Parthenogenesis in plants: Putative functions of *MCM* genes

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Praise be to Allah …

Contents

List of abbreviation

A	Adenine	k	Kilo
A. tumefacience	*Agrobacterium tumefacience*	L	Liter
ATP	adenosine triphosphate	M	Molarity
bp	base pairs	m	Mili
BSA	bovine serum albumin	µ	micro
C	cytosine	MOPS	N-morpholinopropane- sulfonic acid
CaMV	cauliflower Mosaic Virus	mRNA	Messenger RNA
cDNA	complementary DNA	n	nano
D	Dalton	OD	Optical density
DAPI	4,6-diamidino-2-phenylindol dihydrochloride	PAGE	polyacrylamide gel electrophoresis
DEPC	Diethylpyrocarbonate	PBS	Phosphate-buffered saline
DNA	Deoxyribonucleic acid	PCR	polymerase chain reaction
DTT	Dithiothreitol	PMSF	phenylmethylsulfonylfluoride
E. coli	**Escherichia coli**	RACE	rapid amplification of cDNA ends
EDTA	ethylenediamine tetraacetic acid	RNA	Ribonucleic acid
g	gram	SDS	Sodium dodecyl sulfate
G	guanine	T	Thymidine
GUS	β-glucuronidase	T-DNA	Transferred DNA
HEPES	N-[2-Hydroxyethyl] piperazine-N`-[2ethansulfonic acid]	TdT	Terminal deoxynucleotidyl transferase
IPTG	Isopropyl-β-D-thiogalactopyranoside	Tris	Tris-hydroxymethylaminomethane
Caps	(3-[cyclohexylamino]-1-propane-sulfonic acid)		

1. Introduction

1.1. Harnessing apomixis- another green revolution

Sexual reproduction is not the only possibility for plants to generate progeny. In a number of species of about 40 angiosperm families, including grasses, sunflowers and roses, an asexual mechanism called apomixis shortcuts the sexual pathway to produce fertile seeds. Apomictic species produce progenies that are genetic copies of themselves, thus preserving the maternal genotype. Since the discovery of apomixis in 1845 (Asker and Jerling, 1992), it has been regarded as a rare and eccentric breeding system of angiosperms. Recently, this view has changed dramatically and the implementation of apomixis for major crop plants is considered to have economic and social benefits exceeding those of the green revolution (Vielle-Calzada et al., 1996; Grossniklaus et al., 1998). Clonal apomictic derivatives of important sexual crop plants could facilitate the fixation of hybrid effects by the development, mass production and maintenance of elite hybrid genotypes. Genetically pure seed lots could be produced from apomictic species without the physical isolation that is now required. Breeding and production of hybrids will be feasible, allowing the development of genotypes better adapted to abiotic and biotic environmental stress conditions and better tailored for end production uses. The application of transgenic transformants will be facilitated due to the 100% seed transmission of hemizygous transgenes by the apomicitic pathway, obviating the need of inbreeding to obtain transgenic homozygotes. Furthermore, apomictic transformants may exhibit less sterility from tissue culture-induced somaclonal variation, which often results from disturbed meiosis. In species with high genetic load, the level of hybrid heterosis attainable under apomixis will exceed that attainable by sexual means. Once freed by apomixis from the constrains on chromosome number imposed by meiotic sterility, it will be far easier to seek genetic synergism at higher than diploid levels, that is, to minimize mono-allelism (homozygosity, at the diploid

level) and maximize multiallelism (heterozygosity, at the diploid level). Moreover, with meiotic sterility eliminated, crops might be developed more readily from new interspecific and intergeneric hybrids (Vielle-Calzada et al., 1996).

1. 2. Sexual and apomictic gametophytes

1. 2. 1. Development of the gametophyte in the sexual model plant Arabidopsis

The sexual gametophyte development of *Arabidopsis* is the common model for the sexual gametophyte development in angiosperms (Smyth et al., 1990; Reiser and Fischer, 1993). The first visible gynoecial and stamen primordia develop from the central dome. The gynoecia primordium elongates to develop into an open ended oval cylinder, owing to the cessation of growth at the central invagination. The gynoecium consists of six layers composed of vacuolate and meristematic cells. At this stage, two main vascular bundles begin to differentiate in medial positions in the cylinder. Before anthesis, the gynoecium completes the main growth aspect and the ovules develop integuments and complete megagametogenesis. The megasporangium (nucellus) originates at the megasporophylls (carpels). The megaspore mother cell (MMC) differentiates within the nucellar part of the ovule. Meiosis in a selected MMC results in four haploid megaspores. Three of them degenerate. The left over embryo sac cell gives rise by mitotic divisions and differentiation to the female gametophyte or embryo sac (megaprothallium). Normally an embryo sac consists of 8 haploid cells: The egg cell proper surrounded by two synergids at the micropylar end of the embryo sac, two central polar cells and three antipodal cells. Double fertilisation of the egg cell and the polar cells leads to the formation of a diploid zygote and the triploid endosperm cell, respectively.

The first cell division of the zygote generates the embryo proper and the suspensor. Further embryogenesis includes characteristic phases known as globular, heart, torpedo and cotyledon stages. The triploid endosperm differentiates into a storage tissue and the integuments form the seed coat. A stalk-like structure, the funiculus, connects the ovule to the placental region of the gynoecium.

Due to the alternation of generations typical for the plant life cycle, the mature ovule is composed of tissues differing in the ploidy level of their cells. While the embryo sac is haploid, representing the female gametophytic phase of the life cycle, the rest of the ovule is diploid forming the sporophytic part of the organ. Meiosis of the megaspore mother cell and double fertilisation are the most prominent phenomena in the sexual pathway.

1.2.2. Development of the gametophyte in apomicts

Apomictic seed formation is the result of a complex developmental process characterised by the uncoupling of developmental checkpoints. Thus, embryo sac development is uncoupled from meiosis and both embryogenesis and endosperm development occur without fertilisation. Apomicts exhibit a considerable variability where the apomictic embryos are formed in the ovule by at least two different pathways. A) In sporophytic apomixis, also called adventitious embryony, the embryo arises directly from the nucellus or the integument without the formation of an embryo sac. B) The gametophytic apomixis which is always characterised by the formation of an embryo sac and is further subdivided in diplospory and apospory. In diplospory the embryo sac originates from the megaspore mother cell which is still unreduced. In apospory the unreduced embryo sac is derived from nucellar cells. In both cases the embryo arises autonoumsly from an unreduced cell of the embryo sac. Whereas diplosporic embryo sacs are usually very similar to the meiotic embryo sacs, aposporic ones are

clearly contrasting. Apomixis and sexuality may coexist in a state of balance in the same plant, sometimes in the same ovule, thus the capacity to produce clonal seeds does not mean lack of sexuality. Some apomicts, however, give rise only to progeny that is identical to the mother plant and are therefore regarded as obligate. Others, known as facultative apomicts, represent variable levels of expression of the character. Since apomicts produce viable and generally reduced pollen, the trait can be transferred to a progeny if they are used as male parents in crosses to sexual plants.

Apomixis can be considered as a triade of three interconnected processes:

- Apomeiosis (avoidance of meiosis)
- Parthenogenesis (Fertilisation independent embryogenesis)
- Autonomous or pseudogamous development of the endosperm.

Apomeiosis occurs during the generation of the megaspore. The normal process is disturbed and a mitotic process replaces meiosis in the megaspore mother cell (MMC) resulting in the formation of a megagametophyte, in which all the nuclei, including that of the egg cell, are unreduced (2n). Controlling the process of ovule initiation and the first meiotic division of the megagametophyte might be the first step to develop an apomictic embryo sac. Ethylene production seems to be a trigger for the first meiosis of the megagametocyte in tobacco (Zhang et al., 1993; De Martinis et al., 1999).

Mutants like *ms5*, *tes*, *mel1* and *ask1* cause defects only in male meiosis and MEL1 and ASK1 are possibly involved in proteolytic reactions required for the progression through the meiotic cell cycle. These mutants cause phenotypes of sterility or variable numbers of pollen with abnormal karyotypes (Spielman et al., 1997; Glover et al., 1998; Yang et al., 1999; Bhatt et al., 1999). The recently isolated *dyad* mutant is the only mutant arrested in female meiosis at the dyad stage after meiosis I (Wei-Cai Yang et al., 2000). From these observations it has been concluded that different genes are employed during the male and female meiosis in plants.

Parthenogenesis is the second component of apomixis and means the development of an embryo without fertilisation. The parthenogenesis could be either:

- generative (haploid) if the female gametes are reduced or
- somatic (diploid) if the female gametes are unreduced.

Spontaneous parthenogenesis of reduced egg cells was found in many sexual species of angiosperms and the resulting embryos are usually initiated from the synergid (Kimber and Riley, 1963). Genotypes with stably increased rates of autonomous development of reduced egg cells have been selected in *Zea mays* and *Solanum tuberosum* (Lashermes and Beckert, 1988). In some cases, progenies with reduced chromosome numbers were generated after delayed pollination or wide crosses. Moreover, the autonomous development of reduced or unreduced egg cells was detected from the seed set after pollination with pollen inactivated by X-irradiation, ultraviolet light or treatment with toluidine blue. These results were explained with the so called mentor-pollen or prickle pollination effect. Physical (low and high temperature), chemical and biological means (application of plant extract or using wide crossing) were used to induce parthenogenesis, in *Gossypium hirsutum.* Physical means gave the highest rate of induction of parthenogenesis with mixoploidy progenies (Shi-Qi et al., 1991). In maize the plant response to chemical induction of parthenogenesis was highly dependent on the type of the hybrid. The parthenogenetic F1 plants were identical in their morphology to the diploid plants of the same hybrid, regardless of the chemical applied, and characteristics appeared uniform among individual plants. The cytological investigations show that most of the obtained seed were diploid and small portions were mixoploid, but haploid and tetraploid plant were found as well (Gongshe et al., 1991).

The sporophyte derived from the reduced parthenogentic egg cells are sterile in the first (diploid or amphidiploid parents) or in successive

generations (autopolyploid parents). Therefore, this mode of production may be regarded as a blind alley in evolution. However, if parthenogenesis is connected with apomeiosis it may have a selective advantage in evolution (Nogler, 1984; Koltunow, 1993).

It was demonstrated that parthenogenesis is genetically determined and dominant over sexuality. Doses effects and/or modifier genes are probably involved in the control of parthenogenesis. The data obtained from the Flow Cytometric Seed Screen (FCSS) have shown that there is no close linkage between apospory and parthenogenesis in *Poa pratensis* (Matzk, 2000). Further, an independent genetic control of diplospory and parthenogenesis has been described for *Allium tuberosum* and *Taraxacum officinale* (Baarlen et al., 1999). Occasionally, fertilisation of the unreduced egg cell does occur, and hybrids with a higher level of ploidy, the so called B_{III} hybrids, are formed (Nogler, 1984).

Endosperm formation is the final component required to produce an apomictic seed. Two types of endosperm formation can be distinguished:

- Autonomous endosperm: The central cell develops parthenogenetically, giving rise to endosperm without fertilisation. Autonomous endosperm development can also be induced by certain experimental conditions. Thus, unfertilised ovules of *Viola odorata L* can be used to induce autonomous endosperm formation *in vitro* by the application of 2,4-D as a sole growth regulator. The induction rate is highly dependent on the genotype of the donor plant. However, no cellularization could be observed in the induced endosperm.
- Pseudogamous endosperm: The endosperm is the results of a single fertilisation of the central cell. In some pseudogamous apomicts the diploid egg develops parthenogenetically independently of central cell fertilisation, however, in other species the fertilisation is necessary to induce the parthenogenetic development of the egg cell.

Normal endosperm development is dependent on the correct relative 2:1 proportion of maternal and paternal genome dosage in the endosperm. Reversed male and female proportions result in seed abortion (Ehlenfeldt and Ortiz, 1995). It has been suggested that the endosperm dosage systems are imprinted within the gametes and the same gene may produce different results when inherited through the male or the female gamets (Lin, 1994). However, in some apomictic species like *Tripsacum* specific dosage effects are not required for normal endosperm development (Grimanelli et al., 1998b).

1.3. Apomixis - hypothetical molecular mechanisms

Genetic studies to determine how many genes are involved in the control of apomixis in various species have proved to be difficult for a number of reasons. Most of apomicts are polyploid. Intra-specific or intra-generic crosses are often required as the pollen recipient must be an obligate sexual plant. Such crosses can lead to sterility and variation in fertility in the segregating progeny Apomicts are also heterozygous plants and a wide range of genotypes and phenotypes are produced following crosses to a sexual plant. Reproduction ratios from the F1 population, backcross and succeeding generations need to be obtained to determine the mode of inheritance. Finally, an embryological assessment of the progeny is also required to confirm the apomictic mechanism (Nogler, 1984).

Despite these difficulties it has been established that apomixis in some plants is determined by a major dominant apomixis locus. However, it is possible that such locus might consist of several closely linked genes, encoding for several elements of apomixis (Van Dijk et al., 2000). The single gene model suggests the presence of a single locus to control apomeiosis. According to this theory parthenogenesis is considered as a pleiotropic effect of circumventing meiotic reduction. The avoidance of meiosis in apomictic plants results in faster egg cell formation. Precocious

oogenesis before anthesis prevents fertilisation and could cause fertilisation independent embryogenesis. The attractiveness of a single-gene theory is that it bypasses the evolutionary problem posed by the two-gene model. It would be difficult to bring together two different mutations causing both the circumvention of meiosis and autonomous embryo development. Each mutation alone would lower the fitness.

The molecular data of aposporic apomixis markers suggest that the Apospory Specific Genome Region (ASGR) is located in a genomic region where recombination is repressed and this region may contain a high number of single dose alleles. The level of hemizygosity that have been observed around the apomixis locus suggests that at least a portion of the chromosomal homolog bearing this locus in *P. squamulatum* has been isolated by the lack of recombination for a considerable period of time. The use of RFLP and RAPD analysis of the progenies of crossing between, *Pennisetum glaucum* as a sexual parent and *Pennisetum squamulatum* as an aposporic parent have been shown to be highly specific for apomictic genotypes (Ozias-Akins et al., 1993 and 1998). In this system, no verifiable recombination among 12 apomixis-linked markers could be detected. Furthermore, the ASGR may be partially conserved in several other species of *Pennisetum* where at least two of the same molecular markers are tightly linked to apospory (Lubbers et al., 1994). In diplosporic *Tripsacum* the closest wild apomictic relative to maize, Grimanelli et al. (1998a) used RFLP to detect the dipolospory specific region in the F1 of crossing between *Zea mays* L. as female parent (2n=20) and *Tripsacum dactyloides* as the male parent (2n= 4x= 72). The data suggest that recombination in the chromosome carrying the apomixis gene(s) is strongly restricted at the tetraploid (apomictic) level as opposed to the diploid (sexual) level in *Tripsacum* and maize. Furthermore, other RFLP loci have been located on the specific chromosomes that are not linked to apomixis. These results

suggest that a restricted level of recombination specifically on the segment controlling apomeiosis can be found.

It is well known that gametophytic apomixis is associated with polyploidy. Very few diploid gametophytic apomicts occur in nature and none of the exceptional cases belong to the grass family (Asker and Jerling, 1992). Apomicts were considered to have arisen as an escape from sterility in polyploids of interspecific hybrid origin. The work of Ellenström et al. (1977) support this model. In this work the Ellenström obtained apomictic allopolyploidy of *Raphanobrassica* resulting from hybridisation between duplicated sexual diploid accessions. It was proposed that apomixis could have arisen after autoploidy of 2X outbreeders to form tetraploids in a process that probably occurred in two consecutive steps: 1) the formation of an intermediate triploid through fertilisation of an 2n unreduced and 2) a second fertilisation of an 3n unreduced gamete (Quarin, 1992). Recently, Galitski et al. (1999) provided definitive evidence for the induction of new gene expression as a result of increasing ploidy (from haploidy to tetraploidy) in yeast.

The recessive theory of apomixis is based on the heredity of apomixis in *Ranunculus auricomus.* In this species a cross between a sexual diploid plant and a tetraploid aposporic plant resulted in a first generation with sexual small size plants, suggesting that the control of apospory was recessive. More crosses clearly demonstrated a monogenic control with dominance for apospory. However, the study on the known meiotic mutants predict that homozygosity for apomixis genes would be lethal and that the ratio of apomixis to wild type genes that would generate a viable apomictic phenotype is most likely to occur in polyploids. The results coming from the crossing system of *Pennisetum glaucum* and *Pennisetum squamulatum* revealed a ratio of the progenies of apomictic to non-apomictic plants of 15:12. This ratio fits to a model of tetrasomic inheritance with linked genetic lethality (Ozias-Akins et.al., 1998). From these results, apomixis genes are

proposed to be recessively lethal and can not be transmitted through haploid gametes. This is a plausible explanation because apomixis is always associated with higher ploidy levels. Dihaploids recovered from apomicts were often apomicts but had low fitness. The crosses between the sexual and apomictic plants indicated the involvement of modifiers in addition to the apomixis locus (Baarlen et al., 1999).

1.4. Model systems to study apomixis

Many of naturally apomictic species were used to study the apomictic process. In addition, the use of *Arabidopsis* mutants as a sexual model provides an alternative possibility to identify genes involved in megasporogenesis and embryo sac development.

1. 4. 1 Natural apomictic model systems

Naturally apomictic plants have been used to develop genetic markers linked to apomixis. The cross between a natural apomictic male parent and their sexual relatives provide heterozygous progenies, including apomictic and sexual plants. Such F1 plants could be used as material for genomic marker techniques.

