Chapter 4
Studies on the putative spindle checkpoint gene
AtMAD2
4.1 Introduction

The dyad and duet mutants of Arabidopsis are defective in female and male meiotic cell cycle progression respectively due to defects in chromosome segregation and organization (Siddiqi et al., 2000; Mercier et al., 2001; Agashe et al., 2002; Yang et al., 2003 and Reddy et al., 2003). The cell cycle checkpoints monitor progression from one stage to another stage of the cell cycle. The spindle checkpoint is one such surveillance mechanism, which checks the proper segregation of chromosomes to each daughter cell in mitosis as well as in meiosis. Genes involved in the spindle checkpoint have been extensively characterized in yeast (Shonn et al., 2003). The Mitotic Arrest Deficient (MAD) genes MAD1, MAD2 and MAD3 are active components of the spindle checkpoint mechanism (Gorbsky 1997). The spindle checkpoint is thought to be activated either by the presence of unattached kinetochores during mitotic metaphase or lack of tension between bivalents which are arranged on metaphase 1 plate of meiosis or possibly both (Nicklas et al., 2001). A spo13 yeast mutant undergoes single division meiosis and results in dyads instead of tetrads. The chromosomes in Spo13 show mixed segregation (both reductional as well as equational). The spo13 gene is required for protection of centromeric cohesion in yeast (Lee et al., 2002). Double mutant of spo13 and mad2 results in the formation of tetrad (Shonn et al., 2002). The cell cycle progression defect in spo13 mutant is therefore due to activation of the spindle checkpoint. Knockout of the spindle checkpoint gene Mad2 in a spo13 mutant background relieved the progression defect. In the dyad mutant also, it is possible that the female meiotic progression defect is due to activation of the spindle checkpoint as in spo13. The presence of univalents in prophase can delay cell cycle progression by a MAD2 dependent mechanism (Cheslock et al., 2005). Experiments have been carried out to test this hypothesis.

In case of duet, the number of meiocytes in diakinesis to metaphase I stage were higher than in wild-type (Table: 3.4). Even though bivalent formation takes place, because of lack of proper condensation, mutant
bivalents may not build up appropriate tension at metaphase I. This may result in the activation of spindle checkpoint and causes the meiocytes to arrest at metaphase I. Functional characterization of the role of spindle checkpoint in plants has not been done. Immunolocalization experiments on maize mitotic and meiotic chromosomes revealed MAD2 protein to be attached to the mitotic and meiotic kinetochores (Yu et al., 1999). The wheat homologue of MAD2 also attaches itself to the kinetochores (Kimbara et al., 2004).

The current study was carried out to test whether the progression defect in dyad and duet mutants were due to activation of spindle checkpoint. The study has attempted towards the creation of spindle checkpoint mutant in the background of dyad as well as duet.

4.2 Results

4.2.1 Identification of an Arabidopsis homologue of the MAD2 spindle checkpoint gene

Saccharomyces cerevisiae spindle checkpoint protein Mad2 (NCBI ID: CAA89321) was subjected to BLAST search against AGI proteins (TAIR BLASTP), which led to identification of a putative spindle checkpoint protein AtMAD2 (At3g25980) in Arabidopsis with an e value of 1 e^{-36}. At3g25980 encodes for a protein of 209 amino acids of predicted mass of 23.7 KDa. AtMAD2 is a unique gene in the Arabidopsis genome and does not have high level of similarity to any proteins. Although the immunolocalization data on maize and wheat MAD2 proteins is available, functional studies on plant MAD2 gene is lacking mainly because of lack of a mutant. In this study, efforts were made to identify a mutant for Arabidopsis MAD2 gene, which in turn will be useful to study the progression defects in dyad and duet.

