior to chromosome staining with propidium iodide (PI), the block was treated with DNase-free RNase for 45-60 min at 37°C. Finally, the agarose block was melted on a glass slide, and 15 μl antifade solution Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) was applied to the slide. A coverslip was put on the slide, and nail polish was used to seal the space between coverslip and slide.

Immunolocalization of TtRec8

Immunolocalization was conducted as described by Golubovskaya *et al*. (2006) with minor modifications to monitor the changes of endogenous Rec8 protein in anthers of LDN haploids and the hybrids between LDN and *Ae. tauschii* during meiosis. The primary anti-Rec8 antibody was probed by the secondary Anti-Rabbit IgG (whole molecule)-FITC Antibody produced in goat (Sigma-Aldrich Co., St Louis, MO, USA), and chromosomes were counterstained by propidium iodide (PI). Two negative control experiments were performed to monitor the specificity of the antibodies in meiocytes. In the first negative control, the thin layer of polyacrylamide gel containing meiocytes was

directly incubated with secondary antibody, while in the second one, the thin layer of polyacrylamide gel containing meiocytes was incubated with primary anti-Rec8 antibody that was preabsorbed overnight with fusion polypeptide pGEX-R26 at a molar ratio of blocking peptide to antibody of 50 to 1.

Microscopy

An Olympus BX-51 Phase/Fluorescent Microscope (Olympus Optical Co. Ltd., Tokyo, Japan) was used to sample male meiocytes at different meiotic stages. Confocal microscopy was conducted using a Zeiss Axioplan 2 Imaging Research Microscope equipped with ApoTome confocal component (Carl Zeiss Light Microscopy, Jena, Germany). Two dimensional and three dimensional images were captured and analyzed with the use of Zeiss AxioVision 4 software as described by Cai *et al.* (2010).

Results

Kinetochore orientation of the meiotic chromosomes in the hybrids of LDN DS with LDN

The F_1 hybrids involving LDN DS lines were produced from the crosses of LDN 1D(1B) × LDN, LDN 2D(2A) × LDN, LDN 2D(2B) × LDN, LDN 3D(3A) × LDN, LDN 4D(4B) × LDN, LDN 6D(6A) × LDN, LDN 6D(6B) × LDN, LDN 7D(7A) × LDN, and LDN 7D(7B) × LDN. Primarily, two univalents (unpaired chromosomes), including a D-genome chromosome and an A- or B-genome chromosome substituted by the D-genome chromosome, were observed at metaphase I in each of these hybrids. The rest of the chromosomes mostly paired as bivalent in the hybrids (Figure 5.1).

Sister kinetochores of most univalents were found oriented amphitelically (bipolar attachment) and sister kinetochores of all bivalents oriented syntelically (monpolar attachment) at metaphase I. Clearly, each of the two univalents in the hybrids of LDN 1D(1B) × LDN, LDN 2D(2A) × LDN, LDN 2D(2B) × LDN, LDN 4D(4B) × LDN, LDN 6D(6A) × LDN, LDN 6D(6B) × LDN, LDN 7D(7A) × LDN and LDN 7D(7B) × LDN were attached by the microtubules emanating from opposite poles at metaphase I (Figure 5.1, a1, b1, c1, e1, f1, h1, j1 and l1). The two paired chromosomes in each of the bivalents were connected to each other by chiasmata and attached by the microtubules emanating from opposite poles at metaphase I. At anaphase I, amphitelically-oriented univalent chromosomes in the hybrids of LDN 3D(3A) × LDN and LDN 6D(6A) × LDN failed to segregate and stayed at the equatorial plane (Figure 5.1, d1 and g1), while bivalent chromosomes normally segregated and migrated to opposite poles (Figure 5.1). Occasionally, equational division of the sister chromatids in the amphitelically-oriented univalent chromosomes was observed at anaphase I in the hybrids of LDN 6D(6B) × LDN and LDN 7D(7B) × LDN (Figure 5.1, i1 and k1).