Diplospory has been studied in *Tripsacum* as an apomictic parent and the closely related maize as a sexual parent as well as in *Taraxacum*, where the diplospory locus seems to be located on a single chromosome (Mogie, 1992). The comparative mapping between maize and *Tripsacum* showed that maize contains an orthologous apomixis locus like in *Tripsacum* (Grimanelli et al., 1998a). Two maize mutants with a diplospory-like phenotype have been isolated and demonstrated to lack meiosis (Curtis and Doyle, 1991; Golubovskaya et al., 1992).

The apomictic *Hieracium* species have special anatomical and genetically characters that make them a favourable model to study apomixis at the molecular level. *Hieracium* exhibits a facultative apospory and

autonomous endospermy (Koltunow, 2000). *Hieracium* is bisexual and the apomixis, sexuality and sterility were scored following decapitation of the anthers and stigmas from the florets in the immature capitulum, thereby preventing self-pollination. The coexisting of sexual and apomictic processes in the *Hieracium* ovule raises questions related to the dependence of two reproductive modes upon each other and their relationship with the surrounding cells of the ovule (Koltunow, 2000). *Hieracium* species are polyploid ranging from triploid to octaploid (Tutin et al., 1976). Apomixis in *Hieracium* was inherited as a monogenic dominant trait, which could also be transferred by both haploid and diploid male gametes (Nogler, 1995: Bicknell et al., 2000). The diploid *H. piloselloides* derived from the triploid origin exhibit defects in ovule development affecting the funiculus and ovule curvature, implying that alternations to the mode of apomixis may have resulted from changes in ovule development (Koltunow, 2000). The natural apomictic models which are used to study the apomixis are summarized in the following table:

Species	Apomeiosis type	Family	Inferred Genotype	Evidence for suppression recombina-tion	Most closely linked molecu lar marker	Reference
Apomeiosis						
Ranunculus auricomus	Apospory	Ranunculaceae	Aaaa	_	_	Nogler, 1984
Panicum maximum	Apospory	Poaceae	Aaaa	_	_	Savidan, 1982
Pennisetum squamulatum	Apospory	Poaceae	Aaaa	Yes	0 cM	Ozias-Akins et al., 1998
Brachiara decumbens	Apospory	Poaceae	Aaaa	?	1.2 cM	Pessino et al., 1998
Paspalum simplex	Apospory	Poaceae	Aaaa	Yes	0 cM	Pupilli et al., 2001
Hieracium piloselloides	Apospory	Compositae	Aaa	_	_	Bicknell et al., 2000
Hieracium aurantiacum	Apospory	Compositae	Aaa	_	_	Bicknell et al., 2000
Tripsacum dactyloides	Diplospory	Poaceae	Aaaa	Yes	0 cM	Grimanelli et al.,1998a, 1998b
Erigeron annuus	Diplospory	Compositae	Aaa	Yes	0 cM	Noyes and Rieseberg, 2000
Taraxacum officinale	Diplospory	Compositae	Aaa	?	4.4 cM	Van Dijik et al., unpublished
Partheno-genesis						
Poa partensis	Diplospory	Poaceae	Pppp	?	6.6 cM	Barcaccia et al., 1998
Erigeron annus	Apospory	Compositae	Ppp	No	7.3 cM	Noyes and Rieseberg, 2000

Table 1.
Inheritance of elements of gametophyte apomixis in natural model plants.

1. 4. 2. Mutants in sexual model species

Other recent attempts to harness apomixis are based on the molecular analysis of mutants that exhibit one or more components of apomixis.

The floral mutant *pistillata* (*pi*) exhibits male sterile phenotype. The mutant has been screened for fertilisation independent seed development (Chaudhury et al., 1997) and three genes have been identified called *fis1*, *fis2* and *fis3*. Similar work of Ohad et al. (1996) found an mutation named *fie* (fertilisation independent endosperm) by screening the male sterile *pop1 Arabidopsis* mutant. Genetic and phenotypic studies suggest that *FIE* and *FIS3* are allelic. On the other hand the *FIS1* gene was found to be allelic to the *MEDEA* gene which has an effective role during maternal embryo development (Grossniklaus et al., 1998). In *fie* (*fis3*) mutant ovules, endosperm develops up to the syncytium stage, but in *fis1* and *fis2* mutant ovules, the endosperm develops further to the multicellular stage. The diploid ovule tissues surrounding the mutant female gametophyte grow and develop into seed coat like structure which is similar to those of wild type plants. Without fertilisation *fie* or *fis3* mutant do not develop an embryo, although *fis1* and *fis2* mutants initiate embryo development in the absence of fertilisation. However, when *fie*/+ or *fis*/+ plants are pollinated with normal pollen to allow normal seed development, half of the seeds carrying the mutant allele have embryo that shows abnormal development, arresting at the heart or torpedo stage. The *FIE*/*FIS3* gene encodes a protein with strong similarity to the subgroup of Polycomb group protein and contains WD repeats. FIS1 is the same like MEDEA, belongs to another subgroup of polycomb group protein which contain a SET domain and a CXC cysteine rich domain. In *Drosophilae* and mammals, polycomb group protein are required for long term repression of homeotic genes. Furthermore, the WD repeat polycomb group proteins are known to physically interact with SET domain polycomb group protein. The FIE and MEDEA proteins may therefore be part of a complex that determine the expression pattern of regulatory genes that control seed development. Another suggestion proposes the interaction between FIE and FIS2. The *FIS2* gene encodes a

zinc finger protein which provides the possibility for interactions with polycomb group protein (Luo, 1999; Yadegari et al., 2000).

The *lec1 Arabidopsis* mutant exhibits a seed maturation deficiency. The isolation and ectopic expression of *LEC1* gene in *Arabidopsis* results in the formation of embryo structures directly from the leaves without any pre-treatment with auxin or any other tissue culture requirement (Lotan et al., 1998). At the RNA level, the ectopic expression of *LEC1* initiates the embryonic programs in the vegetative tissues, such as leaves. The *in situ* hybridisation of the *LEC1* transformed plants demonstrate the presence of embryo specific cruciferin RNA (Harada et al., 1998). However, the *dyad* mutant of *Arabidopsis* is the only mutant arrested in female meiosis at the dyad stage after meiosis I (Wei-Cai Yang et al., 2000)

In *Tripsacum* the *afd1* mutant can not produce an embryo sac and the megaspore mother cell is unable to act as unreduced megaspore. Another mutant, *el1*, results in the production of a variable number of unreduced but fertilised gametes that give rise to triploid embryos. Both mutant loci have been mapped, the *afd1* locus is located on maize chromosome 6L and *el1* is found on chromosome 8L. Both 6L and 8L contain mapped loci linked with diplospory.

1. 4 . 3. The Salmon system of wheat

The Salmon system consists of three isogenic homozygous but alloplasmic lines (Matzk et al, 1995). In the Salmon lines the short arm of chromosome 1B of wheat is substituted by the short arm of the rye chromosome 1R. Plants having this nucleus and the *aestivum*-cytoplasm of wheat are completely sexual. However, the transfer of the Salmon nucleus into the cytoplasm of *Aegilops caudata* (cS) or *Aegilops kotschyi* (kS) results in male sterile plants with the capacity of parthenogenesis. The parthenogenesis capacity of the primary alloplasmic Salmon lines amounted to 20 % (Tsunewaki et al., 1990). The system has been improved to a degree of

parthenogenesis of approximately 90% (Matzk et al., 1995). The lines cS and kS are identical concerning the degree and temporal expression of parthenogenesis. The three lines were screened for gene products that appear in the ovules of both parthenogenesis lines but are lacking in the isogenic sexual line, or *vice versa*, at defined developmental stages before, during and after anthesis. Candidate genes have been observed by 2-D polyacrylamid gel electrophoresis (Matzk et al., 1997) and by subtractive hybridisation and messenger display (Balzer et al., 1996). Candidate genes have been identified, an embryo sac specific α tubulin gene, a gene encoding pollen allergen and a *MCM2* gene. The *MCM2* gene is implicated in DNA replication and might be play a role in the initiation of autonomous embryo development.

The parthenogenetic Salmon lines offer a suitable experimental system to induce apomixis, because one component of apomixis (parthenogenesis) is already present, only the mutational switch to apomeiosis is required to complete apomixis. The amphihaploid plants of the parthenogenetic lines offer a suitable basis for such an approach. There are a lot of nucellus cells with the potential to form female gametes also within the ovules of amphihaploids, however, sporogenesis will be interrupted due to disturbances in chromosome pairing and segregation in meiosis I. Therefore, female gametes can arise only *via* a by-pass of meiosis, for instance by apospory or diplospory. Such egg cells should develop embryos autonomously as a consequence of the parthenogenetic capacity, which is inherent in these lines. There is evidence that also amphihaploid sporophytes are able of autonomous embryo formation if functional embryo sacs are present. That means, an induced apomeiosis may be detected by a screen for embryos by means of the auxin test. Thus, a suitable experimental system as well as an efficient screening method are available.

The application of differential cDNA methods (subtractive hybridisation and differential display) for the parthenogenetic and sexual

line of the Salmon system shows the upregulation of a *MCM2* gene (Bäumlein et al., 1996) in the parthenogenetic gynoecia.

1.4.4. MCM *proteins as DNA replication factors*

MCM genes (minichromosomal maintenance) are a gene family of six members *MCM2-7* which were first identified in the budding yeast and the fission yeast either as genes required for replication of minichromosomes or genes required for the progression of the cell division cycle (Takahashi et al., 1994; Okishio et al., 1996). These proteins are evolutionary conserved from yeasts to human. They are considered as universal replication initiators in eukaryotes. Dahmann et al. (1995) and Diffley (1996) show that members of the MCM proteins family participate as a complex in the recognition and binding to the origin of replication of the chromosome and play a role in DNA unwinding. The activation of MCM proteins seems to be controlled by Cdc7 and Cdc28 protein kinases. These protein kinases ensure that DNA replication occurs only once at a specified time in every cell cycle. A comparison of the amino acid sequences of members of the MCM2-7 protein family suggests that there are several regions of conservation. The largest and most conserved region is a stretch of about 200 amino acids in the central region, which includes an element that is similar to the A motif of the Walker-type nucleoside triphohsphate binding sequence (Koonin, 1993). A potential zinc finger motif of the type $CX_2CX_{19}CX_2C$ is found at the N-terminal conserved region of MCM2. A variation of this motif is found in MCM4, MCM6 and MCM7. MCM3 contains a non-conserved region at the C-terminal end which is similar to the nuclear localisation sequence (NLS) of transcriptional factor SWI5 which regulates the expression of cell-cycle specific genes (Young et al., 1997). Further studies on the NLS of MCM3 indicate that it is essential for the translocation of MCM3 into the nucleus. Similar studies of the *Saccharomyces pombe* MCM2 protein suggest that MCM2 also contains an NLS that is essential for its translocation and

perhaps cotranslocation of other members of the MCM2-7 family (Tye, 1999). The MCMs associate with the chromatin during G1 phase and dissociate from chromatin during S phase (Young et al., 1997; Aparicio et al., 1997). These cyclic association between MCMs and chromatin in G1 cells and their dissociation from S chromatin may suggest their role as an replication licensing factor.

In *Drosophila* embryos *MCM2* seems to follow the normal expression pattern like in the rapidly dividing cells (Treisman et al., 1995). In early embryos the replication is mediated by the maternal *MCM* transcripts, which disappear in the latter stages in embryo development. Later the expression shows difference according to the developing tissues. The *MCM6* expression in the embryo of *Drosophila* shows two submembers, firstly a maternal *MCM6* expressed in early embryo stages and then substituted by a zygotic *MCM6* transcript. In *Arabidopsis*, the *prolifera* mutant was defined by the use of enhancer trap transposon mutagenesis technique. This technique demonstrated the requirement of *PROLIFERA* for both gametophytic and sporophytic development (Springer et al., 1995). The use of the GUS reporter gene which was ligated to the promoter region of the *PROLIFERA* shows expression throughout the early leaf primordia but lost the activity in the distal domain of immature leaves. Staining was observed in root tips, but not in differentiated root tissue. GUS was also expressed throughout young flower buds, but became restricted to the carpels and finally to the ovules (Springer et al., 2000).

The alternation of generation is a complex process that started with the formation of embryo sac and ended by the embryogenesis process which starts directly after the fertilization. The understanding of the regulation mechanism that control embryo sac formation and the parthenogenetic development of the egg cell will help to harness apomixis. The possibility to study genes that control these two process is available by using Salmon wheat system. The isogenic lines of Salmon system exhibit different

developmental process. Parthenogenesis and sexuality present two alloplasmic but isogenic lines. However, the absence of embryo sac in the parthenogenetic haploid plants and embryo sac presence in the same line after conversion to diploid by using colchicines. These genetic similarities will minimize the false results which usually associated with differentially display methods.

2. Material

2.1 Primers and 0ligonucleotides

Oligonucleotides used as primers for sequences

Primer Name	Primer Sequence
M1	5`-CCCAGGACAGTTTCCACA-3`
M2	5`-GGGGTACATTTCAGTCTACC-3`
M3	5`-GGAAGTGATACTTCTGAATGATC-3`
Mcm4	5`-GCCAGCAGGAACAATCCC-3`
M5	5`-GGAGGATGGCGTCAGTTG-3`
M6	5`-GTTTCTGTAATTCTTGTGGA-3`
M7	5`-GCTATACAGTTTGGTATTTGG-3`
M8	5`-GCCACAGTGGTGGAGG-3`
M10	5`-GGGGTCATCGGCTGATG-3`
M110	5`-CTCCTTGACCGTGCTGC-3`
M11	5`-CCGGAAATTCTGCAGACAA-3`
M17	5`-GAGGATGAGAGGAACCTGG-3`
M18	5`-GCCAACCTTATTTTACAACCA-3`
M26	5`-GTGACCCACTCTCGAGTGTTC-3`
M27	5`-CTGACCGTCTCCATTTATCAAAAGAC-3`
M28	5`-GTTGCATGATTAGCATTTAA-3`
M37	5`-ATTAAGGACAACATTTCTTCG-3`
M38	5`-TTATGTGCAATGCTAGTTATACGG-3`
M47	5`-GCATTTGCTGTTGAATCTGTTC-3`
M48	5`-GATAGAGTTGTAGCTGACGAGGAGGATG-3
P2G15	5`-GTTGAAGAGGTCCTCCCCATC-3`

Oligonucleotides used as primers for 5`RACE

R1 primer	5`-CCCTGAATGCCAATCCAAAGGT-3`
A1 primer	5`-AGTATGAGTCGGTTGGGTTAGACG-3`
Smart II primer	5`–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3`
UMP Smart primer	5`–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3`
NUP Smart primer	5`–AAGCAGTGGTATCAACGCAGAGT–3`

Oligonucleotides used as primers to isolate the MCM2 gene of Saccharomyces cerevisiae

Yeast/Bam primer	5`TGGATCCATGTCTGATAATAGAAGACGTAGACGTGAG
Yeast/Xho primer	5`CTCGAGTTAGTGACCCAAGGTATAAATTGCGAAAGAC

Oligonucleotides used as primers to isolate wheat MCM2 cDNA

MCM2-5FUS	5`-GGGGTACCCCTCAATGATTACCGAAGAATGG-3`
MCM2-3FUS	5`-GCTCTAAGACCGCAACTGGATGCCTGATGATC-3`

Oligonucleotides used as primers to amplify full length wheat MCM2 cDNA

W/Xho primer	5`-CCCTCGAGACACGCGTCCTCCACAG-3`
W/ECOR primer	5`-GGAATTCTGAGGTCGATTGAGTTGTTATG-3`

Oligonucleotides used as an adapter for Srf1 restriction site

Bgl/Srf	5`-GATCTGCCCGGGCG-3`
Srf/Nhe	5`-CTAGCGCCCGGGCA-3`

Oligonucleotides used as primers to confirm plant transformation

EGpromo	5`-ACGTGGACGGTGGAGATTTTT-3`
EGPRO/FM2	5`-TCCAGGTCGAGCACAGTCAA-3`
EGPRO/Lec	5`-GAAGACGAAGAGCCACCACCAACA-3`
EGPRO/WM2	5`-TGGACCTTTGGATTGGCATTCA-3`
EGPRO/ ZM6	5`-AGTTGCCTGTATTCCTGCTTTGGTA-3`

Oligonucleotides used as primers for cDNA subtraction

Adapter 1

```
5`- CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3`
                                        3`-GGCCCGTCCA-5`
```

Adapter 2

```
5`-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3`
                                    3`-GCCGGCTCCA-5`
```

PCR primer 1 5`-CTAATACGACTCACTATAGGGC-3`

Nested PCR primer1 5`-TCGAGCGGCCGCCCGGGCAGGT-3`

Nested PCR primer2 5`- AGCGTGGTCGCGGCCGAGGT-3`

2.2. Enzymes and kits

Amersham Buchler, Braunshweig	Megaprime DNA labelling kit
Biolabs, Beverly Ma, USA	restriction endonucleases, T4-DNA ligase, alkaline phosphatase, klenow enzyme
Boehringer/Mannheim GmbH	restriction endonuclease, T4 ligase, alkaline phosphatase, Klenow enzyme, Taq DNA polymerase
Clontech Laboratories, CA, USA	smart PCR kit, YEX press yeast expression system
Gibco-BRL, Gaithersburg MD, USA	5`-RACE system for rapid amplification of cDNA ends, Superscript II RNase H$^-$ Reverse Transcriptase