4.2.2 Isolation of full length cDNA of AtMAD2 and identification of gene structure

The cDNA of AtMAD2 was amplified by RT-PCR using primer combinations MDHLF1 and MDHLR. The amplicon was cloned into pMOS
blue vector and sequenced. Comparison of genomic DNA and the cDNA sequence revealed 7 exons and 6 introns. The gene structure of \textit{AtMAD2} is in complete accordance with the predicted gene structure of the At3g25980 locus. The exons are 249, 16, 131, 121, 104, 122 and 63 bp and the introns are 88, 101, 82, 94, 95, and 81 bp in size. Coding DNA sequence ranges from 174 to 803, which suggest the 5' UTR to be 173 bp. The \textit{AtMAD2} gene potentially encodes a protein of 209 amino acids and with a predicted mass of 23.7 KDa. A conserved domain (CD) search identified a HORMA domain (for \textit{Hop1}, \textit{Rev7} and \textit{Mad2}), which lies from amino acid position 13 to 201 (Bauer and Bryant 2004). The HORMA domain has been suggested to recognize chromatin states that result from DNA adducts, double stranded breaks or non-attachment to the spindle and acts as an adaptor that recruits other proteins. MAD2 is a spindle checkpoint protein, which prevents progression of the cell cycle upon detection of a defect in mitotic spindle integrity (Aravind and Koonin 1998). \textit{Arabidopsis thaliana} MAD2 protein was aligned with \textit{Zea mays} and \textit{Oryza sativa} homologues of MAD2 protein. \textit{Arabidopsis} and maize MAD2 proteins are of 209 amino acids each whereas the rice MAD2 protein is of 208 amino acids. Multiple sequence alignment by ClustalW software (http://www.ebi.ac.uk/clustalw) has identified significant similarity among the three proteins. Out of 209 amino acids, 170 a.a are identical to each other (Fig. 4.1: B). A higher level of conservation at protein level among the plants of different taxonomic groups in angiosperms (based on sequence availability) suggests conservation of MAD2 function in plants.

4.2.3 A test of the function of \textit{AtMAD2} by RNAi

The RNAi strategy was employed to test the basis for the meiotic progression defect in \textit{dyad} and \textit{duet} mutants. A silencing construct for \textit{AtMAD2} was made as described in Chapter 2.

4.2.3.1 Genotyping of \textit{AtMAD2} RNAi plants

Transgenic plants harboring the \textit{AtMAD2}-RNAi construct would contain the \textit{AtMAD2} cDNA construct in their genome along with the native
Figure 4.1 A: Gene structure of AtMAD2 (At3g25980).

Figure 4.1 B: Alignment of AtMAD2 with maize and rice MAD2 proteins.

Arabidopsis thaliana * Identities
Zea mays — HORMA Domain
Oryza sativa

Figure 4.1 C: Expression analysis of AtMAD2 in different tissues.

1: 100 bp ladder
2, 3 and 4: Wild-type leaves, Inflorescence and Pistil cDNA used as a template
5: Genomic DNA used as a template
6: No template –Ve control
AtMAD2 genomic locus. Therefore, amplification with AtMAD2 gene specific primers (AMD2F3 and AMD2R3) will give two amplicons, one of 550 bp size, which is from the native genomic locus, and another one of 290 bp size which is the cDNA amplicon from the RNAi cassette. Wild-type genomic DNA template served as negative control for this experiment, where it amplified only the genomic locus. DNA was extracted from 29 KanR plants and used as template for the PCR. These T1 generation plants were genotyped with respect to dyad allele. The CAPS marker KKL, which flanks dyad was used to genotype plants (Agashe et al., 2002). Plants homozygous for dyad (n=6) were selected for further analysis (data not presented).

4.2.3.2 Expression analysis of AtMAD2

The expression of AtMAD2 mRNA in inflorescence from six dyad/dyad;AtMAD2 RNAi T1 plants were compared to wild-type by semi-quantitative RT-PCR. The AtMAD2 cDNA amplification could not be detected in five out of the six plants that were examined (Fig. 4.2: A). The plants in which AtMAD2 mRNA levels were reduced were analyzed further at the phenotypic level.

4.2.3.3 Phenotypic analysis of ovules

In angiosperms, the embryo sac contains an egg cell, two synergids and three antipodal cells [Fig. 4.2: B (a)]. However, in dyad which is defective in female meiotic progression, ovules contain two enlarged cells in place of a functional embryo sac [Fig. 4.2: B (b)]. The ovules of dyad/dyad;AtMAD2 RNAi plants also showed two enlarged cells, similar to the case of dyad mutant. Small proportions of triads and tetrads were also observed in these plants, which were similar to the case of mature ovules of dyad [Fig. 4.2: B (c and d)]. This observation indicates that though the level of AtMAD2 mRNA was reduced in the dyad mutant, the female meiotic progression defect was not relieved.