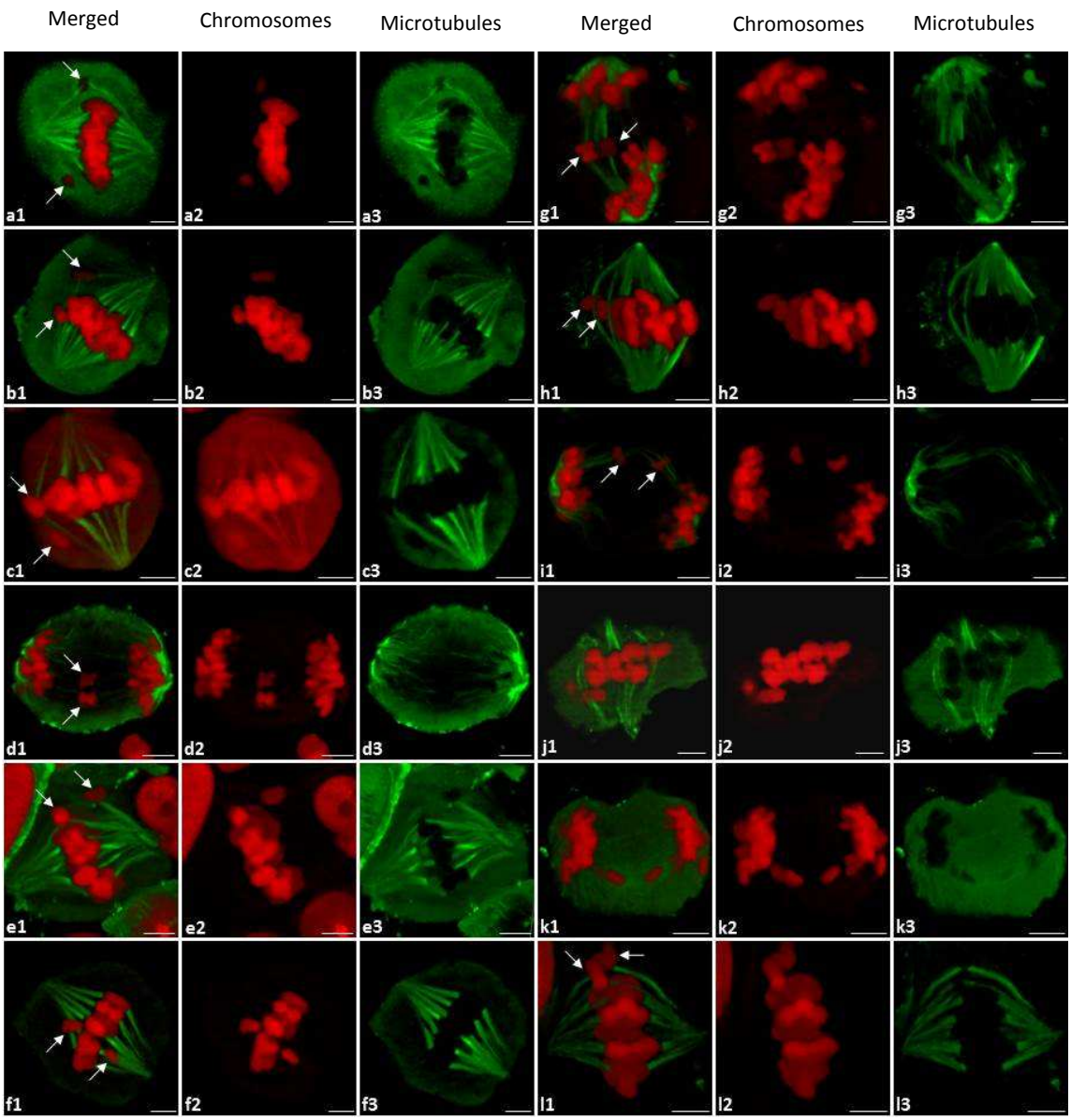

Figure 5.1. Kinetochore orientation and segregation of paired and unpaired chromosomes in the hybrids between LDN DS and LDN at meiosis. Chromosomes are shown in red and microtubules are shown in green. a1-a3: LDN 1D(1B) × LDN, b1-b3: LDN 2D(2A) × LDN, c1-c3: LDN 2D(2B) × LDN, d1-d3: LDN 3D(3A) × LDN, e1-e3: LDN 4D(4B) × LDN, f1-g3: LDN 6D(6A) × LDN, h1-i3: LDN 6D(6B) × LDN, j1-j3: LDN 7D(7A) × LDN, and k1-l3: LDN 7D(7B) × LDN. Arrows point to unpaired chromosomes. Scale bars: 5 μm.

Kinetochore orientation in the haploids and interspecific hybrids

The LDN haploids were generated by pollinating LDN with fresh maize pollen. The interspecific crosses were made from the LDN DS lines LDN 1D(1A) and LDN 1D(1B) with 'Gazelle' rye and *Ae. tauschii* RL5286, respectively. Interspecific hybrids of LDN 1D(1A) with 'Gazelle' rye and *Ae. tauschii* RL5286, and LDN 1D(1B) with *Ae. tauschii* RL5286 were obtained, but not from the cross of LDN 1D(1B) with 'Gazelle' rye. All the chromosomes appeared as univalents in LDN haploid, and most of the chromosomes appeared as univalent in the hybrids. One bivalent was observed in the hybrids of LDN 1D(1A) and LDN 1D(1B) with *Ae. tauschii* RL5286, which were believed to be formed between two 1D chromosomes in the hybrids (Figure 5.2). The univalents mostly oriented amphitelically and bivalents oriented syntelically at metaphase I in these hybrids (Figure 5.2).