Invitrogen, Leek, Holland	thiofusion system, TA cloning kit (PCR II 2.1), pYES-2 yeast expression system
Promega, MadisonWI, USA	pGEM-T cloning kit
Qiagen, Hilden	QIAEX gel extraction kit, Qiagen plasmid isolation kit, lambda midi kit
Stratagene, Heidelberg	restriction endonuclease, pfu polymerase
USB, Cleveland OH, USA	restriction endonuclease, Klenow enzyme, T4 polynucleotide kinase
Takara Shuzo Co., Ltd.	SUPREC^{TM}-02 column

2.3. Chemicals

Aldrich,	Sodium carbonate, Sodium bicarbonate, Sodium chloride, $Tween_{20,}$ Potasium chloride, Sodium monophosphate, Sodium diphosphate, diethylamine.
Amersham Buchler, Braunschweig	[α^{32}P] dATP, [α^{32}P] dCTP, nylon-membrane (Hybond-N$^{+)}$
Biometra, Göttingen	chloroform, phenol, phenol-chloroform, ATP, BSA, dNTPs, SDS
Difco, Detroit MI, USA	bacto®-agar, bacto®-trypton, yeast extract
Duchefa, Brüssel, Belgium	Murashige-Skoog whole medium solid substance, rifampicin, kanamycin, hygromycin, carbenicillin
Fluka, Buchs (Schweiz)	DEPC
Gibco-BRL, Gaithersburg MD, USA	agarose, 1Kb DNA ladder, DNA molecular weight ladder, EDTA
Invitrogen, Leek, Holland	RM basis media, induction basis medium
Kodak, Rochester NY, USA	X-Ray film, diapositive films
Merck, Darmstadt	ethanol, ethidium bromide, formamide, HEPES, Magnesium cloride, Sodium acetate, Sodium hydroxide, Sodium-dihydrogen phosphate, di-Sodium hydrogenphosphate, trichloroacetate, Tris.
Metabion,	DNA oligonucleotides
MWG-Biotech	DNA oligonucleotides
Roth, Karlsruhe	formaldehyde, glycerol, isopropanol, Lithium chloride, Sodium chloride
Schleicher&Schuell, Dassel	blotting papper GB 002, nitrocellulose membrane BA 85
Serva, Heidelberg	X-Gal, Sodium citrate, Tween20, tetracyclin, coomassie blue G. Ponceau

	stain
Sigma, louis MO, USA	gelatin, IPTG, mineral oil, maltose, MOPS
Molecular Probes, Eugene, Oregon, USA.	propidium iodide

AGS, Heidelberg	DNA gel-electrophoresis tanks
Appligene, Illkirch, France	Slab Gel dryer
BioRad, München	Gene-Pulser
Biotec Fischer, Reiskirchen	phero-stab. 200 electrophoresis power supply
Du Pont, Bad Homburg	sorvall centrifuge RC 5C
Eppendorf, Hamburg	mastercycler®5330 (DNA- thermocycler), thermomixer 5436 and 5437, cold centrifuge 5402
Perkin Elmer	Thermocycler, 9700.
GFL, Burgwedel	hybridisation oven, water bath
Heraeus, Osterode	Shaker(Vortex Genie 2™), centrifuge (Biofuge 13)
Hofer, San Francisco CA., USA	transfer electrophoresis unit
Pharmacia, Freiburg	photometer, ultrospec plus
Polaroid, Offenbach	MP-4 camera
Raytest, Straubenhardt	FUJI BAS imager, imaging plates
Stratagene, Heidelberg	UV-Stratalinker® 1800, NucTrap® probe purification columns
Zeiss, Jena	stereo microscope stemi 200C
Zeiss, Oberkochen	axiophot, axioscope, microscope photometer MPM-200, filter block I(#48791, UV-H365)

2.4. Media

LB 10 g NaCl, 5 g Tryptone, 5 g yeast extract for 1 L (pH 7.4)

M9 salt 6 g Na_2HPO_4 (anhydrous), 3 g KH_2PO_4, 0.5 NaCl, 1 g NH_4Cl

NZY 5 g NaCl, 2 g $MgSO_4x7H_2O$, 10 g Caseine-Hydrolysate, 5 g yeast extract for 1 L (pH 7.4)

SOC 0.58 g NaCl, 0.186 g KCl, 20 g tryptone, 5 g yeast extract, 2 mL of 2 M glucose (add after autoclaving) for 1 L (pH 7.4)

TBY 5 g NaCl, 5 g $MgSO_4x7H_2O$, 10g tryptone, 5 g yeast extract for 1 L (pH 7.4)

YEB 0.5 g $MgSO_4x7H_2O$, 5 g beef extract, 5 g peptone, 5 g saccharose, 1 g yeast extract for 1 L (pH 7.0)

YPD 20 g peptone, 10 g yeast extract for 1 L

SD-minimal media:

100 ml	20% Glucose
25 ml	40x YNB Medium
10 ml	Vitamin mix
2 ml	Histidine 1 gm/100 ml
2 ml	Tryptophane 1gm/100 ml
3 ml	Leucine1gm/100 ml
10 ml	Uracil 200 mg/100 ml
10 ml	$(NH_4)_2SO_4$
Complete the volume to 1 liter with H_2O	

Vitamin mix	
40 µg	Ca-D pantothenat
40 µg	thiaminiumdichlorid
10 µg	nicotinacid
40 µg	pyridoxine-HCl
0.4 µg	biotin
400 µg	myo-inositol
Complete the volume to 100 ml with H_2O	

All solid media containing 1.5 % Difco-agar

Plant growth media

GM	0.5 x MS salts, 1x MS vitamins (Murashige and Skoog, 1962), 0.1% MES, 1% sucrose (pH 5.7)

2.5. Bacterial strains : E. coli strains

DH5α	RecA1, endA1, gyrA96, thi-1, hsdR17, (r_K-m_{K+}), relA1, supE44, u80ΔlacZΔM15, Tn10, $(Tet)^r$, (Sambrook et al.,1989)
XL1 Blue	RecA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F'proAB$lacI^qZ$ΔM15, Tn10, $(tet^r)]^c$, (Bullock et al. 1987)
LE391	SupE44, nsupF58, hsdR514, galK2, galT22, metB1, trpR55, lacY1, (Sambrook et al., 1989)
G1698	F^-, λ^-, $lacI^q$, lacPL8, ampC ::P_{trp} cI (Invitrogen)
HMS174(DE3)	F^-, recA, hsdR ($rk_{12}^-mk_{12}^+$) Rif^R(DE3)

2.6. S. cerevisiae strains

Saccharomyces cerevisiae (Bröker strain)	Cl3 ABYS86
Saccharomyces cerevisiae mcm2 mutant	8534MZ MATα, leu 2-3,112,ura3-52, his4-Δ34, mcm2-1,ts

2.7. Software

DNA and protein sequence data were processed using the programs "DNA Star"

3. Methods

3.1. Plant growth

Arabidopsis plants were grown in a greenhouse at a temperature of 20-22 °C or in a growth room at 22°C with 6000 lux of white light for 16 hours. The plants grow at this conditions until the end of maturation (~22 days after pollination). The collected seeds were either sterilised and plated on a selective media or analysed directly by flow cytometry.

3.2. Plant transformation of Arabidopsis thaliana by vacuum infiltration

Transformation of *Arabidopsis* was performed based on the protocol of Bechtold et al. (1993). Plants of *Arabidopsis thaliana* (ecotype Columbia) were grown for three weeks under short day conditions (8 hours light, 16 hours dark) and transferred to long day (16 hours light, 8 hours dark). After three weeks the emerging bolts were cut to induce growth of multiple secondary bolts. Vacuum infiltration of plants with *A. tumefacience* culture was done one week after clipping. Bacteria were grown till $OD_{600} > 2.0$, harvested by centrifugation and resuspended in three volumes of infiltration medium (OD_{600} approx. 0.8). Entire shoots of the plants were submerged into the *A. tumefacience* suspension in a beaker. Vacuum was applied by an oil pump for 5 min and then rapidly released. Plants were removed from the beaker, placed on their side and kept at high humidity under plastic warp for 24 hours, after that they were uncovered and set upright. Seeds were harvested from the siliques, sterilised by Na-hypochloride as described before and plated onto GM selection plates containing 50 mg/ l hygromycin. After two weeks hygromycin resistant plants were transferred to soil, grown up and their seeds were collected. Stable transformation and expression of the constructs were analysed by PCR.

3.3. Basic cloning methods and sequencing

The standard molecular cloning methods (restriction digestion, ligation, DNA and protein gel electrophoresis) were performed according to (Sambrook et al., 1989). The transformation of *E. coli* was performed using the heat–shock procedure (Cohen et al., 1972). Plasmid DNA extraction and purification was done using the Qiagene plasmid kit or according to the fast preparation method by Holmes and Quingly (1981). For isolation of phage DNA the lambda Maxi kit (Qiagene) was used. PCR products were cloned using the pGEM®-T vector system (Promega). DNA fragments were isolated and purified from the agarose gel with the QIAEX Gel Extraction kit (Qiagene). DNA sequence were determined in the Institute für Pflanzengenetik und Kulturpflanzenforschung Gatersleben by dideoxynucleotide chain termination method (Sanger et al., 1977). DNA was detected using Fluorescence-labelled Primers by the A. L. F. Sequencer (Pharmacia LKB) and the Autoread Sequencing kit (Pharmacia). Sequences of longer DNA fragments were determined by primer walking.

3.4. Extraction of plant genomic DNA

The rapid plant DNA extraction was carried out according to Edwards et al., (1991). The leaf tissue (~100 mg) was grinded in liquid nitrogen and then 400 µl of extraction buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 125 mM EDTA, 0.5% SDS) was added and the mixture was shaken for 1 min. The leaf suspension was centrifuged for 5 min at full speed and the supernatant transferred to a new tube containing 300 µl of isopropanol. The DNA was collected by centrifugation for 10 min and washed twice with 70% ethanol and resuspended in 100 µl of H_2O.

3.5. RNA preparation and 5`RACE

Total RNA from wheat gynoecia from the diploid line of the Salmon system was extracted according to the RNA easy kit (Qiagene). Poly (A) RNA was isolated from total RNA using Dynabeads mRNA purification kit (Dynal). The synthesis of cDNA was carried out using the oligo(dT)-primer and superscript reverse transcriptase (GibcoBRL). The purification of single stranded cDNA and the addition of the homopolymeric tail (poly C) to the 3`end of the cDNA was done using the 5`RACE kit (GibcoBRL). PCR amplification was done using the 5`RACE Abridged primer and gene-specific oligonucleotide (Comp primer) present at a distance of 1003 bp from the 5´ end of the incomplete cDNA. The cycling conditions were as follow: denaturation (3 min, 94°C) followed by 35 cycles of denaturation (30 sec, 94°C), annealing (1min, 60°C) and primer extention (1,5 min, 72°C). The PCR products were cloned using the pGEM-T PCR cloning kit (Promega) and checked by sequencing.

The full length cDNA was obtained by digestion of incomplete cDNA with Van91I enzyme. This enzyme restricts the incomplete cDNA in a unique site at 351 bp and digest the 5`RACE product at 502 bp. The two Van91I digested product were ligated to produce the complete cDNA.

3.6. Southern blot hybridisations

Plasmid DNA was prepared using the mini or maxi plasmid isolation kit (Qiagen). For Southern hybridisation DNA was digested with restriction enzymes, separated on a 1% agarose gel in Tris –acetate buffer (Sambrook et al., 1989) and transferred onto a Hybond N^+ membrane using alkali capillary blots (0.4 M NaOH) for 16 hrs. Hybridisation buffer (0.9M NaCl, 50 mM Sodium phosphate, 5 mM EDTA, 0.05% BSA, 0.05% Ficoll, 0.05% PVP,

0.5% SDS) the salmon sperm DNA was added to a final conc. 0.1µg/mL. The filter was prehybridised for 5 hr at 65°C. After the prehybridisation solution was removed, the radioactive probe added to a new hybridisation buffer and incubated for 16 hr at 65 °C according to the procedure described by Sambrook et al. (1989).

Filters were washed twice at 65 °C with 2x SSPE (0.36 M NaCl, 20 mM Sodium phosphate, 2 mM EDTA) with 0.1% SDS and twice with 1x SSPE (0.18 M NaCl, 10 mM Sodium phosphate, 1 mM EDTA) with 0.1% SDS and twice with 0.1x SSPE (18 mM NaCl, 1 mM Sodium phosphate, 0.1 mM EDTA) with 0.1% SDS for 15 min each time. Signals were detected and quantified with a Bio-Imaging analyser BSA2000 (Fuji Photo Film Co. Ltd).

3.7. Western blot analysis

Soluble proteins were separated in a denaturing 8% (w/v) SDS-polyacrylamide gel. After electrophoresis the proteins were transferred in transfer buffer (25 mM Tris, 0.25 M glycin pH 8.3, 20% methanol) to the nitrocellulose membrane (Schleicher and Schuell) at 1.3 mA/cm^2 for 16 hrs. The membrane was blocked using 5% BSA in TBST buffer (10 mM Tris pH 8, 150 mM NaCl, 0.1% Tween$_{20}$) for 2 hrs at room temperature or over night at +4 °C. The monoclonal antibody diluted in TBST and 1% BSA buffer with the dilution recommended by the manufacture, in case of polyclonal Anti-MCM2 antibodies the dilution used was 1:1000. The filters were incubated with the antibodies at a dilution (1:2000) for 2 hrs at room temperature and then washed three times for 15 min each with TBST (in the case of polyclonal serum, washing buffer was TBS and 1% Triton$_{100}$). The secondary antibody (Anti-mouse or Anti-rabbit POD-conjugate, Amersham) was added to the filter in TBST buffer (the dilution was adjusted according to the manufacture) and incubated for 1 hr. After washing with TBST 4 times for 20 min each, the chemoluminescens of the detected antigen was developed with the ECL-System (Amersham).

3.8. Determination of specificity of polyclonal antibodies using ELISA

The following amount of purified wheat MCM2 protein 0.5, 1. 5, 10, 20, 40, 80 µg were diluted in 100µl of coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$) and the pH was adjusted to 9.6 using NaOH or $NaHCO_3$. The total volume was loaded in the ELISA microtiter plate and incubated at 37 °C for 4 hr. The wells were washed 3 times with 125 µl of PBS-Tween (173 mM NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% $Tween_{20}$, pH 7.4). The polyclonal antibodies were diluted 1:25 and 1:50 in PBS-Tween containing 0.5% BSA. 50 µl of the diluted antibodies was added to the well. The wells were washed 3 times with PBS-Tween and 50 µl of antirabbit IgG alkaline phosphatase (Boehringer) with dilution 1:100 was added to each well. The plate was incubated for 2 hr at 37°C and then the substrate was added. The 4-Nitrophenylphosphate was diluted as 1mg/1ml of substrate buffer (10% diethanolamin, 1mM $MgCl_2x6H_2O$) and 100 µl of the diluted substrate was added to each well and the plate was incubated at room temperature for 20 min. The reaction was stopped using 50 µl of 3N NaOH and the OD was measured at 405 nm.

3.9. Bacterial expression of fusion protein

For the synthesis of the wheat MCM2 protein in *E. coli*, two expression systems were used. The thiofusion expression system (Invitrogen) and pET expression system (Novagen).

In the thiofusion system the incomplete cDNA (2635 bp) encoding an about 99 KD protein was amplified using *Pfu* polymerase (Stratagene) and the primer Oligonucleotides MCM2-5Fus and MCM2-3Fus. Since those primers contain the restriction sites for the KpnI and XbaI, the amplified fragment was cloned unidirectionally into the pTrxFus expression vector (Invitrogen). The in frame fusion with the thioredoxin gene sequence was confirmed by partial sequencing.

The synthesis of the fusion protein in the *E. coli* strain GI698 was induced by the addition of tryptophane (final concentration 100 mg/ml) to the bacterial culture grown at OD~ 0.4 at an incubation temperature of 25 °C in the expression medium (1x M9 salts, 0.2% casaminoacids, 0.5% glycerol, 1mM $MgCl_2$, 100 μg/ml ampicillin). In the pET expression system the Asp718/Sac1 cDNA was isolated from the pGEM-T and cloned to Asp718/Sac1 digested pET-17b (Novagen). The in frame fusion with the T7 tag sequence of the expression vector was confirmed by partial sequencing. The synthesis of the wheat MCM2 in *E. coli* strain HSM174 was induced by the addition of IPTG (final concentration 0.4 mM) at the early log phase of the bacterial culture (optical density OD_{550}~0.4) which was incubated at 30 °C in the expression medium (LB medium).n both expression systems used, the expressed protein is mainly found as inclusion bodies.

3.10. Purification and solubilisation of inclusion bodies

The induced cells were harvested by centrifugation and resuspended with TE buffer (20 mM tris pH 8, 2 mM EDTA, 200 μg/ml lysozyme, 10 mM $CaCl_2$). The suspension was incubated at 30 min at 37 °C and then frozen and thawed three times with sonication. The suspension was centrifuged at 13000 rpm for 10 min and the precipitate washed twice with washing buffer (1% Triton, 1% Na-deoxycholate, 0.01%SDS). The partially purified inclusion bodies were solubilised in solubilisation buffer (5 M Guanidium Hydrochloride, 10 mM CAPS pH 9). The soluble product was centrifuged at 13000 rpm for 10 min and the supernatant was dialysed against gradual concentrations of GuHCl and finally dialysed against CAPS buffer.