Similar efforts were made to identify duet mutant plants that had AtMAD2 RNAi cassette by selecting hygromycin resistance transgenic
Figure 4.2 A: Expression analysis of \textit{AtMAD2} gene in wild-type and \textit{dy/dy;AtMAD2 RNAi} plants.

\textit{Wt}: Wild-type; 11, 17, 19, 20, 21, and 25 are \textit{dy/dy;AtMAD2 RNAi} plants;

\textit{Gen}: Genomic DNA used as template.

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\textit{AtMAD2}  
\textit{GAPC}

Figure 4.2 B: Analysis of the ovule phenotype by DIC optics.

A: Wild-type; B: \textit{dyad} mutant; C & D: \textit{dy/dy;AtMAD2 RNAi}; Scale bar: 20 \textmu m.
plants. These were confirmed by doing PCR for the presence of cDNA size amplicon and genotyped with respect to DUET locus. The duet/duet;AtMAD2 RNAi plants (n=4) were also similar to duet single mutant phenotype and defective in microsporogenesis. Detailed analysis on male meiotic chromosomal spreads revealed high proportion of arrested meiocytes with diffused chromatin at metaphase I, which was similar to duet single mutant phenotype (data not presented). The results from the above experiments indicated that either knocking down the spindle checkpoint gene AtMAD2 by RNAi may not completely disturb the function of the gene or that the meiotic progression defects in dyad as well as in duet is not due to the activation of spindle checkpoint. However, one cannot rule out the possibility of an alternate redundant pathway that may be present for the spindle checkpoint. There may be multiple causes for the progression defects observed in the mutants along with spindle checkpoint, in which case relieving spindle checkpoint alone may not be sufficient to relieve the progression defect.

4.2.4 Transposon mobilization strategy to identify a line a carrying transposon in AtMAD2 (At3g25980) gene

It would be required to knock out the function of putative spindle checkpoint gene in dyad and duet mutants to test the hypothesis that the progression defect in these mutants may be due activation of a potential spindle checkpoint. The phenotype of dyad/dyad;AtMAD2 RNAi plants did not differ from that of the dyad single mutant. Similar was the case with duet/duet;AtMAD2 RNAi plants. However, it has been reported that even one single unattached bivalent can induce the spindle checkpoint (Nicklas 1997) and that only unaligned chromosome remains positive for MAD2 (Waters et al., 1998). This implies that a small amount of MAD2 protein is able to activate the spindle checkpoint. RNAi strategy reduces the amount of mRNA but does not completely abolish the function of the gene. This may be the reason that in dyad/dyad;AtMAD2 RNAi plants, though there is significant reduction in mRNA of AtMAD2, it may not serve as a complete knockout. Therefore, a genetic knockout, which completely abolishes the
function of \textit{AtMAD2} gene is required to test the hypothesis. A search in T-DNA insertion lines collection identified SALK\_125904, in which the T-DNA was inserted 184 bp upstream to the transcription start site in \textit{AtMAD2} locus. This position may lies in the promoter region of \textit{AtMAD2} gene and could potentially disturb the transcription. Homozygous plants identified by PCR did not show any significant difference to that of wild-type phenotype. RT-PCR analysis revealed that there was no disturbance in the transcription of \textit{AtMAD2} mRNA in SALK\_125904 homozygous line (data not presented). Therefore, a transposon mobilization strategy was approached to identify plants carrying a \textit{Ds} transposon insertion in the \textit{AtMAD2} gene.