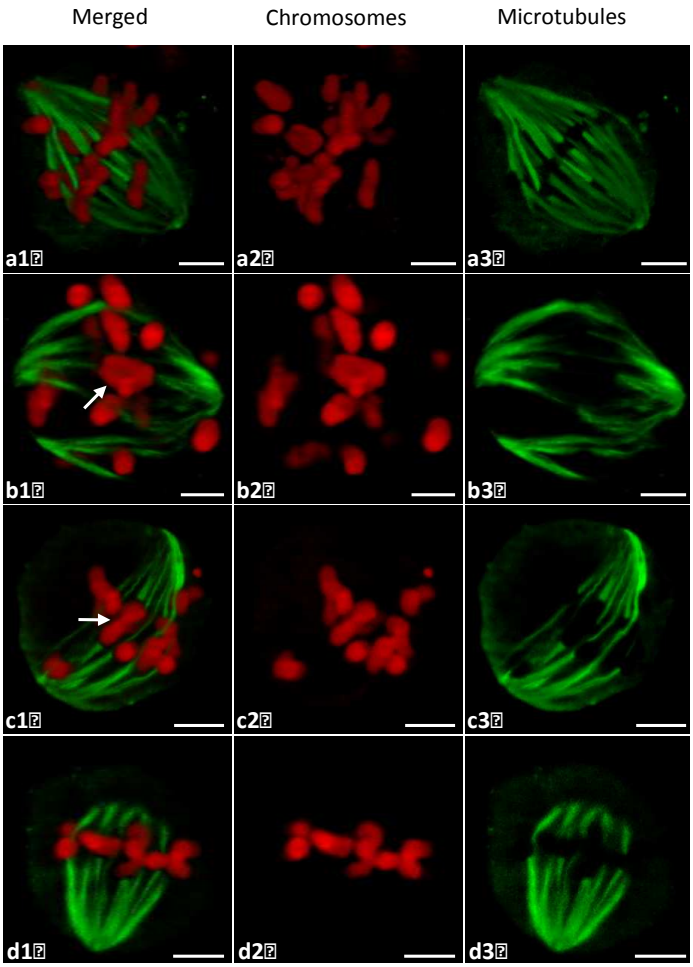

Figure 5.2. Kinetochore orientation of paired and unpaired chromosomes in the interspecific hybrids and LDN haploids at meiosis I. Chromosomes are shown in red and microtubules are shown in green. a1-a3: LDN 1D(1A) × 'Gazelle' rye, b1-b3: LDN 1D(1A) × *Ae. tauschii* RL5286, c1-c3: LDN 1D(1B) × *Ae. tauschii* RL5286, and d1-d3: LDN haploid. Arrows point to bivalents. Scale bars: 5 μm.

Immunolocalization of TtRec8 in LDN haploid and LDN hybrid with *Ae. tauschii*

TtRec8 cohesion protein was found to associate with unpaired chromosomes at early prophase of meiosis I in LDN haploid and hybrid with *Ae. tauschii* (Figure 5.3, a1, b1, d1 and e1). After that, TtRec8 was not detectable as what we observed in LDN

(Figure 5.3, c1, f1 and g1; TtRec8 labeling data on meiotic chromosomes after metaphase I stage not shown). Obviously, *TtRec8* expressed in a similar manner under both haploid and disomic conditions.

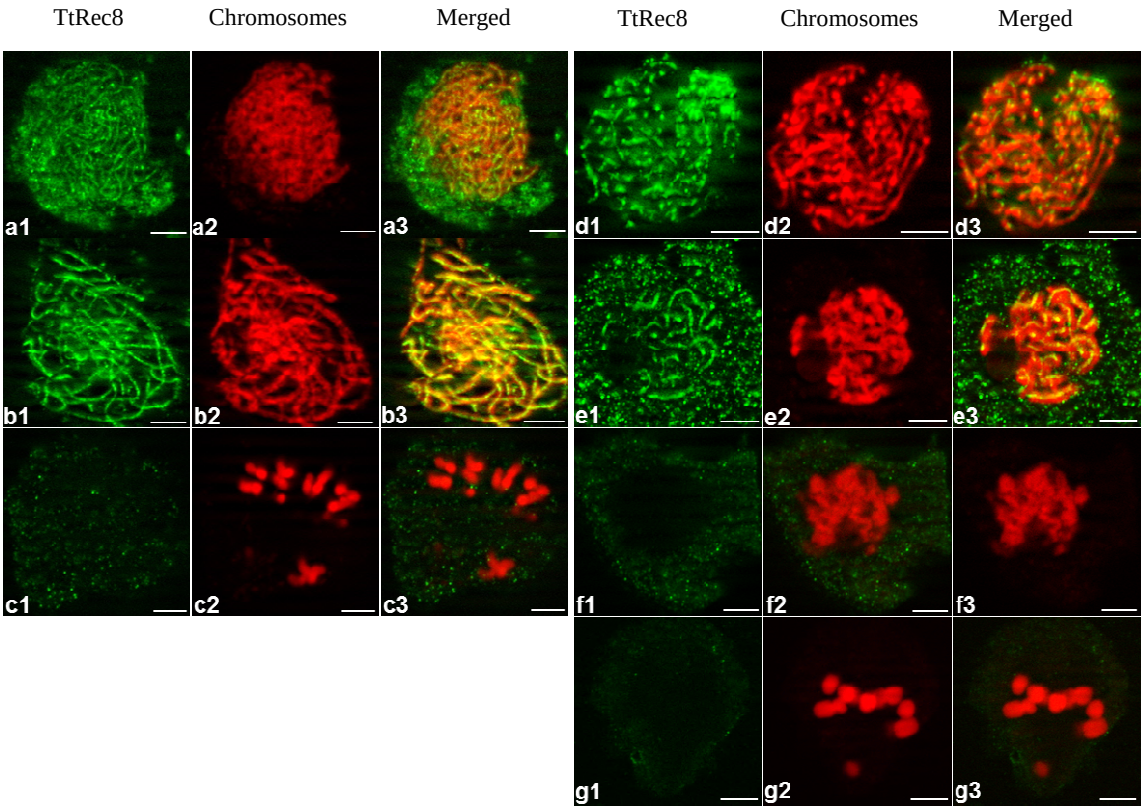

Figure 5.3. Fluorescent immunolocalization of TtRec8 protein on meiotic chromosomes in LDN haploid and hybrid with *Ae. tauschii*.TtRec8 protein is shown in green and chromosomes are shown in red. a1-c3 shows TtRec8 immunolocalization in LDN haploid, and d1-g3 shows TtRec8 immunolocalization the hybrid between LDN and *Ae. tauschii*. a1-a3: zygotene; b1-b3: pachytene; c1-c3: metaphase I; d1-d3: leptotene; e1-e3: zygotene/early pachytene; f1-f3:diakinesis; and g1-g3: metaphase I. Scale bar: 5 µm.