3.11. Isolation of the 99 KD antigen protein

The dialysed protein was centrifuged at 13000 rpm to separate the refolded protein (supernatant) from the insoluble contaminants (denatured

protein). 700 µg soluble protein was mixed with protein sample buffer (70 mM Tris pH 6.8, 0.5 % SDS, 0.002% Bromophenol blue, 6% Glycerol) and separated on the SDS-polyacrylamide gel. After electrophoresis the protein were transferred in transfer buffer (25 mM Tris, 0.25 M glycin pH 8.3, 20% methanol) to the nitrocellulose membrane (Schleicher and Schuell) at 1.3 mA/cm^2 for 16 hrs. The blotted membrane was stained with Ponceau S for 5 min, the dominant 99 KD band was cut. The elution of the purified protein was done by incubating the cut membrane with 0.5 ml of elution buffer (1% Triton X-100 and 50 mM CAPS buffer, pH 9.5). The amount of the purified protein ranged from 80 to 100 µg which was directly used to inject the rabbit.

3.12. Production of polyclonal antibodies against wheat MCM2

The immunized rabbit was injected with 80 µg of purified protein for four times with an interval of 1 month in between. After two weeks from the 3^{rd} injection a serum sample was taken to check the polyclonal specificity. Two weeks after the 4^{th} injection the animal was killed and its blood was collected. The polyclonal serum was obtained by centrifugation of the collected blood and the polyclonal IgG fraction was isolated using protein A sepharose (Amersham Pharmacia). About 1 ml of drained gel was resuspended in 50 ml PBS buffer pH 7.0 and it was degassed for 10 min. the gel slurry was packed in C10/10 column (Amersham Pharmacia) and the gel washed 30 ml of PBS buffer. After washing 3 mg of serum protein was added to the surface of the column and allowed to penetrate through the column by adding some buffer then the column was stood 30 min at room temperature to complete the binding complex between IgG and protein A. After binding time, the column was washed with PBS until the outlet buffer has no protein content, as measured with OD at 280 nm. The elution of IgG was done using 0.1 M of glycine buffer pH 3.0 and the out let was collected in 3 ml collection tubes and the pH was immediately neutralised using a

saturated solution of KOH. The IgG protein was precipitated at 50% ammonium sulphate and the protein was collected by centrifugation. The protein pellet was reconstituted in PBS pH 7.0 and dialysed in the PBS buffer then the protein content was measured and the polyclonal IgG was dispensed at stored at –20 °C.

3.13. Protein extraction from yeast

The Bröker yeast strain (strain of *S. cerevisiae* used for protein expression) or mcm2 mutant strain were transferred with plant MCM2 genes in addition to yeast *MCM2* gene using the YEX-BX vector from CLONTECH. The yeast culture was grown at 28°C on SD-media containing His, Trp and Leu amino acids until OD~ 0.5. The induction of gene expression was started by adding $CuSO_4$ to a final concentration of 0.5 mM. 10 ml of yeast culture were taken before the induction and then every 2 hrs after the induction. The samples were centrifuged and the supernatant were discarded. The pellet was frozen at –80 °C until use. The pellets were grounded in liquid nitrogen and then 0.5 ml of lysis buffer (50 mM Hepes, pH 7.9, 10 mM EDTA 0.5 mM EGTA, 0.5 mM DTT, 1% Triton, 1 mM PMSF, 1 mM dimethylbenzamidin) was added. The homogenised samples were centrifuged and the supernatant tested for the protein content using the method described by Bradford (1977). The protein content for each sample was adjusted to be 1µg/µl in total volume 200 µl . For western blot 25 µg of each sample were fractionated by 8% SDS-PAGE.

3.14. Transformation of Agrobacterium tumefacience

The competent cells of *Agrobacterium tumefacience* (pGV 2260) were prepared using the $CaCl_2$ method. The *Agrobacterium* strain was grown in 50 ml of YEP media at 28 °C until OD_{600}~ 0.5-1.0. The cells were centrifuged at 3000 rpm for 5 min and resuspended in 1 ml of 20 mM of $CaCl_2$. The resuspended cells were aliquot to 100 µl volume. The

transformation with plant expression vectors was done using the thawing-freezing method as described by Höfgen and Willmitzer (1988).

3.15. Total DNA miniprep from Agrobacterium tumefacience

The bacteria were grown in 5 ml YEP at 28 °C for 18 hours and the pellets were collected by centrifugation in a 1.5 ml tube. After suspension in 300 µl of suspension buffer (20 mM EDTA, 50 mM Tris pH 8), 100 µl of 5% sarkosyl in TE and 2.5 mg/ml proteinase K in TE were added. The mixture was incubated at 37 °C for 2 hours, followed by two extractions with phenol, one with phenol-chloroform (1:1) and finally twice with chloroform. The supernatants were precipitated by adding 0.3 M NaCl and two volumes of ethanol, the DNA pellets were washed with 70 % ethanol and resuspended in 50µl TE buffer.

3.16. Yeast cell transformation

The competent yeast cell were prepared using the method of Dohmen et al. (1991). The fresh yeast colony was grown in 5 ml of YPD media at 30 °C with 150 rpm shaking. After 18 hours the culture was transferred to fresh 50 ml media and left to grow until OD~0.7. The cells were centrifuged at 5000 rpm for 5 min and washed with 0.5 volume of washing buffer (1M Sorbitol, 3% Ethylenglycol, 5% DMSO, 10 mM Bicine-NaOH pH 8,3). The cells were resuspended in 0.02 of the started volume in the same buffer and aliquot in 200 µl and kept at –70 °C. For transformation, about 5 µg of the plasmid DNA was added to the frozen competent cells and the cells were incubated at 37 °C for 5 min with shaking. The cells were diluted with 1.4 ml of incubation buffer (40% PEG, 0.2 M Bicine-NaOH, pH 8,3) and incubated at 30 °C for 1 hour. After centrifugation at 4000 rpm, the cells were washed with 1.6 ml of washing buffer (0.15 M NaCl, 10 mM Bicine-NaOH, pH 8.3). The transformed cells were resuspended in the same buffer and plated on the selective SD-agar plates.

3.17. Detection of GUS activity

The whole plant or detached organs were submersed in GUS staining solution (0.3 M mannitol, 50 mM PBS (pH 6.5), 2.3 g/l Murashige and Skoog salts, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM ferrocyanide, 2 mM 5-bromo-4-chloro-3 indolyl glucuronide) with a brief vacuum infiltration, and incubated for 12hr at 37 °C. To remove pigments and for fixation the tissues were washed using increased ethanol concentrations started fro 30% to 70%. A Zeiss stereomicroscope was used for documentation.

3.18. Flow cytometry assay

In yeast experiments, the mutant *mcm2*-yeast strain was transformed with plant *MCM2* genes in addition to empty expression vector as a control. The picked colony was grown in 10 ml of minimal SD- His, Trp, Leu media for 24 hours at 28°C. The cells were centrifuged, washed with H_2O and used to inoculate 50 ml volume of fresh minimal media. The cells were left to grow at 28 °C until OD_{600} ~2.0 then they were centrifuged and washed once with H_2O. The cells suspension was used to inoculate culture that will be grown at 30 °C (permissive temperature) or 37°C (restricted temperature) with or without $CuSO_4$ (final concentration 0.5 mM) starting from OD_{600}~0.5. A 1ml sample was taken at 6 hours, 12 hours, and 24 hours. The cells were centrifuged and suspended in 0.3 ml of cooled water and then – 20 °C ethanol was added to get 70% concentration. The cells were mixed carefully and left in the fixing solution at least 5 hr to over night at +4°C then they were centrifuged and the pellet resuspended in 1 ml PBS saline for 1–2 hr in the presence of 20 µl of RNase . 50 µl of Propidium iodide (1mg/ml) add to 1ml suspension. Before measuring the samples were diluted 1:50 in PBS buffer and used to measure the DNA content.

To determine the DNA contents in the *Arabidopsis* seeds, about 50

seeds were crashed by sand paper and suspended in 1 ml of PBS buffer. The suspensions were stained with 2 µg/ml DAPI in PBS buffer for 15 min. The extracts were filtrated (with 30 µm falcon tube) and stored on ice until measurement. The analyses were done with a FACStar^{PLUS} flow cytometry (Becton Dikinson, San Jose, CA, USA) equipped with argon-laser INNOVA 90-5 (Coherent, Palo Alto, CA, USA) using analyses programme CellQuest.PI fluorescence was excited with 500 mW at 514 nm and measured an the FL 1-channel using a 630 band-pass filter.

3.19. cDNA subtractive hybridisation

The total RNA was isolated from gynoecia of the haploid line of wheat salmon system (driver) and from the isogenic diploid line (tester) using the RNA miniprep kit (Qiagen). 50 µg of total RNA was used to isolate polyA mRNA using the Dynabead mRNA purification kit (Dynal). The synthesis of cDNA was carried out in 25 µl volume using the oligo(dT)-primer and superscript reverse transcriptase (GibcoBRL). The second strand cDNA was synthesised using a mixtures of 0.8 µl of RNase H 1u/µl and 1.2 µl of DNA polymerase (both from Displaysystems), 7.5 µl of polymerase buffer and 2,5 µl of dNTP Mix (5 mM each) in 50 µl volume. The mixture was added to the 25 µl first strand reaction and incubated at 16°C for 2 hours. The synthesised DNA was extracted with phenol/chloroform and precipitated and washed with ethanol. The DNA was digested with Taq I restriction enzyme and the resulting DNA was ligated to the Taq I adapter primers. The Taq I fragments were amplified using *pfu* polymerase and universe primers provided by the kit. The amplified Taq I fragments of both tester and driver were purified using SUPREC^{TM}-02 column (Takara) and the DNA was redissolved in 5.5 µl of H_2O. 1µl of amplified Taq I fragments of tester were diluted 5 times with H_2O ligated to the subtractive adapter 1. Another 1µl from the tester was diluted and ligated to adapter 2, the final ligation volume was 10 µl . the first round of hybridisation has been

done by incubating the 1.5 µl of each adapter ligated tester separately with 1.5 µl of unligated and undiluted Taq I amplified fragment of driver and hybridisation buffer was (20 mM HEPES, pH 7.3, 5 mM EDTA, 0.75 M NaCl). The tubes were prehybridised at 98°C for 1.5 min and incubated at 68°C for 8 hours. The second round of hybridisation was carried out by the addition of 1µl of amplified driver, 1µl of hybridisation buffer and 2µl of H_2O. The hybridisation mixture was incubated at 98°C for 1.5 min. Both tubes from the first hybridisation cycle were collected and mixed immediately with the freshly denatured driver. The second hybridisation mix incubated at 68 °C for 16 hours. The hybridisation mix diluted with 200 µl of dilution buffer (20 mM HEPES-HCl pH 8.3,50 mM NaCl, 0.2 mM EDTA pH 8), heated at 68 °C for 7 min and stored at –20°C. the amplification reaction of the hybridised mixture was carried out using PCR primer 1 amplifies only the subtracted fragments. The PCR product was used for a second PCR reaction using the nested primer to confirm the first amplification. The amplified DNA was cloned using the PCR cloning vector PCR-2 (Invitrogen).

4. Results

4.1. A MCM2 gene with preferential expression in the parthenogenetic wheat line

Subtractive hybridisation was used to isolate genes which are differentially expressed in the parthenogenetic gynoecia compared to the sexual gynoecia of the wheat `Salmon` system. After the subtractive procedure, the fragments were cloned and sequenced. One partial cDNA sequence was recognized as part of a MCM2 gene which is present in all eukaryotes and plays a role in cell cycle regulation. The complete sequence was isolated using both 3` RACE and 5` RACE kits. The obtained sequence information was used to design specific primers: MCM2-5Fus (with an Asp718 cloning site) at the 5`end and MCM2-3Fus (with a XbaI cloning site) at the 3`end. The 2.7 kb of TaMCM2 cDNA was amplified using the gyneocial cDNA library of parthenogenetic plants. The amplified fragment was extracted from the gel and cloned in the pGEM-T cloning vector. The differential expression of MCM2 protein was confirmed by Western blot of protein samples which were extracted from gynoecia of sexual and parthenogenetic wheat lines. This work was done in cooperation with Dr. J. Balzer. As shown in (Fig. 1), the TaMCM2 protein can be detected in the parthenogenetic and sexual lines before and after anthesis. However, the wheat MCM2 (TaMCM2) protein is differentially detectable in the parthenogenetic wheat line during anthesis and absent from the sexual line at this stage.

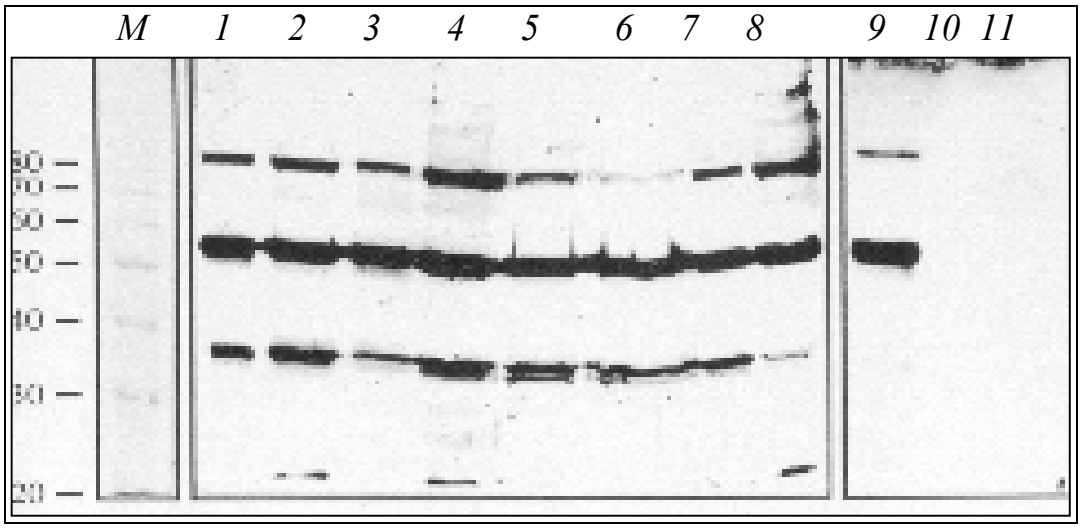


Figure 1

Western blot with polyclonal AB against yeast MCM2 and monoclonal AB against alpha-tubulin.

M- 10 kD ladder	6- aS, gynoecia, Anthesis
1- cS, gynoecia, 1dbA	7- aS, gynoecia, 20haf
2- cS, gynoecia, Anthesis	8- aS, gynoecia, 3daA
3- cS, gynoecia, 1daA	9- cS, gynoecia, 1daA
4- cS, gynoecia, 3daA	10- aS, first leaf, meiosis I
5- aS, gynoecia, 1dbA	11- aS, first leaf, after Anthesis

cS, the parthenogenetic line of the wheat `Salmon` system (caudata Salmon), aS, the sexual line of wheat `Salmon`; daA, day after anthesis; dbA day before anthesis.

4.2. The genomic structure of theTaMCM2 gene

The analysis of the precise genomic structure of the *MCM*2 gene was a prerequisite for the determination of the exon-intron structure and for the localisation of the transcriptional start site of the gene. The screening of the wheat genomic library was performed using a partial clone sequence of *TaMCM2* cDNA as a radioactive probe. The screening resulted in two positive plaques. These phages were picked. Phage DNA was isolated using the maxi phage isolation kit from Qiagen. The phage DNA was digested with SalI and the digested product was fractionated on a gel. The digestion pattern showed an upper band with a molecular weight of more than 10 kb representing the phage arms. In addition, four

more SalI DNA fragments could be detected. All the genomic fragments were gel purified, cloned into the SalI site of the pUC18 vector and sequenced using reverse and universe plasmid primers. The largest genomic fragment was about 6 kb and its sequence showed similarity with the sequence of the isolated cDNA. The comparison between the cDNA sequence and the complete sequence of the 6 kb genomic fragment allowed the determination of the exact exon-intron structure of the gene. However, the extended upstream region of the 6 kb genomic sequence is only 761 bp in length and most likely to short to comprise the complete promoter sequence. The P2G15 primer located 84 bp from the 5`end of the 6 kb fragment was used for the direct sequencing of the upstream region using the phage DNA (Fig. 2). By comparing the sequence of the phage DNA to the sequence of the other three genomic fragments, it was found that the 2.4 kb SalI fragment is positioned in front of the 6 kb fragment. The complete exon-intron structure of the *TaMCM2* gene is given as appendix 1.