4.2.4.1 Characterization of starter lines in which a transposon is inserted in the vicinity of \textit{AtMAD2} gene

\textit{Ds} transposons are known to have a preferential hopping to linked sites. A search was conducted to identify a \textit{Ds} insertion line that was in the vicinity of the At3g25980 locus, which could serve as a launch pad for mobilization of the transposon element. Two \textit{Ds} transposon insertion lines (ET8568 and SET6770) were identified that had the \textit{Ds} element inserted at 3.4 kb and 0.5 kb respectively upstream and down stream to the At3g25980 locus. Since SET6770 was only 0.5 kb away from the termination codon of At3g25980, the probability of mobilization of \textit{Ds} into \textit{AtMAD2} gene was higher. The terminal inverted repeat sequences present at both ends of the \textit{Ds} element are very critical for mobilization. The 5' flanking region of SET6770 line was amplified and sequenced to determine the intactness of \textit{Ds} transposon. It was found that 11 bp at the end of 5' inverted repeat of \textit{Ds} in SET6770 was deleted. Therefore, SET6770 could not be used as a launching pad for the mobilization of the \textit{Ds} transposon.

Line ET8568 was also analyzed for the intactness of transposon ends. A three bp (CAG) deletion was observed at the 5' end. The transposon had inserted in the genomic locus adjacent to CATG. This resulted in ‘CATG’ sequence at the end of transposon instead of a ‘CAG’. This situation raises the following questions. 1. Can the particular \textit{Ds} transposon hop or not? 2. If it can hop, what is the frequency of hopping?
During the course of hopping either insertion site duplication or deletion of some bp (around 8 bp) termed as transposon foot printing occurs. This feature of *Ds* transposon was used to determine the extent of hopping nature of *Ds* in the ET8568 line. A line homozygous *Ds* and presence of *Ac* was genotyped and selected to study mobilization nature. If transposon mobilizes in a specific cell, that particular cell lacks the transposon but has a very high probability of possessing a footprint. All cells developing from this particular cell in which excision has taken place will be genotypically identical to each other. The size of the somatic patch depends upon time of excision event during development. If the excision occurred early during development, it results in a large soma clonal patch and if it is later in development, it results in a smaller patch. If in some soma clonal patches *Ds* has excised, the DNA of these cells can act as template for these gene specific primer (ET8568F and ET8568R) on either side of transposon insertion. Since the excision patch tissue is low and varies with stage of excision when compared to the total tissue used for DNA isolation, the amplification may not result in a ethidium bromide visible band. Such amplified product further can be amplified in secondary PCR using nested primer on either side of the *Ds* transposon (ET8568IF and ET 8568IR). On the other hand if there is no excision the secondary PCR would also be negative for PCR amplification. With this strategy one such amplicon was obtained in a secondary PCR with *Ds/Ds; Ac* genotype. The sequence analysis identified a two bp (AC) addition at the site of *Ds* insertion when compared to wild-type sequence. From this result it could be concluded that in the ET8568 line, *Ds* transposon possessed excision ability (Fig. 4.3: A).

4.2.4.2 Analysis of excision ability of the *Ds* transposon line ET8568

Efficiency of transposon excision was assessed using the following strategy. If the transposon element had excised out, a PCR will be negative with *Ds* and gene specific primers (Ds5-2 and ET 8568R) for a genotype heterozygous for presence of *Ds* from *F₂* plants (*Kan^R* and *NAM^R* plants) derived from a cross between *Ds/Ds* (ET8568); +/- X +/-; *Ac/Ac*. Out of 100 plants tested, four plants were negative for amplification. The PCR was
Figure 4.3: Transposon mobilization strategy into *AtMAD2*.

**A: Identification of excision ability of *Ds* transposon in the ET8568.**

1: 100bp ladder
2 and 4: 1° PCR with ET8568F and ET8568R, *Ds/Ds*; *Ac* as template
3 and 5: 2° PCR with diluted PCR products of 2 and 4 as template using ET8568IF and ET8568IR

![Diagram of excision process](image)

1: 100bp ladder
2 and 4: 1° PCR with ET8568F and ET8568R, *Ds/Ds*; *Ac* as template
3 and 5: 2° PCR with diluted PCR products of 2 and 4 as template using ET8568IF and ET8568IR

**B: Identification of excision efficiency of transposon in the ET8568.**

Arrows indicate excision

*Kan*\(^R\) and *NAM*\(^R\) *F*\(_2\) plants DNA as template; *Ds5-2* and ET8568R as primers