Discussions

Kinetochore orientation and chromosome cohesion regulate chromosome segregation during meiosis. Normally, sister kinetochores of meiotic chromosomes

oriente syntelically and the cohesion along the chromosome arms are removed prior to anaphase I, facilitating reductional division of homologous chromosomes at meiosis I. However, cohesion protein around centromeric regions persist to hold sister chromatids together untill anaphase II. The syntelic orientation of paired homologous chromosomes creates a pulling force towards opposite poles by microtubules, which counteracts with the persistence of cohesion as well as chiasmata between homologues. As the cohesion between homologous chromosomes is removed and chiasmata are resolved, microtubules emanating from opposite poles pull paired homologous chromosomes (bivalents) toward opposite poles. The paired homologous chromosomes (bivalents) in LDN and its hybrids with LDN DS and *Ae. tauschii* underwent normal meiosis as described above. However, sister kinetochores of unpaired chromosomes (univalents) oriented amphitelically (*i.e.* bipolar attachment) and did not undergo normal reductional division as the paired homologous chromosomes (bivalents) at meiosis I in the same meiocytes of the LDN × LDN DS and LDN DS × *Ae. tauschii* hybrids (Figures 5.1 and 5.2). It seemed that physical interaction, or called synapsis/pairing, between homologous chromosomes was essential to ensure syntelic orientation of sister kinetochores (*i.e.* monopolar attachment) and subsequently regular reductional division of chromosomes at meiosis I in LDN and its hybrids with *Ae. tauschii* and rye. Without synapsis or pairing, sister kinetochores of univalent chromosomes oriented amphiteclically, but cohesin appeared to retain around the centromeric regions in most of the meiocytes observed. Evidently, synapsis or pairing was a crucial meiotic event to steer kinetochore orientation in LDN and its hybrids with *Ae. tauschii* and rye. In fact, chromosomes themselves have been found to play significant roles in some meiotic events, such as chromosome 1A, 2A, 4A, 5A, 5B and

6B in tetraploid LDN, in addition to meiotic genes/proteins (McKim and Hawley, 1995; Paliulis and Nicklas, 2000, Xu and Joppa, 2000b).

Rec8 has been found involved in the genetic network responsible for kinetochore orientation in addion to functioning as a meiotic cohesin (Watanabe and Nurse, 1999; Yokobayashi *et al.*, 2003; Chelysheva *et al.*, 2005; Zhang *et al.*, 2006; Shao *et al.*, 2011; Yuan *et al.*, 2012). Here in this study, sister kinetochores were found to orient differently with paired and unpaired chromosomes in the same meiocytes that contained functional TtRec8 (Figure 5.3). In other words, meiotic synapsis/pairing mediates kinetochore orientation in LDN wheat. However, it is unknown whether TtRec8 protein is involved in the regulation of kinetochore orientation in LDN. Further studies are underway for a better understanding of TtRec8 in this particular genotype (*i.e.* LDN wheat) that undergoes haploidy-dependent UMCD (Cai *et al.*, 2010).

TtRec8 is located on chromosome 1A and 1B, respectively, in tetraploid wheat (present study). Wheat chromosome 1D also contains a *Rec8* homoeoallele, designated *TtRec8-D1*. The interspecific hybrids of LDN DS 1D(1A) and 1D(1B) with *Ae. tauschii* harbor the homoeoallele *TtRec8-A1* and *TtRec8-B1*, respectively, in addition to *TtRec8-D1*. However, meiotic kinetochores and chromosomes behaved in the similar manner in both hybrids, suggesting these three homoeoalleles might individually play a similar role in kinetochore orientation and chromosome segregation if they did mediate these meiotic processes.