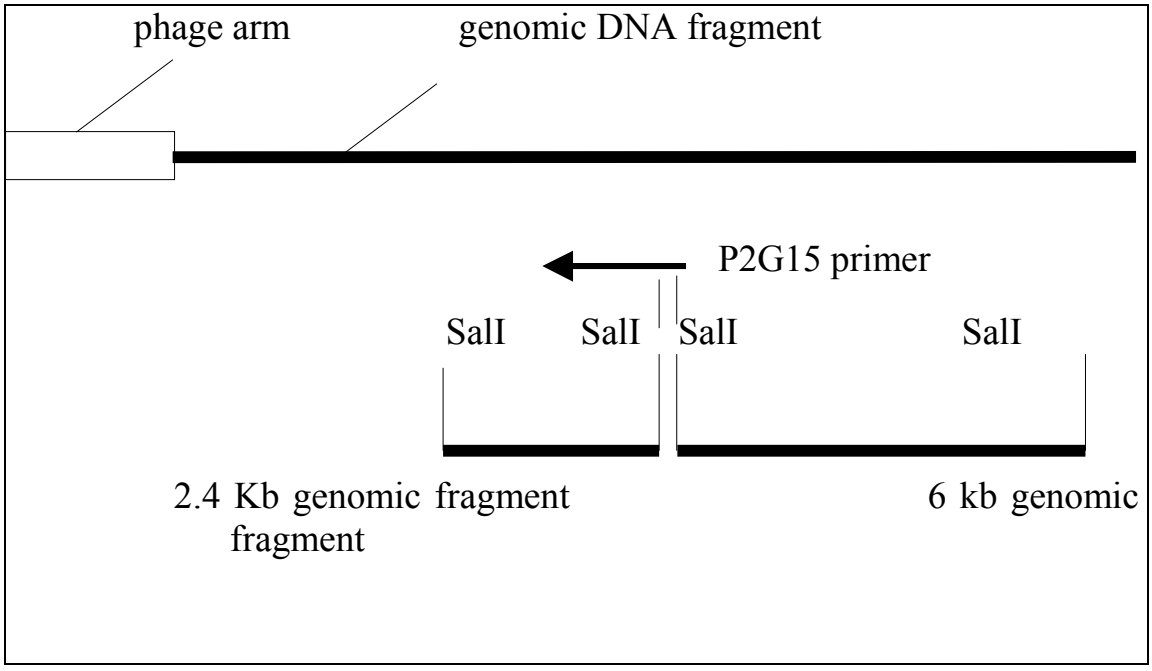


Figure 2
Schematic structure of the genomic *TaMCM2* gene fragment. The P2G15 primer was used to sequence the recombinant phage DNA to determine the upstream adjacent fragment.

4.3. 5`RACE of the TaMCM2 cDNA

The comparison between the amino acid sequence of the TaMCM2 protein and the amino acid sequence of the *Vicia faba* MCM2 (VfMCM2) protein (kindly provided by Dr. H. Weber, IPK-Gatersleben) shows strong homology (Fig.3). However, the sequence comparison also suggested that about 70 amino acids are still missing at the 5`end of the *TaMCM2* cDNA.

Figure 3
Alignment between the amino acid sequences of TaMCM2 and VfMCM2. The underline sequences represent the amino sequences of the sequenced peptides of TaMCM2.

The RACE techniques provide the most common technique to get the full length cDNA. In the Smart RACE of 5` technique (CLONTECH), the sequence of the terminal 5`region of the gene is amplified using the template

switching mechanism of the MMLV enzyme to add a primer sequence at the 5`end of the mRNA. We have used this technique to amplify the cDNA with the Smart 5`primer and the gene specific primer R1 position at 980-1001bp (Fig. 4). The PCR product was tested using the nested gene specific PCR primer A1 at 55- 78 bp and the NUP Smart primer. The product of smart PCR are usually multiple bands representing the population of full length cDNA, incomplete cDNA and non-specific bands. The nested PCR should amplify only the specific cDNA. Nevertheless, the amplification product still contain some non specific DNA. To eliminate these DNA unspecific fragments, the nested PCR product was fractionated on an agarose gel and blotted for Southern hybridisation using the *TaMCM2* cDNA as a probe. Five different clones of the amplified product were sequenced and their sequences were generally the same except for some nucleotide changes which may be referred to polymerase mistakes. The missing sequence at the 5` end was detected as a new exon in the genomic sequence. There are two ATG codons at about 200 bp upstream of the 5`untranslated sequence of the complete cDNA. However, there are two stop codons TAA and TGA in frame, located upstream of the presumptive translation initiation site. Therefore, the indicated ATG is considered to represent the N-terminus of the TaMCM2 protein.

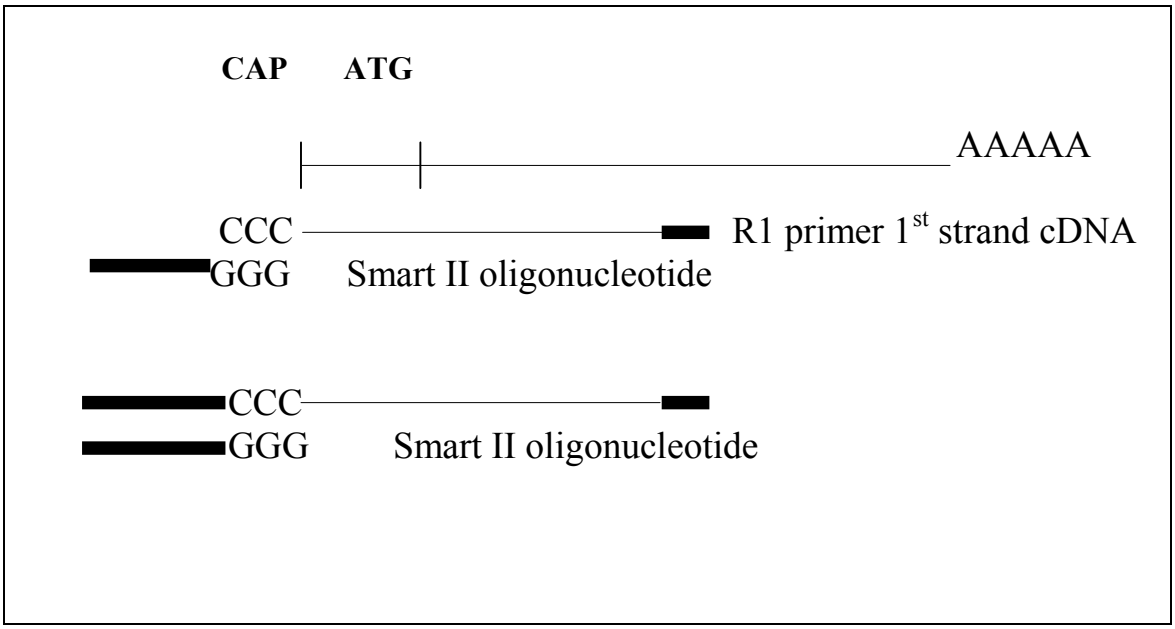


Figure 4

The primer extension of *TaMCM2* using A1 primer was followed by the SMART template switching technique using the Smart II oligonucleotides. The complete sequence of *TaMCM2* gene and the corresponding amino acids sequences is shown appendix 2.

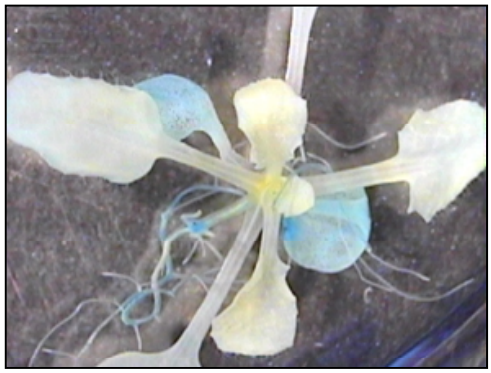
4.4. Expression of TaMCM2 promoter/GUS gene in transgenic Arabidopsis plants

The complete sequence of the wheat 2.4 kb genomic fragment demonstrates the presence of 201 bp of the genomic *TaMCM2* gene sequence (representing the first exon, the first intron and a part of the second exon) in addition to the 2.2 kb of the 5`flanking region. To study the expression pattern of the *TaMCM2* gene, the 2.4 kb fragment was transcriptionally fused to the GUS reporter gene and used for the transformation of *Arabidopsis* plants using the binary vector pGA482 and *Agrobacterium* mediated transformation. As shown in Fig. 5, the GUS staining shows the expression in parts of the cotyledons and in the hypocotyl region. There was no detectable colour in the apical meristem or in young leaves. The second appearance of GUS staining is found in sepals of young flowers before anthesis. After the differentiation of the stamen, the GUS expression was recorded in the anther stalk and in the anther locule. The

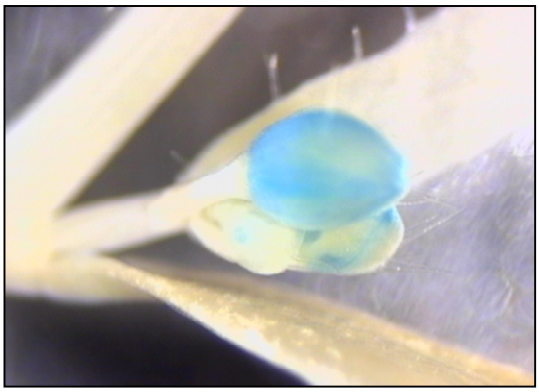
GUS staining could be detected in the anther through and after maturation of pollen grains. The histological sections of the gynoecium show no difference between the transformed and untransformed plants caused by GUS expression in the embryo sac structure. The GUS staining was absent during the early and late embryogenesis as shown in Fig. 5 (I and L).

A

B

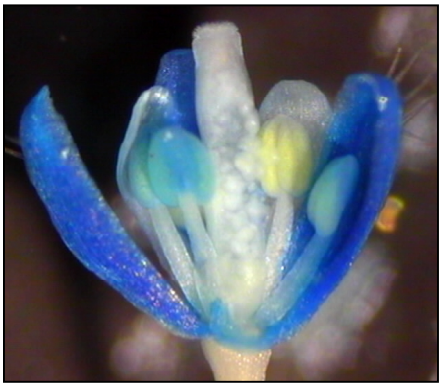


C



D

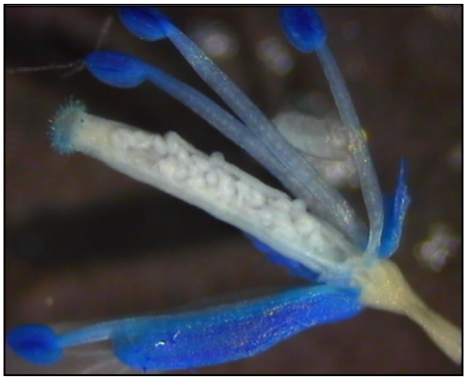
E



F

G

H



I

L

Figure 5.

The activity pattern of *TaMCM2* gene promoter in transgenic *Arabidopsis* plants using the GUS reporter gene.
Panel A and B - the GUS expression in the cotyledons leaves and hypocotyl.
Panel C, D, and E- the expression in different parts of young flowers.
Panel F, G, and H- show the expression in different parts on the flowers at anthesis.
Panel I and L - the expression at early stages of embryogenesis and at immature seeds respectively.

4.5. Expression of TaMCM2 in E. coli

The MCM2 protein has been suggested to function as a regulator of the cell cycle and determines the entrance into the S phase. In addition, there is preliminary evidence suggesting a function of MCM2 for the initiation of embryogenesis without fertilization. Expression and isolation of the TaMCM2 protein is a prerequisite for the generation of polyclonal antibodies. The antibodies will be used to evaluate the expression pattern of the *TaMCM2*-gene inside the ovule structure.

4.5.1. Thioredoxin expression system

The thiofusion expression system allows the efficient expression of foreign proteins in *E. coli* reaching up to 40% of total cell protein. The presence of the thioredoxin moiety appears to confer a better solubility to formerly insoluble heterologous proteins (Holmgren, 1985; La Vallie et al., 1992). Therefore, the 2.7 kb *TaMCM2* cDNA was fused in frame to the thioredoxin start codon of the pTrx expression vector. For this purpose the 2.7 kb fragment present in the pGEM-T vector was digested with the restrictases Asp718 and XbaI. The insert fragment was gel purified and ligated into the Asp718/XbaI digested expression vector. The expression of the fused *MCM2* cDNA is controlled by the λ phage PL promoter which

itself is controlled by the λ phage CI repressor. Since the repressor gene is regulated by the trp promoter, the addition of tryptophan induces the expression of the fused protein. The G1698 competent cells were used as the expression host. This strain is recommended to express the recombinant protein at a temperature below 30°C. The culture was induced at OD_{600} ~ 0.4 and samples were taken just before induction and then every 2 hr for a total period of 16 hr. The cells were centrifuged and the proteins were checked in the supernatant, periplasmic space and in cell lysate using the clarified culture medium, the osmotically shocked cells and total cell lysate, respectively. The SDS-PAGE fractionation of 30 mg protein demonstrate that the fused protein is exclusively present in the inclusion bodies (Fig. 6). The inclusion bodies were partially isolated with lysis buffer. After solublisation and refolding, the protein was incubated with ThioBond Resin for the purification of thioredoxin TaMCM2 fusion product. The eluted proteins were separated on SDS-PAGE and showed a main band with a molecular weight of 110 kD in addition to minor bands at lower molecular weights. The purification using ThioBond resin gave a low yield which was about 30 μg for each 1 L of culture as estimated by the intensity of the Coomassie stained band.

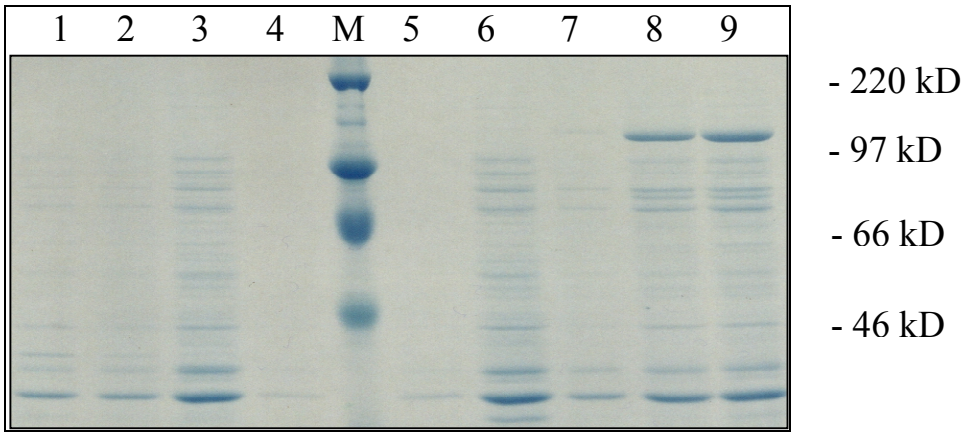


Figure 6.
SDS-PAGE of the expressed MCM2 protein using the thioredoxin expression system.

1- untransformed culture at induction
2- untransformed culture at after 2hr
3- untransformed culture at after 4 hr
4- untransformed culture at after 6 hr
M- high and low protein molecular weight
5- transformed culture at induction
6- transformed culture after 2 hr
7- transformed culture after 4 hr
8- transformed culture after 6 hr
9- transformed culture after 20 hr

To separate the thioredoxin moiety, the purified protein was cleaved with enterokinase which recognises the sequence Asp-Asp-Asp-Asp-Lys. Enterokinase hydrolyses the peptide bond between Lysin and the next amino acid. The cleavage product shows an unspecific pattern with 60 kD band instead of the expected 100 kD band, in addition to low molecular weight bands (Fig. 7). This result suggests the unspecific digestion of the isolated TaMCM2 by enterokinase. The resin purified MCM2 protein was used to produce polyclonal antibodies. Unfortunately, this antibodies exhibit a high degree of unspecific reactions.

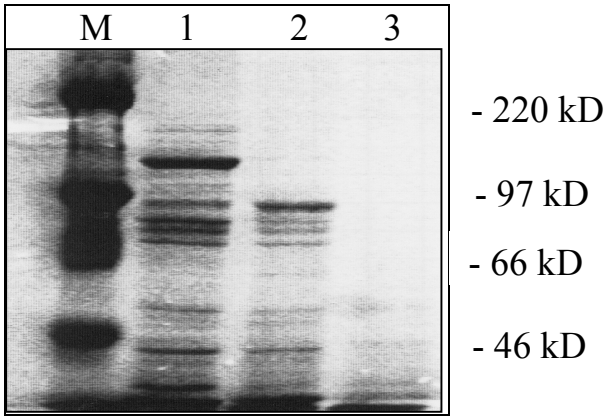


Figure 7.
The SDS-PAGE shows the enterokinase digestion products after the indicated incubation time.
(M) molecular weight marker, (1) solubilised and refolded inclusion bodies, (2) after 30 min incubation with enterokinase , (3) after 1hr incubation with enterokinase.

The purification of the refolded protein using Sephacryl S-200 HR gel filtration did not result in protein purity required to produce a high specific polyclonal antibody due to the presence of other protein bands, as it is showed in figure (8). The western blot of the collected fractions using monoclonal antibody against the thioredoxin moiety indicates that the associated proteins are degraded products of the TaMCM2 (Figure 9).

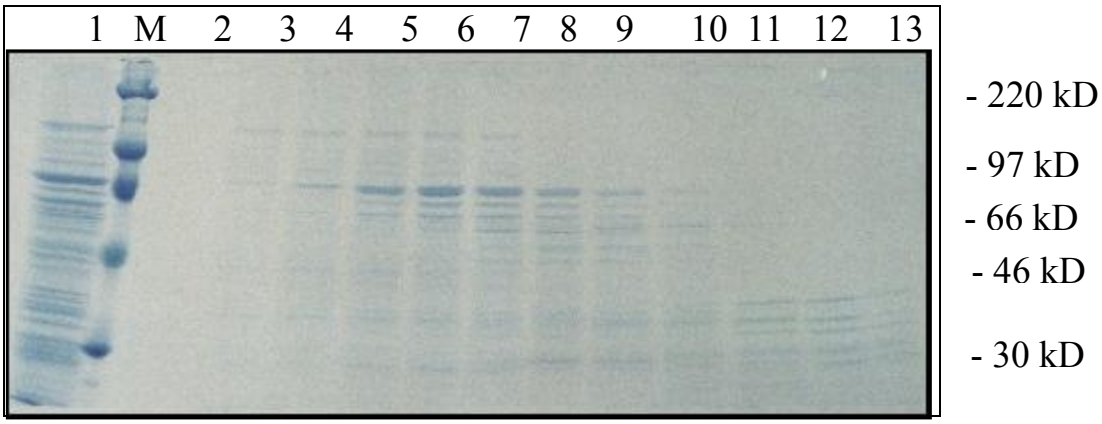


Figure 8.
SDS-PAGE representing the eluted fractions of refolded MCM2 after Sephacryl S-200 HR gel purification.
(1) the refolded MCM2 protein, (M) the protein marker, (2-13) the eluted fractions of the gel filtration.