![Diagram of excision process](image)

1: 100bp ladder
2 and 4: 1° PCR with ET8568F and ET8568R, *Ds/Ds*; *Ac* as template
3 and 5: 2° PCR with diluted PCR products of 2 and 4 as template using ET8568IF and ET8568IR
Figure 4.3: Transposon mobilization strategy into \textit{AtMAD2}

C: PCR screening strategy

ET8568 homozygous line (Kan\textsuperscript{R}) \times \textit{Ac} expression line (NAM\textsuperscript{R})

\[ \begin{array}{c}
\varphi \\
\downarrow \\
F_1: Ds/+; Ac/+ \\
\downarrow \\
F_2 (n=4,500): 25 \text{Kan}^R \text{and NAM}^R \text{plants as a single pool for PCR}
\end{array} \]

\begin{itemize}
  \item \textbf{Primers} \hspace{1cm} \\
    1: RMAD2FM \\
    2: MDFLFI \\
    3: T6770R \\
    4: MAD2RM
\end{itemize}

\begin{itemize}
  \item \textbf{Primer combinations in 1\textsuperscript{st} and 2\textsuperscript{nd} PCR} \hspace{1cm} \\
    1\textsuperscript{st} PCR: 1&Ds5-1 \text{ and } 1&Ds3-1 \\
    1&Ds3-1 \text{ and } 3&Ds5-1 \\
    3&Ds3-1
\end{itemize}

\begin{itemize}
  \item \textbf{D: Confirmation of the positive pool in 3\textsuperscript{rd} PCR} \hspace{1cm} \\
    DNA from Pool No.139 used as template. A size difference correspond to Ds5-2 and Ds5-3 annealing sites can be observed
\end{itemize}
repeated thrice for the particular four DNA samples with appropriate controls. Quality of DNA from these four plants was good as it could amplify for the GAPC locus. Also, these four plants were positive for PCR with kanamycin gene specific primers (KANF and KANR). In this experimental design, excision events occurring from homozygous plants could not be detected but homozygous plants also have same potential to excise from the native locus. Taking the homozygous plants also into consideration, the percentage excision was 8%. The results from this experiment identified that germinal excision efficiency of the ET8568 Ds transposon was 8% (Fig. 4.3: B).

4.2.4.3 Generation of an F₂ population to screen for mobilization of Ds into AtMAD2

The F₂ population was generated by crossing homozygous ET8568, as the female parent and homozygous Ac line as the male parent. Genotype of the parent plants used for cross-fertilization experiment was confirmed by PCR. A total of 4000 F₁ seed was obtained from nearly 100 crosses. Out of these, 3000-3500 F₁ plants were grown and seed were collected as pools. A total of 65 pools were generated where each pool constituted seed from 50 individual F₁ plants. Transposon excision occurs in the F₁ generation. The F₁ plants were self-fertilized which resulted in the F₂ generation. Seeds from the F₂ generation were selected for kanamycin and NAM (Napthalene Acetamide) resistance to get germinal excision events and to segregate away the Ac element. Kanamycin acts as a reinsertion marker/presence of Ds at original donor site whereas NAM selection was exploited as a negative selection to segregate away the Ac element. The segregation against Ac element is important so as to pick stable insertion lines and to avoid picking false positives arising from somaclonal excisions during PCR screening. A total of 4500 KanR and NAMR seedlings were selected from 65 families and grown as pools of 25 plants in one Petri dish for one week. A PCR based screen was proceeded by isolating DNA from 25 cotyledonary leaves from each seedling in the Petri dish. The reconstitution experiment for PCR ability has revealed that the amount of
DNA was a limiting step in identifying an insertion. SET6770 line was used for reconstitution experiments. One cotyledonary leaf of SET6770 line was mixed with 24 wild-type cotyledonary leaves for DNA isolation. This DNA was used to test the ability of PCR to detect the presence of SET6770 insert (data not presented).