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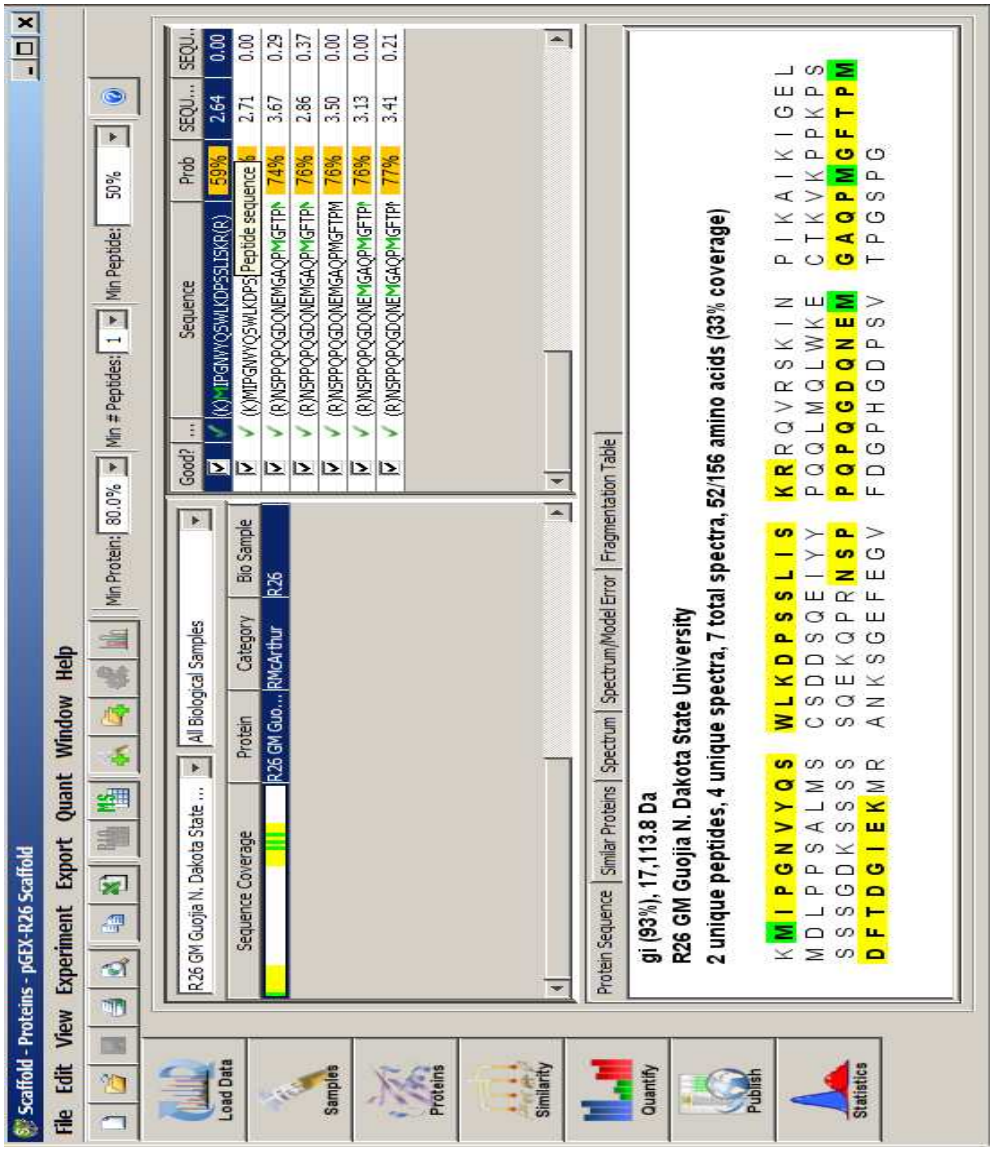
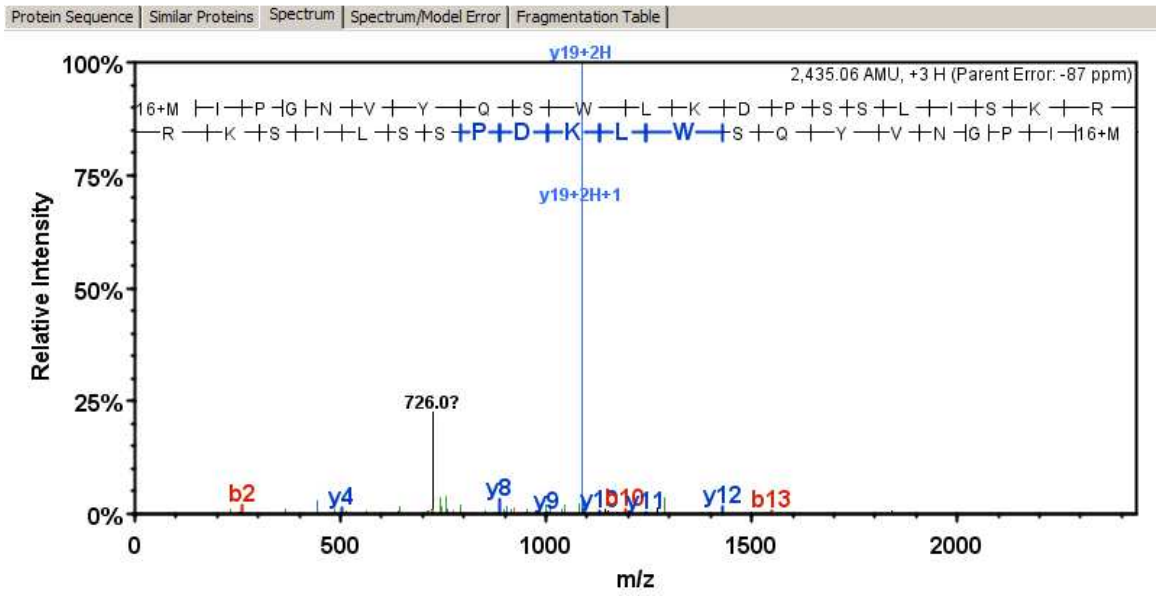
APPENDIX A. PROTEIN ID VERIFICATION OF pGEX-R26 POLYPEPTIDE