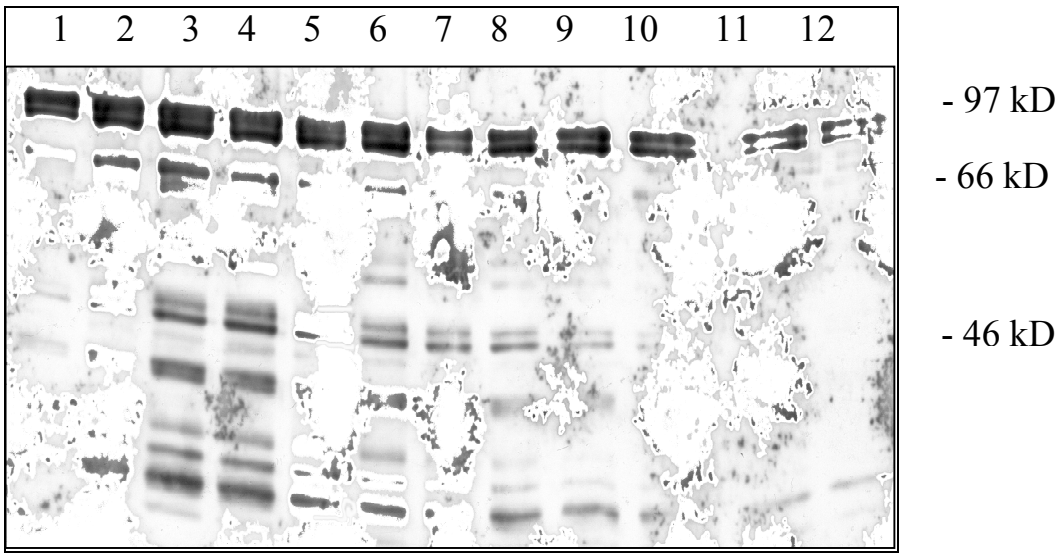


Figure 9.
Western blotting of the eluted fractions using monoclonal thioredoxin antibody. The lanes 1-12 represent the collected fractions shown in the previous figure.

4.5.2. Expression of TaMCM2 cDNA using the pET expression system

The pET expression system is one of the efficient prokaryotic expression systems; the desired product can comprise more than 50% of the total cell proteins. The pET-17b vector was used to direct the expression of *TaMCM2* cDNA. In this system, the target gene is controlled by the T7 promoter which is recognised by the T7 RNA polymerase. The host strain contains a chromosomal copy of the T7 RNA polymerase gene under the control of the LacUV5 promoter, and the expression is induced by the addition of IPTG. The advantage of the pET-17b vector over the pTrex thioredoxin vector is the absence of a fusion protein part in the pET-17b vector. The expressed target protein is fused to only 11 N-terminal amino acids of the T7 tag which is used to detect the expressed protein by western blot or by ELISA. The *TaMCM2* cDNA was isolated from the pGEM-T vector by the digestion with the Asp718/SacI restriction enzymes. The gel isolated fragment was fused behind the T7 promoter using the Asp718/SacI sites. The expression was induced by IPTG and the expressed protein was

localised exclusively as inclusion bodies as shown by SDS-PAGE of cell fractions (Fig. 10 A and B).

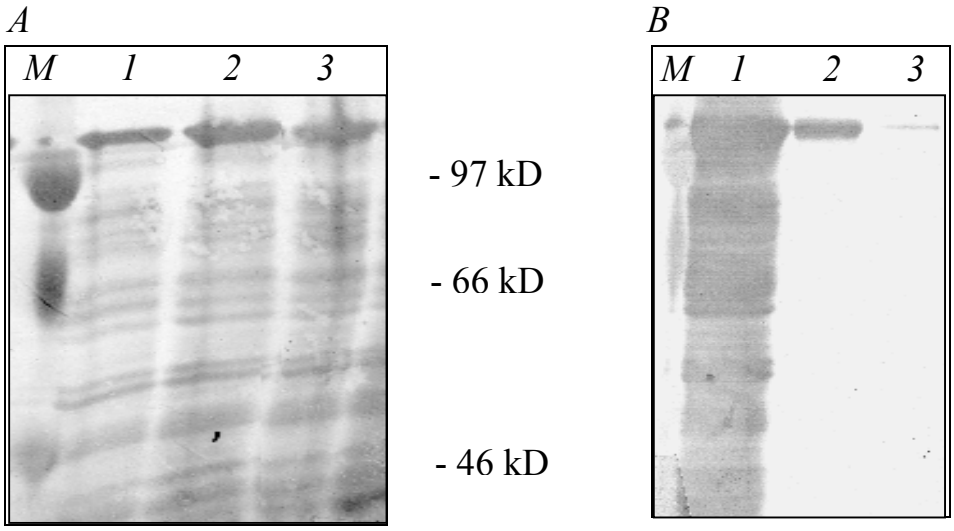


Figure 10.

Western blot to demonstrate expression and purification of MCM2 protein using pET system.
(A) Western blot of the induced bacterial culture (lanes 1, 2, 3) represent 10µg protein of total cell lysate of samples that were taken at 2, 4, 8 hr.
(B) Western blot of 10 µg (lane 1), 1µg (lane 2) and 0.1µg (lane 3) of purified and refolded MCM2 protein. The monoclonal antibody against the T7 tag was used for detection.

4.5.3 Purification of the synthesised TaMCM2 *protein*

The total cell lysate was washed with detergent mixture to solubilise the cell membranes. The inclusion bodies did not solubilise with washing buffer as shown by the SDS-gel (Fig. 10 B). The solubilisation of inclusion bodies was almost complete in 5M guanidium hydrochloride in CAPS buffer pH 9. The refolding of the solubilised protein was done by a gradual dialysis against PBS buffer. The refolded proteins were fractionated by SDS- PAGE, blotted on a membrane and eluted by triton. The eluted band was visualised by SDS-PAGE as a single band at ~100 kD and was analysed by amino acid

sequencing. The amino acid sequence of three different peptides could be analysed. These are:

1. I T N L P V Y D Q I R N
2. F T V N V E Q T I
3. S I A P S I Y G H E

All three can be found in the translated cDNA sequence demonstrating the specificity of the expressed protein (Fig. 3).

4.6. Generation of polyclonal antibodies against E. coli synthesised TaMCM2

The purified MCM2 protein was four times injected into rabbit. Each dose contained 80 µg of purified protein. After the fourth injection, the IgG of immunised rabbit serum was isolated using the affinity of protein A. The antibodies concentration was found to be 8.5 µg/µl. The ELISA test of the purified antibodies shows a linear relation between increasing amounts of antigen concentration and antibody binding. As shown in Fig. 11, the saturation point was found at a concentration of 20 µg of purified protein, indicating that the antibody might be suitable for a reliable histoimmunoassay. Western blotting was used to confirm the specificity of the purified polyclonal antibodies. Therefore, different concentrations of purified antigen were run on a SDS PAGE. Total cell protein of the non-transformed *E. coli* strain was used as a negative control. After blotting the filter was stained with antibodies (dilution 1:2000) and developed by the ECL system. As shown in Fig. 12, the antibodies recognise a purified protein amount as low as 10 ng.

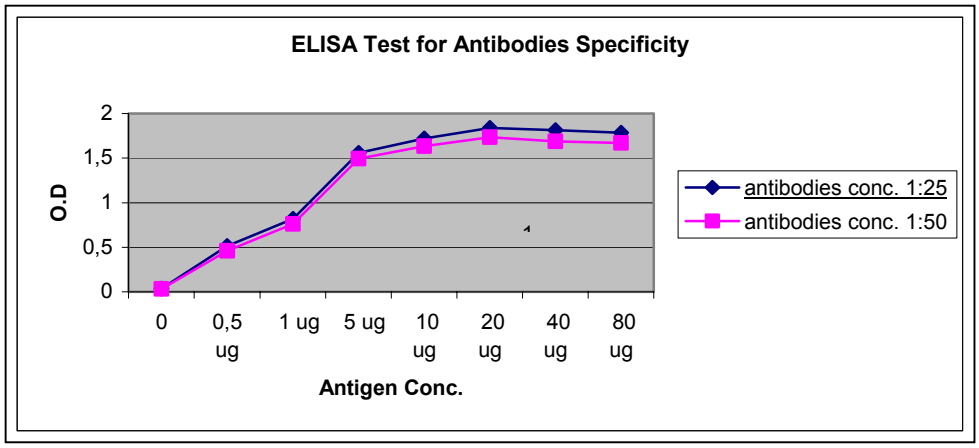


Figure 11.
ELISA to test the specificity of the polyclonal antibodies.

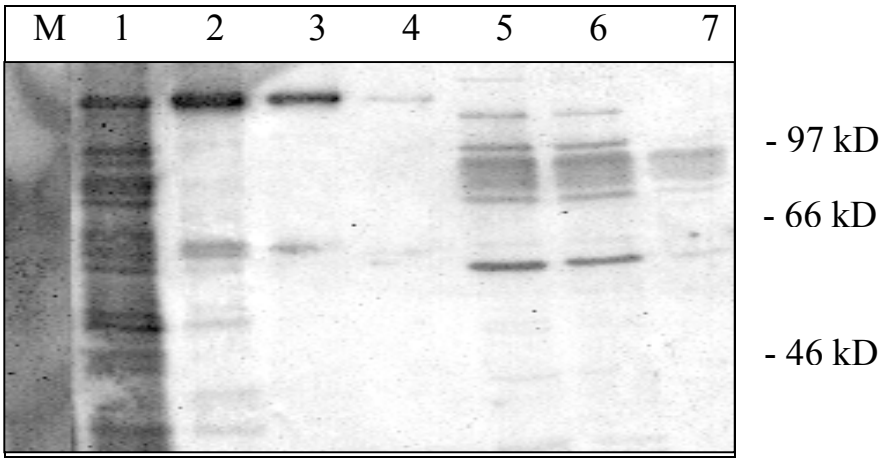


Figure 12.
Western blot of different concentrations of purified MCM2 antigen and detection with the purified polyclonal antibodies.
Lane 1- 1 µg solubilised inclusion bodies
Lane 2- 1 µg purified antigen
Lane 3- 100 ng purified antigen
Lane 4- 10 ng purified antigen
Lane 5- 10 µg bacterial lysate
Lane 6- 5µg bacterial lysate
Lane 7- 1µg bacterial lysate

4.7. Detection of TaMCM2 *in wheat gyneocium*

The production of polyclonal antibodies with reasonable specificity is a prerequiste to demonstrate the accumulation pattern of MCM2 protein inside the different structures of wheat ovule. Western blotting was used to proof the ability of antibodies to detect the differential accumulation of TaMCM2 in the ovary tissue of the parthenogenetic line of wheat. 15µg protein each of wheat gynoecia, wheat leaf and *Arabidopsis* leaf were applied on the SDS-PAGE and blotted on a membrane. In the lane with the ovary protein there are two bands at closely related molecular weights, the upper one has the right molecular weight of native TaMCM2 while the lower band has a molecular weight similar to the antigen band which resulted from the expression of incomplete *TaMCM2* cDNA (ca. 100 kD). In the case of leaf protein, the polyclonal antibodies recognise one band with a molecular weight similar to the native MCM2 protein, but there is no evidence for the presence of the second band. The intensity of bands in case of the wheat gynoecia are much higher than in case of the leaf. The purified antigen used in this experiment was used as a standard to estimate the MCM2 concentration in the wheat gynoecia, which is approximately 150 ng for each 15 µg of total protein (Fig. 13). The band which appeared in the leaf extract seems to be lower in molecular weight than those present in the ovary extract. This may be interpreted as an additional modification (e. g phosphorylation) associated with MCM extracted from wheat gynoecia.

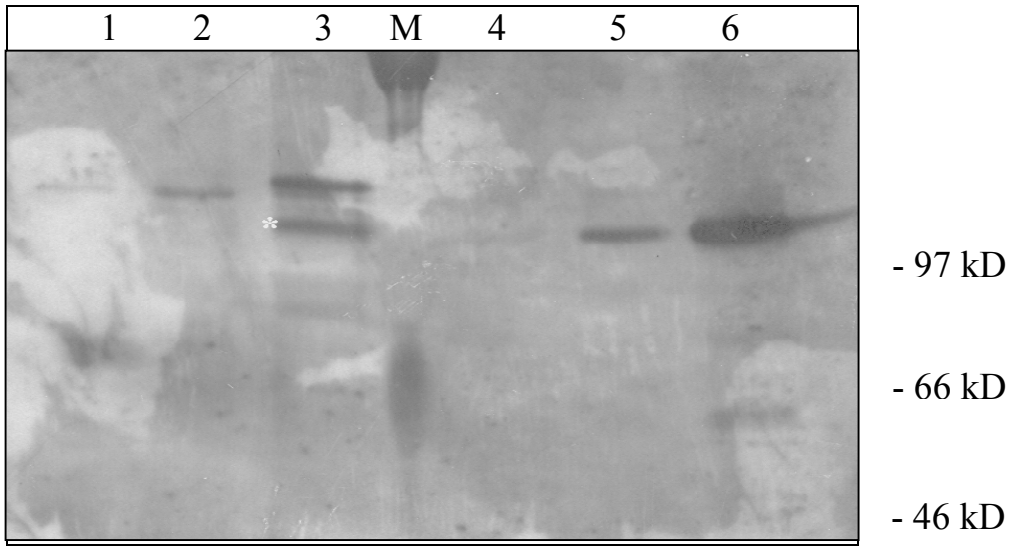


Figure 13
Western blotting of protein samples extracted from plant leafs and gynoecia and detected with the polyclonal antibodies.
Lane 1- protein extracted from the *Arabidopsis* leaf
Lane 2- protein extracted from the wheat leaf
Lane 3- protein extracted from the parthenogenetic gynoecia (cS), shows a reacted protein band of TaMCM2 at (~100 kD), in addition to a cross reacted protein at lower molecular weight (*).
Lane M- molecular weight standard
Lane 4- the purified *E. coli* expressed protein of *TaMCM2* (10 ng)
Lane 5 - the purified *E. coli* expressed protein of *TaMCM2* (100 ng)
Lane 6 - the purified *E. coli* expressed protein of *TaMCM2* (1000 ng)

The tested antibodies was planed to detect the preferentially expressed MCM2 in the ovary of the parthenogenetic lines of Salmon system of wheat. The demonstration of subcellular localization of MCM2 within the embryo sac structure will improve the understanding of TaMCM2 role during parthenogenesis development. The gynoecia of the sexual line (aS) and the parthenogenetic line (cS) were collected at anthesis. The staining of histological sections using anti-TaMCM2 antibodies did not confirm the preferentially expression of *TaMCM2* in the parthenogenetic wheat line (cS) comparing to the sexual line. However, the specificity of the polyclonal was compared to the control serum as shown in Fig. 14.

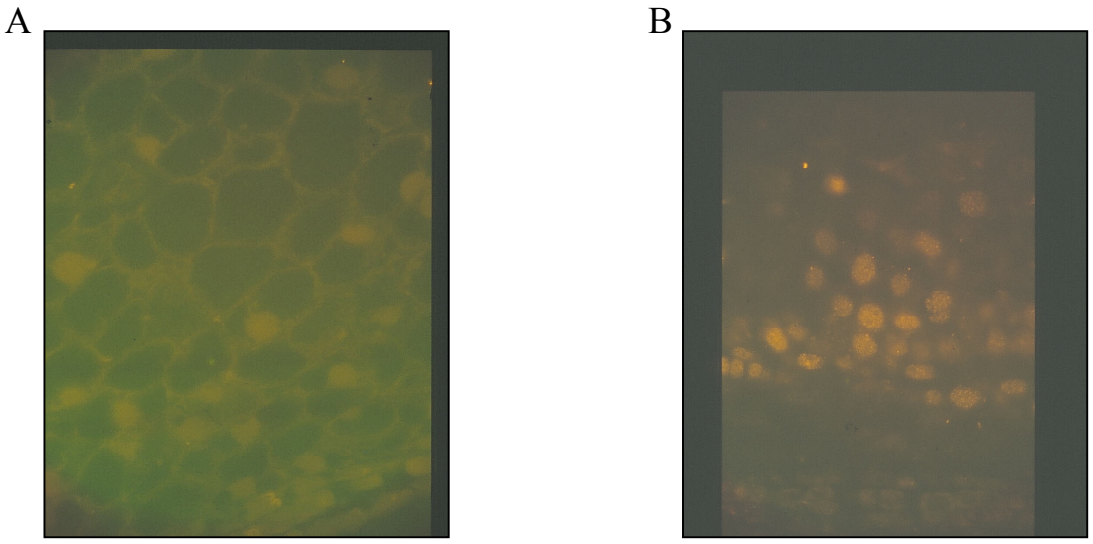


Figure 14.
Protein localization in the gynoecia of Salmon system of wheat.
(A) Staining of the parthenogenetic gynoecia using control serum (cS).
(B) Staining of gynoecia of the parthenogenetic (cS) using anti-TaMCM2 Antibodies.