Since the quantity of DNA was a limitation, remaining 3000 Kan\textsuperscript{R} and NAM\textsuperscript{R} seedlings were transplanted into pots and allowed to grow at a density of 25 plants per pot. Primary inflorescences (n=25) from each pot were pooled to isolate DNA using midi prep protocol. There were a total of 120 pools that were generated, which were then screened by PCR to identify a pool that may have the insertion.

It is known that a transposon can reinsert in any orientation for a particular insertion event. In order to identify an insertion the following PCR based screen was employed. Four sets of PCR were performed for each pool. They were: 1. Gene specific forward (RMAD2FM) and Ds5-1, 2. Gene specific forward (RMAD2FM) and Ds3-1, 3. Gene specific reverse (SET6770R) and Ds5-1 and 4. Gene specific reverse (SET6770R) and Ds3-1. The third and fourth primer combinations act as confirmations for the first and second primer combinations along with serving as assay system in case of a deletion of transposon ends during insertion. Since the target template is expected to be 1/25 plants, it would be difficult to achieve visible ethidium bromide staining amplicon in the primary PCR. Therefore, a secondary PCR was set up using nested primers for each combination. The template used for secondary PCR was a 1:25 dilution of the primary PCR product. The nested primer combinations in secondary PCR were as follows: MDFLF1 and Ds5-2 for first combination, MDFLF1 and Ds3-2 for second combination, MAD2RM and Ds5-2 for third combination and MAD2RM and Ds3-2 for fourth combination (Fig. 4.3: C). Out of the 120 pools screened by the above mentioned strategy, three pools gave amplicons in the secondary nested PCR. These amplicons were validated to be true by setting a tertiary PCR using nested primers.
In a tertiary PCR screen two out of three pools were positive for amplification with an expected size difference between secondary and tertiary PCR screen amplicons (Fig. 4.3: D). The amplicons obtained from secondary PCR were sequenced with Ds specific primers (Ds5-2 and Ds5-3). Sequence analysis revealed that these primers read through AtMAD2 locus, which confirmed the genuine amplification from a line where transposon had remobilized into the AtMAD2 locus. An interesting observation after sequencing the amplicon obtained from pool number 139 with Ds5-2 was that while hopping ET8568 transposon had excised along with 110 bp of At2g35960 locus sequence adjacent to Ds transposon and reinserted in the AtMAD2 locus. The ET8568 transposon end point mutation (5' end conversion of CAG to CATG as described earlier) was also observed upon sequence analysis.

The plants in the positive pools had completed their lifecycle by the time of sequence analysis. Therefore seeds were collected carefully from all the 25 individual plants and germinated on medium supplemented with NAM and kanamycin. Double resistant seedlings were selected and tissue material collected for DNA extraction. All the seedlings derived from 75 plants belong in to three pools were tested for the presence of insert by using Ds and AtMAD2 specific primers. Unfortunately none of the plants tested were positive for amplification. The experiment was repeated twice for all the 75 DNA samples with appropriate positive controls for DNA quality and for PCR. The amplicons obtained from the PCR screening might have originated from somaclonal excision. It may be possible that the allele produced by insertion in AtMAD2 gene may be a gametophytic lethal and transmission to next generation was low (see discussion).

A simultaneous screening strategy with two Wisconsin Ds lox lines (Wisc_258_E07 and Wisc_474_D04) where, the Ds lox was inserted near to AtMAD2 locus was also done. In this method of mobilization of transposon, an excision marker was available (hygromycin resistance). Only one starter line was progressed to the F2 generation. The other starter line Wisc_474_D04 had a translocation, which was apparent from the semi
sterile phenotype. The homozygous starter line (Wisc_258_E07) was crossed with Ac expression line. There were no hygromycin resistant plants obtained in the F₂ generation, which suggests an absence of excision. The results were further confirmed by Dr. P Krysan in a personal communication to Dr. Imran Siddiqi, that in some of the starter lines the Ds transposon did not excise. The experiments performed on transposon mobilization strategy in order to identify a plant carrying an insertion in the AtMAD2 locus would be helpful for future screenings. These include issues regarding the screening process, optimum number of plants that can be handled in a given time, experimental design and transposon excision ability.