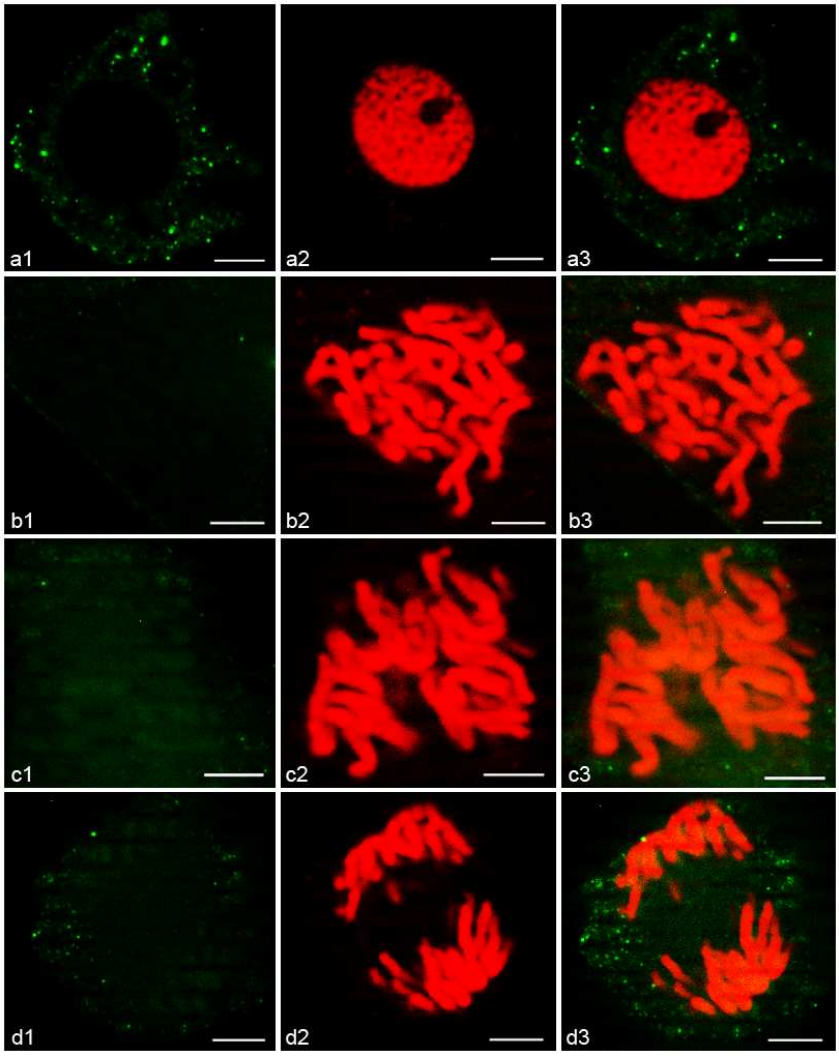


Verification of polypeptide pGEX-R26 by Protein ID assay. LC-MS/MS data are collected from the peptide mixture generated by proteolytic digestion of pGEX-R26 polypeptide sample, and the MS/MS spectra are searched against the database of the deduced amino acid component of pGEX-R26. The result shows 33% coverage of the database, as highlighted in yellow.

APPENDIX B. LC-MS/MS SPECTRA OF POLYPEPTIDE pGEX-R26 FRAGMENTATION

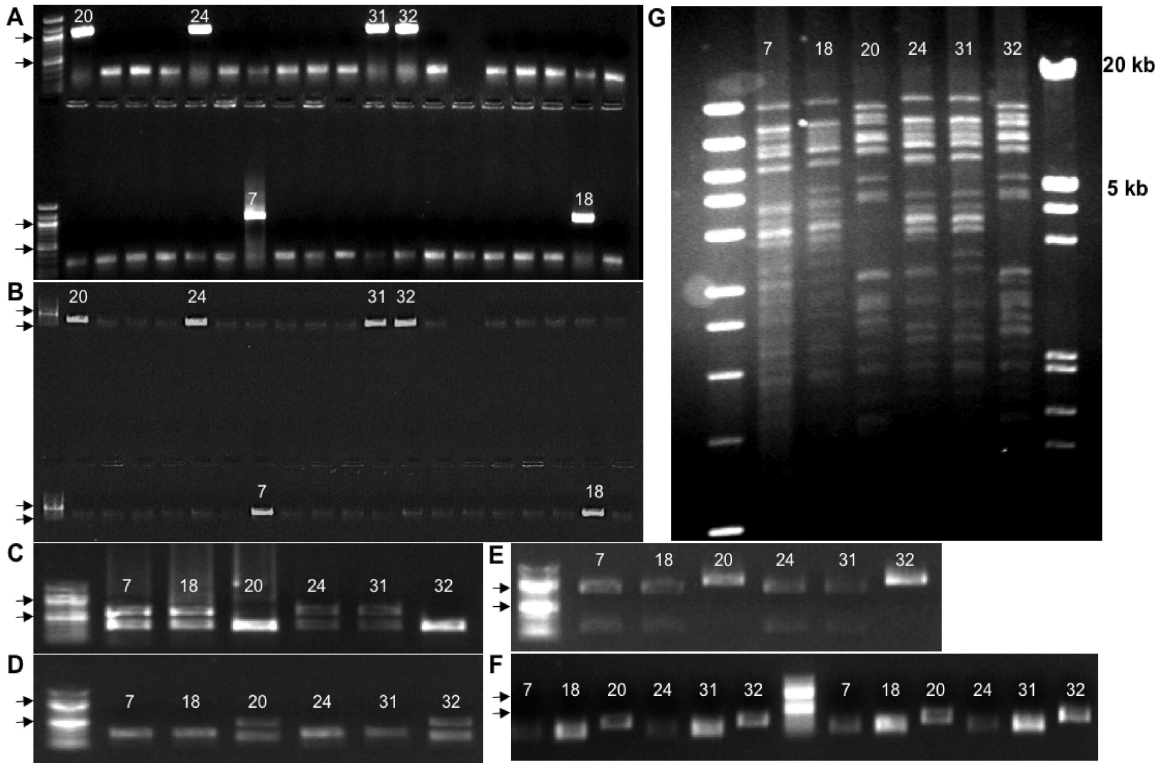


APPENDIX C. DETECTION OF TtREC8 PROTEIN IN SOMATIC CELLS



Detection of TtRec8 protein (a1-d1, green) in somatic cells (a2-d2, red) within meiotic anthers of LDN. a3-d3 are merged pictures. The mitotic cells are within the same slides as fluorescent immunolocalization of TtRec8 on meiotic chromosomes of LDN. a1-a3: prophase; b1-b3: prometaphase; c1-c3: metaphase; and d1-d3: anaphase. Scale bar: 5 µm. No TtRec8 protein was visually detected along the chromosomes during mitosis in LDN.