4.8. The mcm2 mutant of S. cerevisiae as a eukaryotic model

There is a high degree of similarity between the members of the *MCM* gene family, especially within each subfamily as shown in Fig. 15.

All members of eukaryotic MCM2 proteins possess three highly conserved domains as shown in Fig. 16. Therefore, the *mcm2* mutant of yeast might be used as a good tool to study the effect of the expression of different *MCM2* genes. The mutant (8534 MZ MATα Leu 2-3, 112, Ura 3-52, his 4-Δ34, mcm2-1,ts) can normally grow below 30°C but growth is inhibited at 37°C. The *TaMCM2* and *VfMCM2* genes were used to check the possibility whether these higher plant genes can complement the *mcm2* yeast mutant. Two different yeast expression systems have been used to test the effect of *MCM2* gene over-expression on the growth rate of the mutant and the wild type *S. cerevisiae* (CL3 ABY 586). The effect of the yeast *MCM2* gene overexpression on the growth of the mutant strain has been analysed previously (Yan at al., 1993). The result showed that over-expression of

yeast *MCM2* could complement the mutant as concluded from the increased colony size.

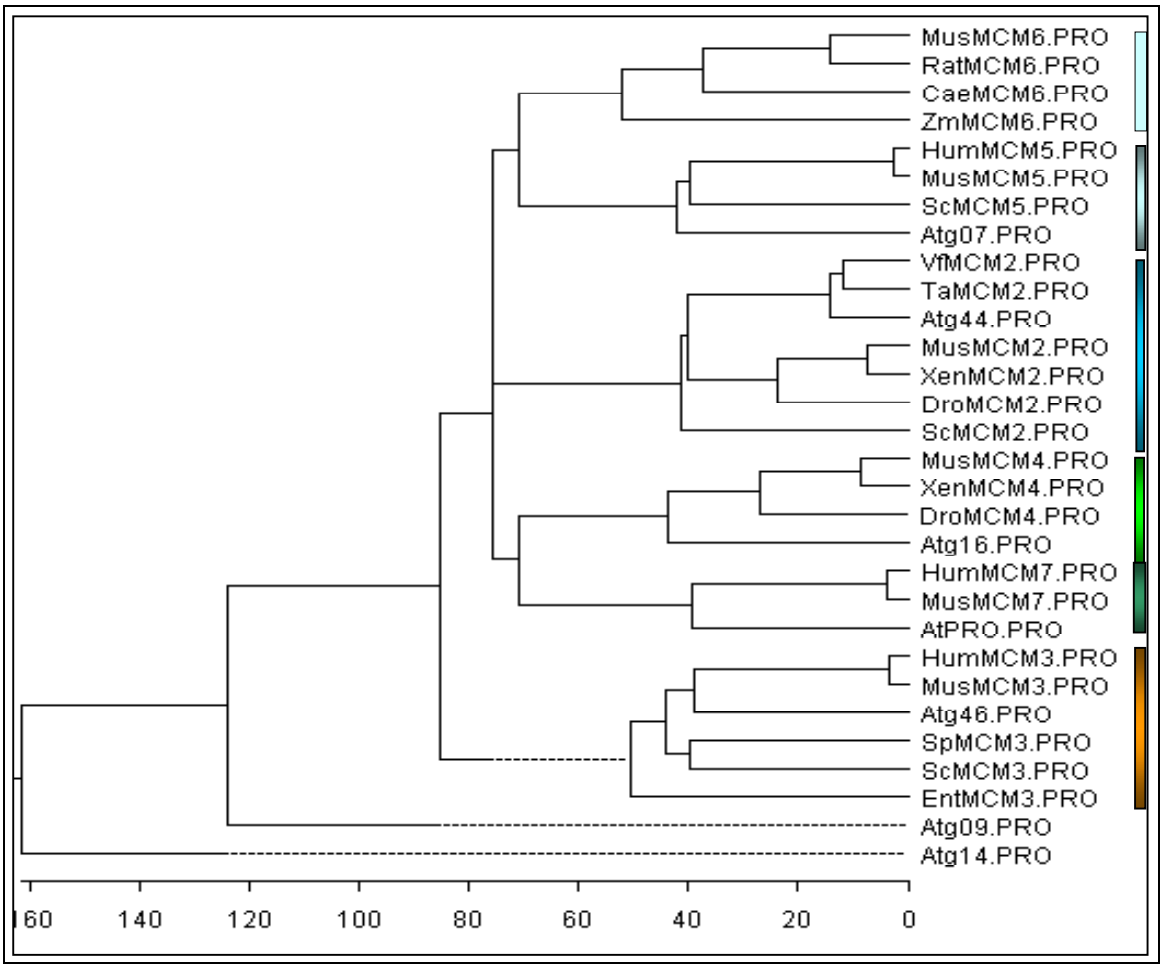


Figure 15.

The phylogenetic tree of MCM-related amino acid sequence. The abbreviations are, Cae (*Caenorhabditis elegans*), Dro (*Drosophila melanogaster.*), Ent (*Entamoeba histolytica*), Hum (*Homo sapiens*), Mus (*Mus musculus*), Rat (*Rattus norvegicus*), Schc (*Saccharomyces cerevisiae*), Schp (*Schizosaccharomyces pombe*), Ta (*Triticum aestivum*), Xen (*Xenopus laevis*), Zea (*Zea maize*), Vf (*Vicia faba*), AtPRO (*Arabidopsis thaliana PROLIFERA* gene). The Atg07, Atg09, Atg14, Atg16, Atg44, and Atg46 are annotate genes which are detected by screening of *Arabidopsis* genome with the AtPRO protein sequence.

ATP binding motif

```
TaMCM2  523- VKSIAPSIYGHEDIKTAIALAMFGGQEKNVKGKPRLRGDINCLLLGDPGTAKSQFLKYVE
VfMCM2  504- VKSIAPSIYGHDDIKTGIALAMFGGREKNVEGKHRLRGDINVLLLGDPGTAKSQFLKYVE
ScMCM2  498- ISSMAPSIYGHRDIKTAVACSLFGGVPKNVNGKHSIRGDINVLLLGDPGTAKSQILKYVE

TaMCM2  583- KTGHRAVYTTGKGASAVGLTAAVHKDPVTREWTLEGGALVLADRGICLIDEFDKMNDQDR
VfMCM2  564- KTGQRAVYTTGKGASAVGLTAAVHKDPVTREWTLEGGALVLADRGICLIDEFDKMNDQDR
ScMCM2  558- KTAHRAVFATGQGASAVGLTASVRKDPITKEWTLEGGALVLADKGVCLIDEFDKMNDQDR

TaMCM2  643- VSIHEAMEQQSISISKAGIVTSLQARCSVIAAANPVGGRYDSSKTFTQNVELTDPIISRF
VfMCM2  624- VSIHEAMEQQSISISKAGIVTSLQARCSVIAAANPIGGRYDSSKLFTQNVELTDPIISRF
ScMCM2  618- TSIHEAMEQQSISISKAGIVTTLQARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRF
```

Zn finger motif in MCM2 proteins **(**$C\text{-}X_2\text{-}C\text{-}X_{19}\text{-}C\text{-}X_2\text{-}C$)

```
TaMCM2  371- KYDCSKCGTVLGPFFQNSYTEVRVGSCPECQSK
VfMCM2  352- KYDCSKCGAILGPFFQNSYSEVKVGSCPECQSK
DmMCM2  311- KYDCVKCGYVLGPFVQSQNTEIKPGSCPECQST
ScMCM2  344- KFNCLKCGSILGPFFQDSNEEIRISFCTNCKSK
             *  * ***  **** *    *     *    *
```

Figure 16
Highly conserved protein domains found in MCM2 proteins present in the *Triticum aestivum* MCM2 (TaMCM2), *Vicia faba* MCM2 (VfMCM2), *Drosophila melanogaster* (DmMCM2) and *Saccharomyces cerevisiae* MCM2 (ScMCM2).

4.9. S. cerevisiae MCM2 gene isolation (ScMCM2)

To calibrate the experimental analysis of the mutant complementation, the over-expression of the *ScMCM2* gene in wild type yeast was included in the experiment. The *ScMCM2* sequence as derived from the Yeast Genome Data Base was PCR amplified with proof reading *pfu* polymerase. A BamHI site containing upper primer and a XhoI site containing lower primer were used to isolate the complete *ScMCM2* coding sequence (Fig. 17). The 2.6 kb PCR fragment was digested with BamHI/XhoI and ligated to the pPCR-2 vector plasmid and used to transform *E. coli*.

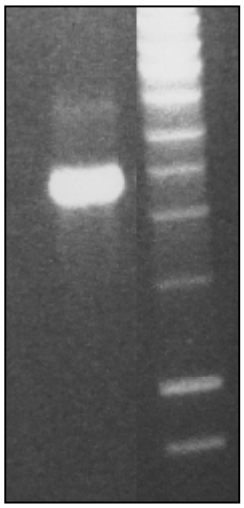


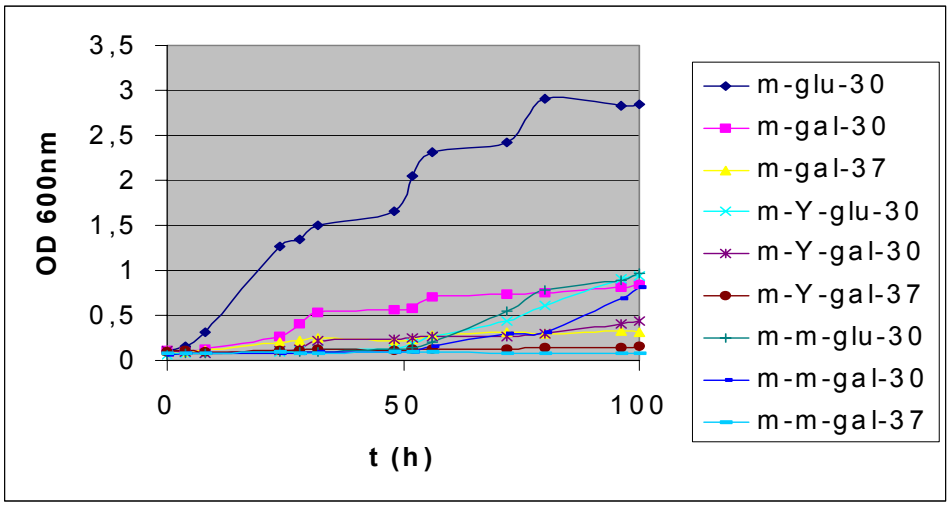
Figure 17
The PCR product of the 2.6 kb *ScMCM2* gene was directly amplified from *S. cerevisiae* genomic DNA.

A positive clone was used for the isolation of the *ScMCM2* gene containing BamHI/XhoI fragment, which was ligated to the YEX-BX yeast vector and used for the transformation of *E. coli*. A positive clone was used to transform both the yeast wild strain (CL3ABY 586) and the *mcm2* mutant strain.

4.10. Cloning of MCM2 genes in pYES-2 and pYEX-BX for the expression in yeast

The pYES2 vector plasmid was used for the expression in yeast. It contains a yeast galactose enhancer and promoter as well as the 2μ origin of replication for a high copy number (10–40 copies per cell). The plasmid further contains transcriptional termination signals of the CYC1 gene. There is no fusion part and the translation will start from the ATG codon of the introduced gene. The expression of the introduced gene is induced by the transfer of the cells to a media that contains galactose as the sole carbon source. However, the suppression effect of galactose on the growth rate of the mutant cells (Fig. 18), suggested the use of another expression system with a moderate effect of the inducer. The YEX-BX is a high copy number yeast expression vector (10 to 50 copy per a cell) and contains a Cu^{++} ion induced promoter. The limited number of restriction sites in the polylinker of this vector requires the blunt end cloning of the MCM2 genes (TaMCM2, VfMCM2 and ScMCM2).

A



B

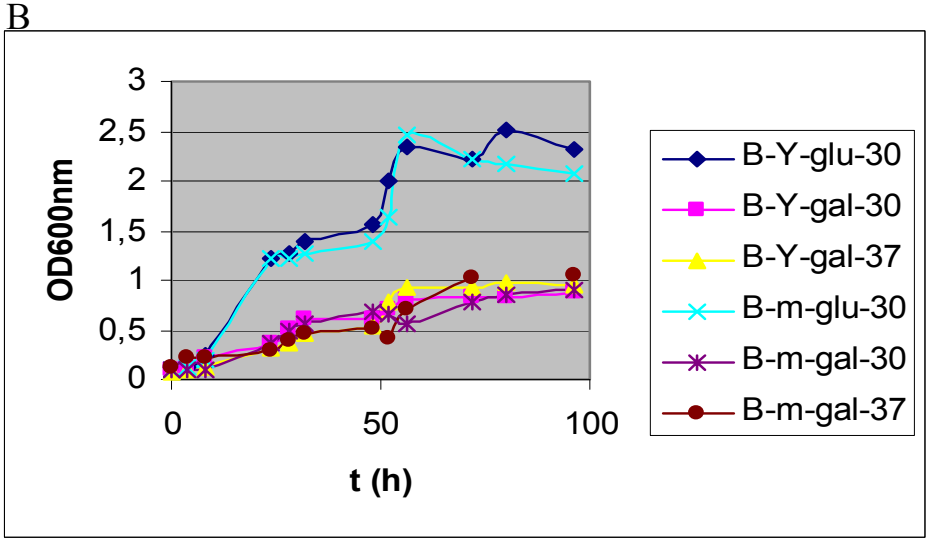


Figure 18
The effect of *TaMCM2* gene over-expression on the growth rate of wild type and *mcm2* mutant yeast at permissive (30°C) and restricted (37°C) temperature using pYES-2.
The mutant strain (m) was grown at 30°C or 37°C using glucose or galactose (inducer) as a carbon source.
The expression strain (B) was grown at 30°C or 37°C using glucose or galactose (inducer) as a carbon source.
Y, are the cells transformed with the expression vector YES-2. m are the cells transformed with *TaMCM2*.

The *TaMCM2* gene was amplified using EcoRI/XhoI primers and the amplified fragment was ligated into the pGEM-vector. The 2.9 kb of the

amplified *TaMCM2* cDNA was digested with EcoRI/XhoI and gel purified. The isolated fragment was ligated to the pYEX–BX vector digested with EcoRI/SalI to generate a compatible linker. The *ScMCM2* gene was amplified using primers containing BamHI/XhoI restriction sites and genomic DNA of *Saccharomyces cerevisiae*. The amplified 2.6 kb of the *ScMCM2* gene was cloned into the YEX-BX-vector using BamHI/XhoI sites. The 3kb *VfMCM2* cDNA was fused into the Ziplocus vector. It was removed from the vector by digestion with NotI/SalI, treated with Klenow enzyme and ligated to the blunt end YEX-BX-vector digested with PvuII. The ligated products of *TaMCM2*, *VfMCM2* and *ScMCM2* were transferred to *E. coli*. Positive clones were cultivated, used to purify the recombinant plasmids which were then used to transform both the yeast expression strain and the *mcm2* yeast mutant.

4.10.1. Overexpression of plant MCM2 genes in wild type yeast and mcm2 yeast mutant

Both, the *mcm2* mutant and wild type were used as an expression strain for the *MCM2* genes(*TaMCM2*, *VfMCM2* and *ScMCM2*). The empty vector was transformed as control. The transformed cells were grown until OD_{600}~ 0.6 on SD-media supplemented with the amino acids Trp, His and Leu. The cells were centrifuged and transferred to the same volume of media supplemented with $CuSO_4$ to a final concentration of 0.5 mM. Then 2 ml samples were taken every 3 hours. The cells were lysed and the different cell fractions were analysed by SDS-PAGE and by western blot using TaMCM2 polyclonal antibodies (Fig. 19 A and B). The western blots showed that the yeast MCM2 protein was accumulated to a high quantity in the soluble cell fraction of wild type strain, as can be seen by the dominant band in SDS-PAGE. The western blot analysis of *S. cerevisiae* cells which were transformed with the *TaMCM2* or *VfMCM2* did not show any detectable protein accumulation (Fig. 19 C).The reason for this is not clear yet. The

western blot of *ScMCM2* expression in the *mcm2* mutant showed that the mutant strain can not produce ScMCM2 protein in an efficient way (Fig. 19).