4.3 Discussion

The spindle checkpoint is a surveillance mechanism that ensures metaphase completion before anaphase could begin. One of the most studied spindle checkpoint genes is MAD2, which encodes a ~24 kDa protein. A variety of evidences indicate that the signal for the spindle checkpoint emanates from the kinetochores. As soon as the chromosomes are attached to the spindle, MAD2 levels diminish at the kinetochores until the next cycle (Yu et al., 1999). The availability of free microtubule binding sites or absence of tension on the kinetochore causes MAD2 to be recruited to kinetochores where it activates the spindle checkpoint. MAD2 localization studies reveal that it is microtubule unattachment and tension between homologous chromosome in a bivalent at kinetochores during meiosis that serves as a signal for activation of the spindle checkpoint (Nicklas et al., 2001).

The phenotype of dyad/dyad AtMAD2 RNAi ovules is similar to that of the dyad single mutant. It can be inferred from this experiment that meiotic progression defects is not relieved by disrupting AtMAD2 mRNA using RNAi. This may be due to multiple checkpoints that may be operational in arrested cells and knocking down one may not be sufficient to relieve the progression defect. However, we could not exclude the possibility of effectiveness of RNAi for the silencing of particular gene. Since AtMAD2 RNAi cassette was driven by CaMV 35S promoter, the expression
of this promoter may not be effective in megaspore mother cell. It is quite likely that in the RNAi plants against AtMAD2 gene, the mRNA levels for AtMAD2 are reduced but did not yield a complete knockout. According to published reports, it is known that a single unattached kinetochore at metaphase plate can activate spindle checkpoint (Nicklas et al., 2001). Even though the RNA levels were reduced in AtMAD2 RNAi transgenics, the low level of protein present in these plants might serve as signal for activation of the checkpoint. There may be some other parallel pathways present to regulate proper segregation of chromosomes in plant meiosis, which haven't yet been discovered. The evidence for presence of the spindle checkpoint in plant meiosis comes from maize MAD2 localization studies which reveal that MAD2 protein is present on both mitotic as well as meiotic kinetochores (Yu et al., 1999). Recent studies indicate a DNA damage checkpoint is effective in both mitosis and meiosis in Arabidopsis (Preuss et al 2003). Taking these two observations together, it may be possible that the checkpoint mechanisms are conserved through different organisms and a similar mechanism operates both in mitosis and meiosis with little differences. Since interfering with the AtMAD2 RNA level in dyad does not relieve the progression defect, spindle checkpoint may not be the cause for the progression defect. The present objective of testing the meiotic progression defect in dyad can be rigorously tested by creating a double mutant of dyad and AtMAD2 genetic knockout. Therefore, efforts have been made to create AtMAD2 knockout by employing transposon mobilization strategy. The transposon mobilization strategy has been successfully worked out for targeted local saturation mutagenesis in Arabidopsis. In this system Ac was expressed under a heat inducible promoter and an antibiotic resistance marker for excision event is available (Nishal et al., 2005). The heat inducible expression of Ac provides a regulated expression of transposase. The availability of an excision marker simplifies the screening work where a Ds excision line can be selected. We checked the position of starter lines and unfortunately out of the 40 lines reported, none of them were in the vicinity of AtMAD2 locus. More number of starter lines needs to developed to get a starter line near the gene of interest. In the transposon mobilization strategy the intactness, excision
ability and efficiency of excision were assessed. However, still an insertion in the *AtMAD2* locus could not be identified. This could be attributed to various reasons. The amplicon that was obtained could be from a NAM sensitive plant and therefore a product of a somatic excision event. This possibility was ruled out by doing a PCR using NAM specific primers. All the 25 individual plants from positive pools were negative for NAM specific PCR. Another reason could be the plant might have died after collection of tissue for pool-PCR. However one could not rule out the possibility that the transmission rate of the allele is very low through the gametophyte. Spindle checkpoint gene may be an essential gene required during the gametophyte generation at the time of haploid mitoses. This may be the probable reason for lack of insertion line in *AtMAD2* locus out of the 3, 30, 000 lines in the *Arabidopsis* mapped insertion line database. In this scenario, it would be required to identify the individual plant from the positive pool in the same generation itself. This information would be useful for any further such screens.