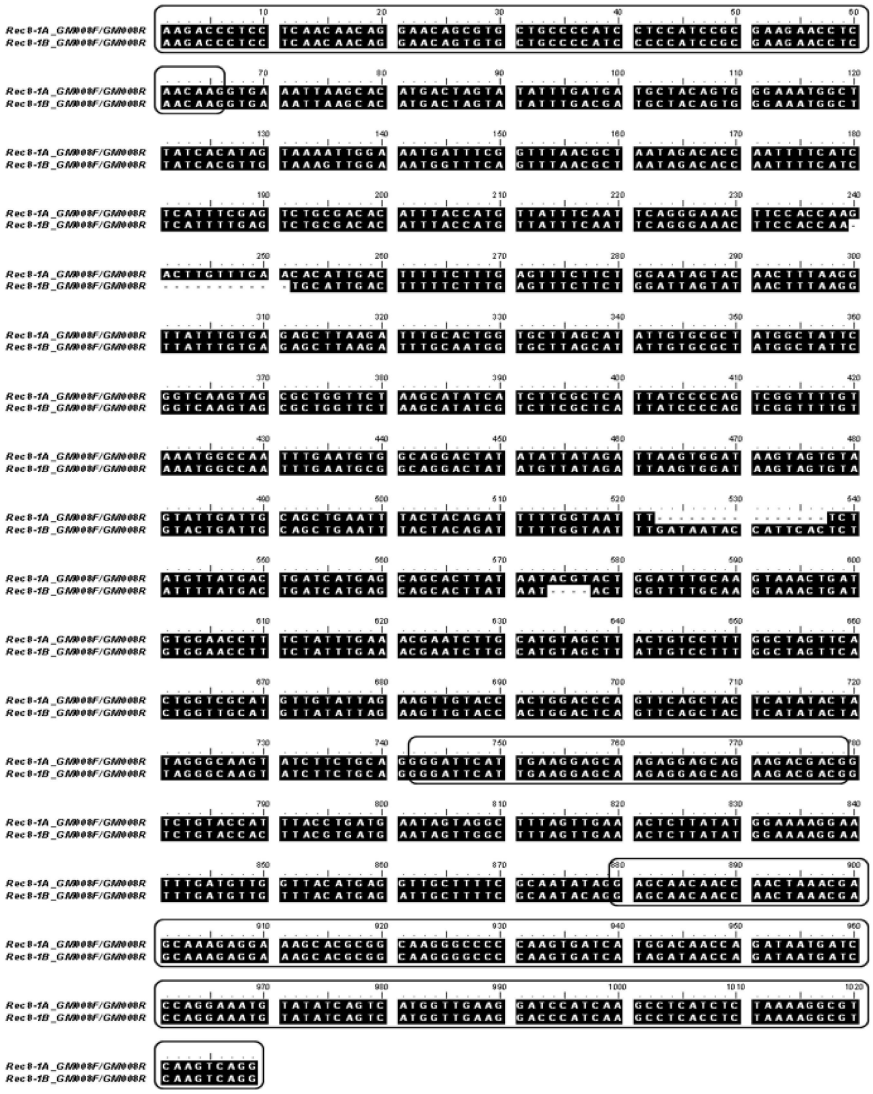
APPENDIX D. IDENTIFICATION OF BAC CLONES THAT RESPECTIVELY CONTAINS *TtREC8-A1* AND *TtREC8-B1* WITH PCR, CAPS AND FINGERPRINTING



Identification of BAC clones that contains *TtRec8-A1* and *TtRec8-B1*, respectively, by PCR, CAPS and fingerprinting methods. Two arrows on the left side of A, B, C, D, E and F indicate the 500 bp and 1 kb size markers, respectively. 5 kb and 20 kb size markers are shown on the right side of G, separately. A-B) PCR amplifications of positive BAC clones screened by bulked probes containing four *TtRec8* cDNA segments by *TtRec8*-specific primer pairs GM008F/GM008R and GM065F/GM065R, respectively. Results from both A and B consistently show that BAC clone No.7, 18, 20, 24, 31 and 32 harbor partial or complete genomic DNA sequence of *TtRec8*, though they cannot be differentiated into two groups based on size. C-E) BAC clones No.7, 18, 20, 24, 31 and

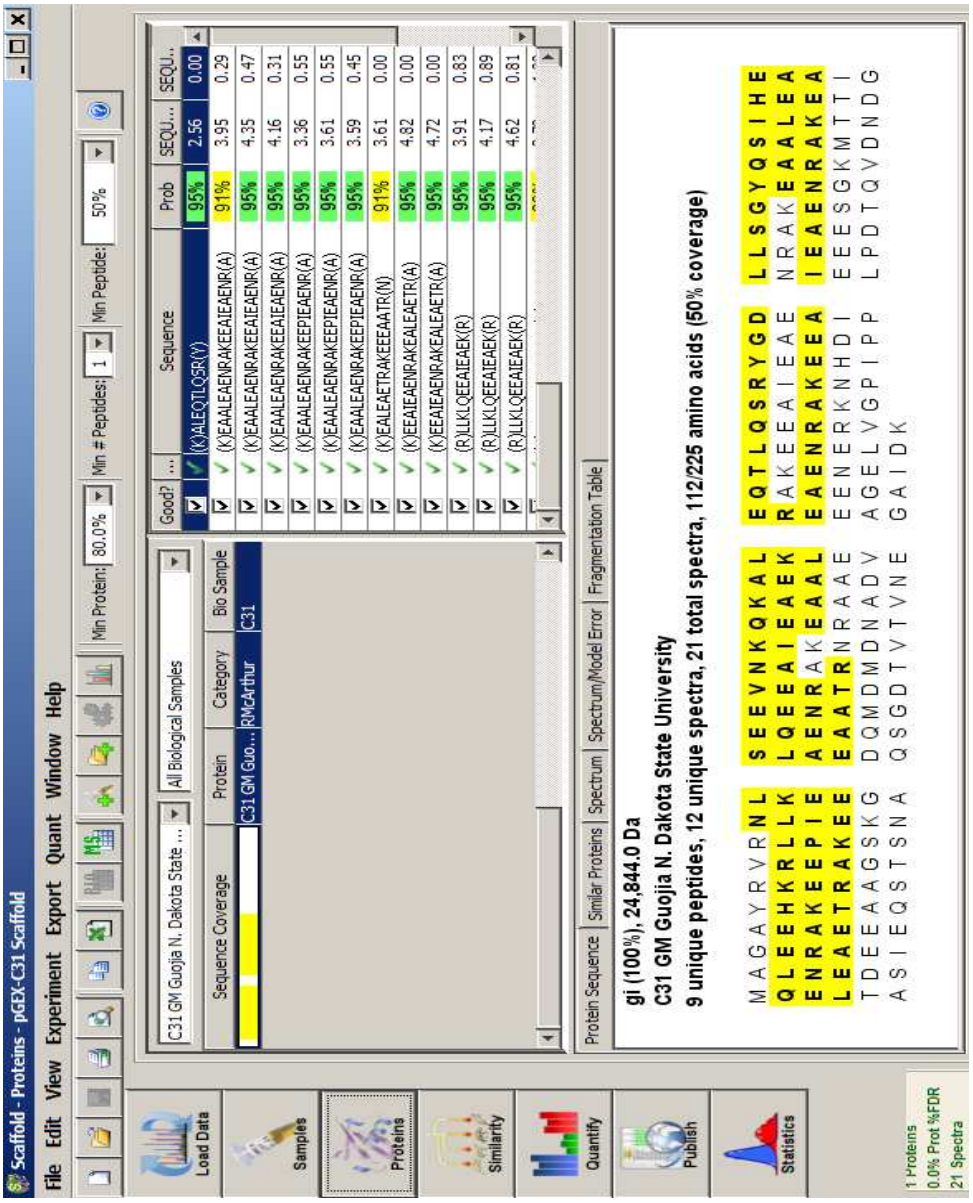
32 are PCR amplified by *TtRec8*-specific primer pair GM008F/GM008R, followed by *Rsa*I, *Taq*I and *Dde*I digestions, separately. F) BAC clones No.7, 18, 20, 24, 31 and 32 are PCR amplified by *TtRec8*-specific primer pair GM065F/GM065R, followed by *Alu*I and *Dde*I digestions, respectively. Using CAPS methodology, *TtRec8*-containing BAC clones could be differentiated into two groups based on different digestion patterns, *i.e.* BAC clone No. 20 and 32 share same pattern, and BAC clone No. 7, 18, 24 and 31 have the other, suggesting the two groups represent BAC clones harboring either *TtRec8-A1* or *TtRec8-B1* homoeoalleles, respectively. G) DNA were extracted from BAC clone No. 7, 18, 20, 24, 31 and 32, followed by *Hin*dIII digestion. The DNA fingerprinting result again proves BAC clone No. 20 and 32 contain one homoeoallele of *TtRec8*, while BAC clone No. 7, 18, 24 and 31 contain the other homoeoallele.

APPENDIX E. NUCLEOTIDE SEQUENCE COMPARISON BETWEEN SEGMENTS OF *TtREC8*



Nucleotide sequence comparison of GM008F/GM008R primer pair amplified segments between the two homoeoalleles of *TtRec8*. Black boxes highlight the exon regions and the rest sequences are introns. Only 2% (5 out of 245 nucleotides) sequence difference was found in exonic regions and 8.1% (62 out of 769 nucleotides) difference was in intronic regions, indicating higher sequence similarity was in exonic regions than in intronic regions between *TtRec8-A1* and *TtRec8-B1* within this segment.

APPENDIX F. PROTEIN ID VERIFICATION OF pGEX-C31 POLYPEPTIDE



Verification of polypeptide pGEX-C31 by Protein ID assay. A) LC-MS/MS data are collected from the peptide mixture generated by proteolytic digestion of pGEX-C31 polypeptide sample, and the MS/MS spectra are searched against the database of the deduced amino acid component of pGEX-C31. The result shows 50% coverage of the database, as highlighted in yellow.

APPENDIX G. LC-MS/MS SPECTRA OF POLYPEPTIDE pGEX-C31 FRAGMENTATION