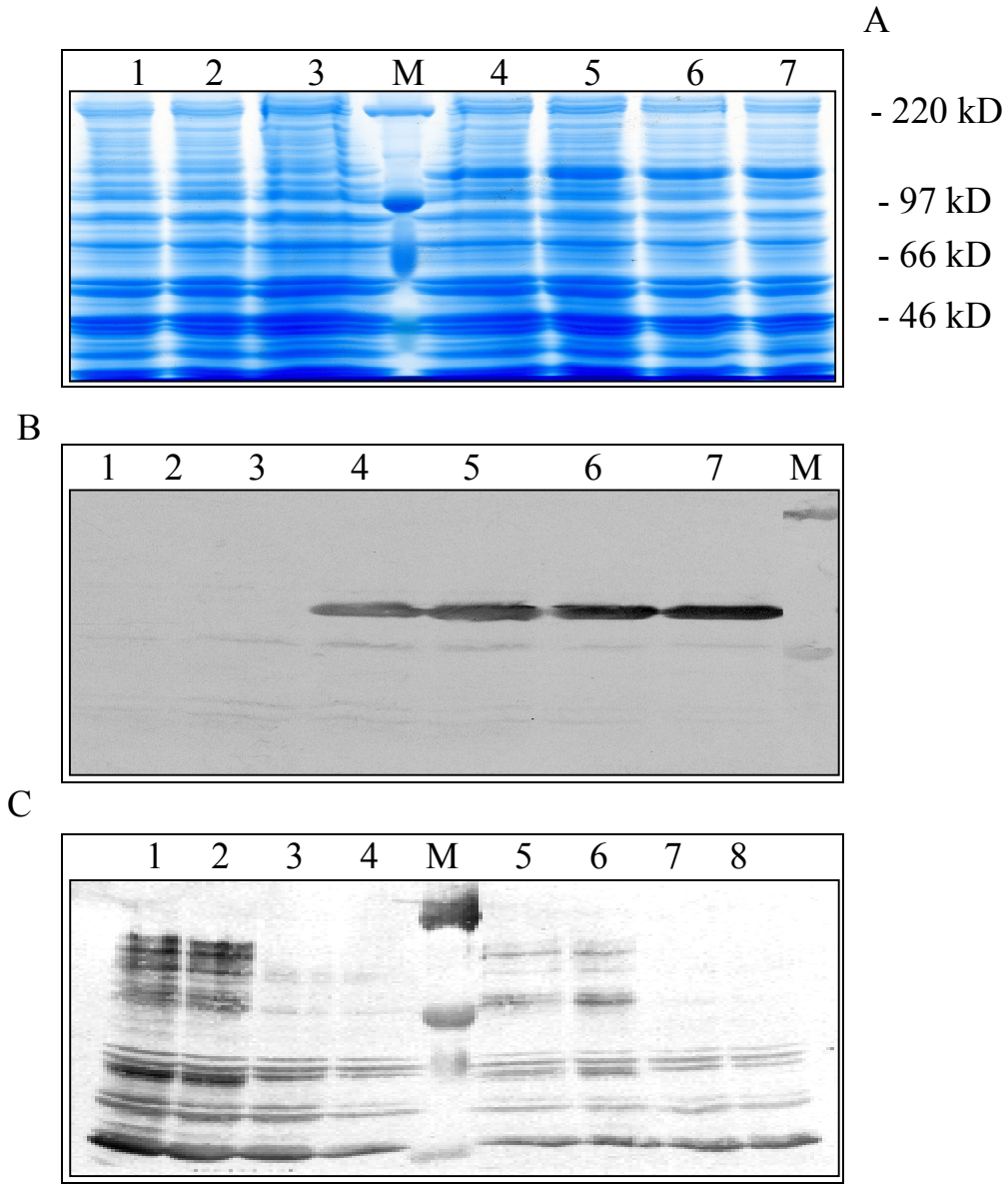


Figure 19

Expression of yeast and plant *MCM2* genes in (expression strain) of *S. cerevisiae.*

(A) SDS-PAGE of protein extracts of the expression strain transformed with the *ScMCM2* gene cloned in the vector YEX-BX.

Lane 1- non transformed control, before induction

Lane 2- non transformed control, after 3 hr induction

Lane 3- non transformed control, after 5 hr induction

Lane M- molecular weight standard

Lane 4- expression of the ScMCM2 gene, before induction

Lane 5- expression of the ScMCM2 gene, after 3 hr induction

Lane 6- expression of the ScMCM2 gene, after 5 hr induction

Lane 7- expression of the ScMCM2 gene, after 7 hr induction

(B) Western blot of the gel shown in (A) using polyclonal antibodies against TaMCM2 protein

(A) Western blot of protein extracts of the expression strain transformed with the genes *TaMCM2* and *VfMCM2* cloned in the vector YEX/BX

Lanes 1,2- two independent clones of *TaMCM2* transformants, before induction

Lanes 3,4- two independent clones of *VfMCM2* transformants, before induction

Lane M- molecular weight standard

Lanes 5,6- two independent clones of *TaMCM2* transformants, after 3 hr induction

Lanes 7,8- two independent clones of *VfMCM2* transformants, after 3 hr induction

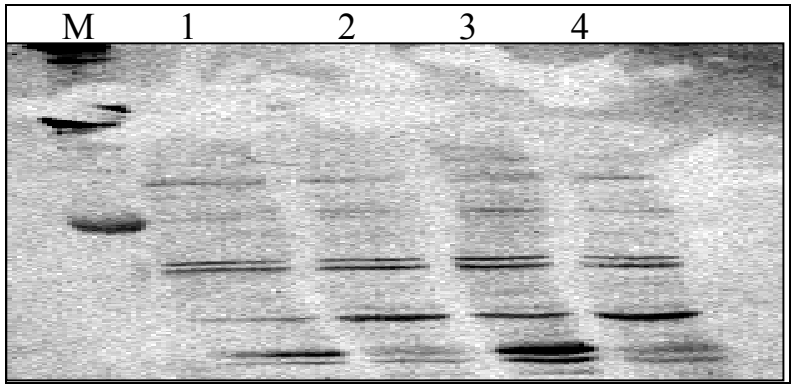


Figure 20.
Western blot using polyclonal antibodies against
TaMCM2 protein and protein extracts of the *mcm2* mutant strain transformed
with the *ScMCM2* gene cloned in the vector YEX/BX.

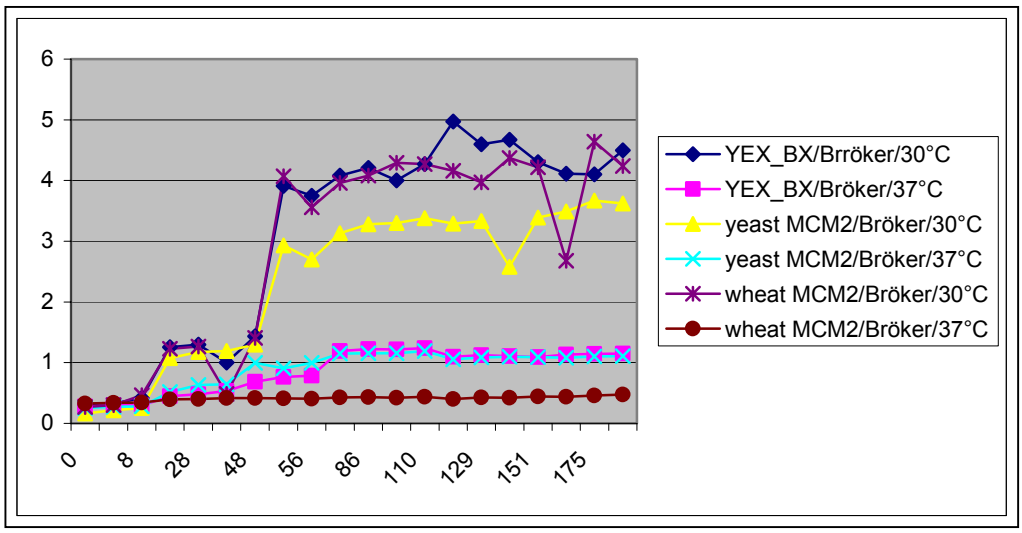
Lanes 1,2- two independent clones of *ScMCM2* transformants, before induction

Lanes 3,4- two independent clones of *ScMCM2* transformants, after 3 hr induction

4.10.2. The over expression of MCM2 genes in yeast and their effect on the growth pattern

To detect the complementation effect of different MCM2 genes, two strategies were followed: 1) complementation effect on the growth rate and 2) complementation effect on the DNA content. Both the expression and the *mcm2* mutant strains were transformed with the empty YEX-BX vector and the corresponding *ScMCM2* and *TaMCM2* constructs. The cells which were transformed with either empty expression vector YEX-BX or with the same vector which is ligated with ScMCM2 or TaMCM2. The transformed cells were grown until OD_{600}~ 0. 5 on SD-media supplemented with the amino acids Trp, His, Leu, and the cells were centrifuged and used to inoculate new cultures supplemented with Cu^{++} to a final concentration 0.5 mM. The new culture was started at OD ~0.2 and incubated either at 30 °C (permissive temperature) or 37 °C (restrictive temperature) and the growth rate was followed using the OD at 600 nm. As demonstrated in Fig. 21, the growth rate of the mutants at 30 °C shows no difference in growth rate patterns between the cells which were transformed with the YEX-BX and the corresponding constructs of *ScMCM2* and *TaMCM2*. Nevertheless, the growth pattern of all transformants were clearly affected at 37 °C (restricted temperature) there is no indication for the complementation effect of the *MCM2* transformants.

A



B

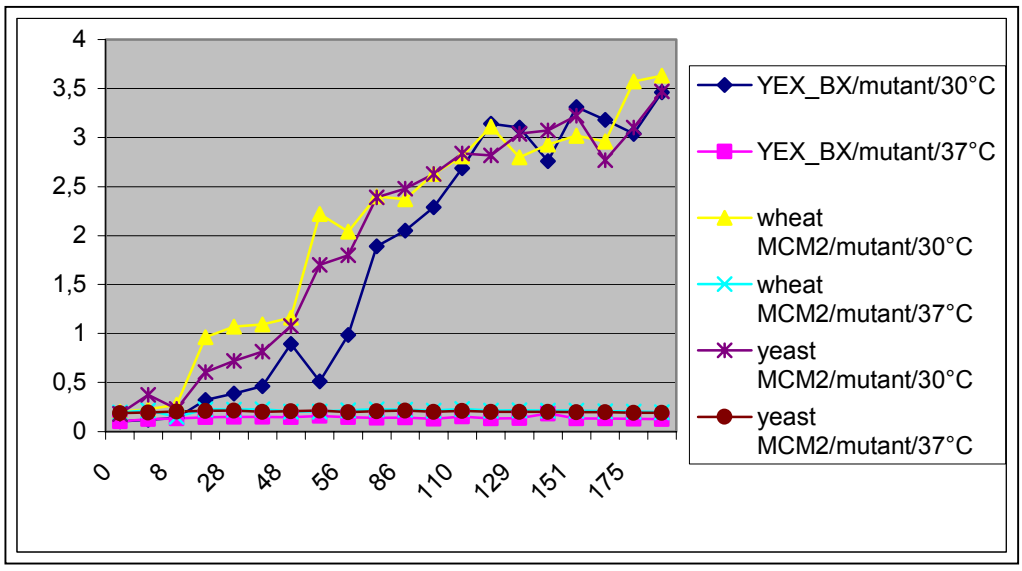
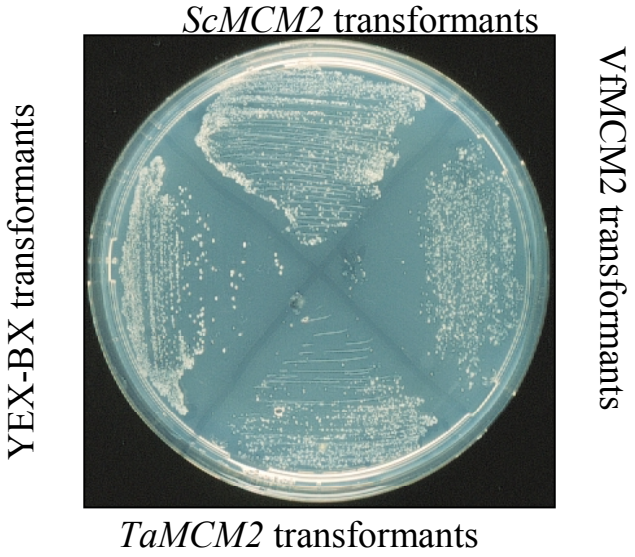


Figure 21.
The effect of over expression of *ScMCM2* and *TaMCM2* on the growth curve of wild and mutant type yeast using YEX-BX expression system.
(A) The Bröker strain was grown at 30°C or 37°C on Cu^{++} inducing media.
(B) the mutant strain was grown at 30°C or 37°C on Cu^{++} inducing media.

However, the complementation test on the solid media gave a better result as shown in Fig. 22. The *ScMCM2* gene proofed its complementation effect for

the mutant strain which was grown at 37°C. in contrast, the both *TaMCM2* and *VfMCM2* genes did not support the growth of the mutant strain at 37°C.

A



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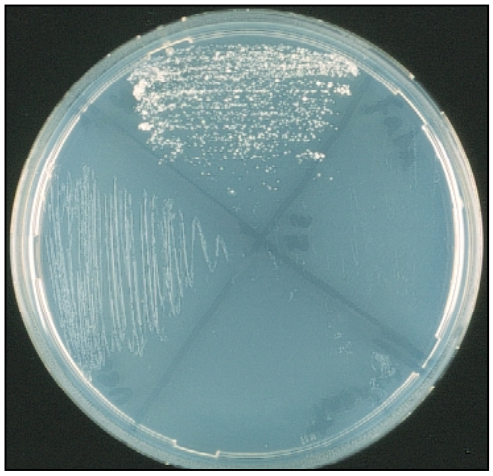


Figure 22

MCM2 genes over expression phenotypes in the *mcm2* mutant with the using of YEX-BX expression vector.

(A) streaking cultures of the transformed mutant with *ScMCM2*, *VfMCM2*, and *TaMCM2* were grown at 30°C.

(B) the replicate plate which was grown at 37°C.

4.10.3. MCM2 genes over expression and DNA content

Since the *mcm2* mutant exhibits a delay of the S phase and increment of DNA content; the use of the flow cytometry technique could help to detect the complementation effect of over expression on the cell division and the DNA content of the mutant (Yan et al., 1991; Liang, et al., 1999). To study the temperature effect on the mutant cell division, several constructs were used to control the experiment. The transformed cells with YEX-BX expression vector as well as its recombinant constructs with *ScMCM2*, *TaMCM2* and *VfMCM2* were started to grow in a culture at early logarithmic phase with OD~ 0.5. The over expression of *MCM2* genes were induced by the presence of Cu^{++} and the DNA content was determined in cultures that were grown at 30°C and 37°C. The mutant strain which was grown at 30°C shows 1n DNA content for the cell majority, while the cells which were grown at 37°C show 2n DNA content for the majority of cells. However, in the case of mutant transformant with *ScMCM2*, shows an increased portion of the 1n DNA content when it grew at 37°C. The DNA content pattern of the mutant transformants with the plant *MCM2* genes show no difference than the DNA content pattern of the mutant.

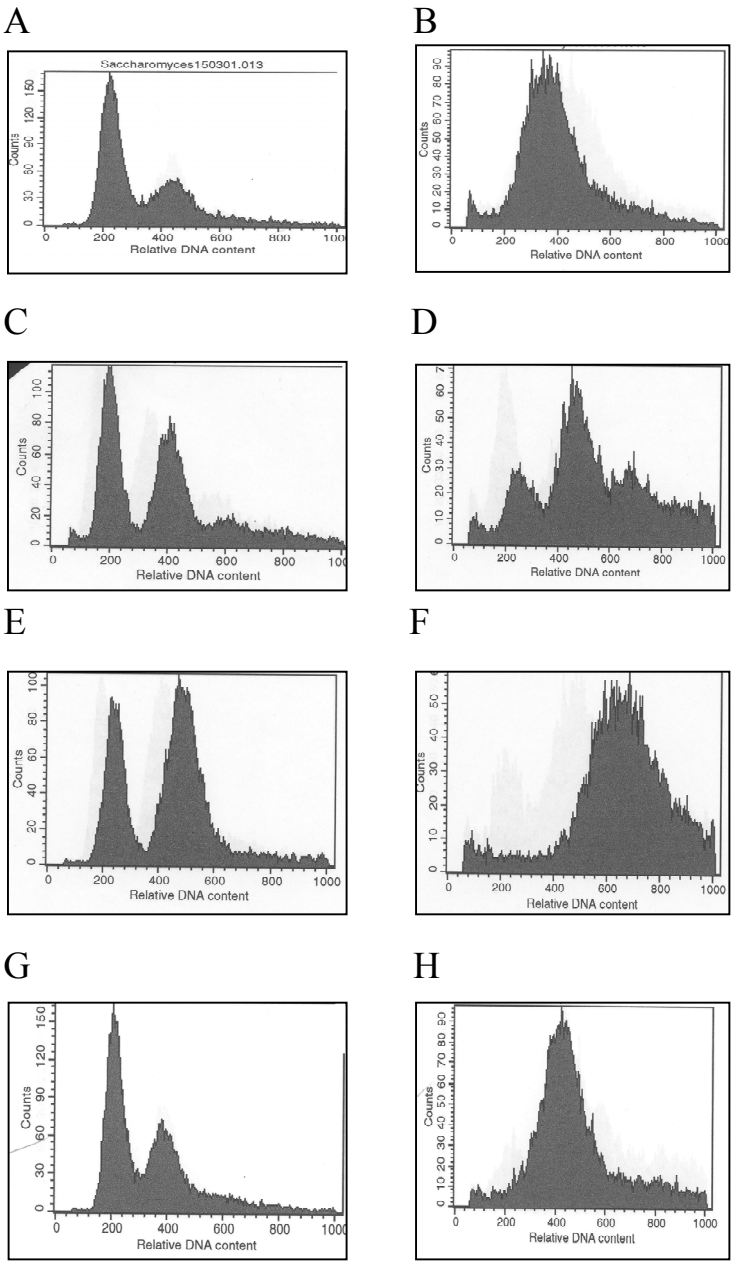


Figure 23.
Flow cytometric DNA content determination in the *mcm2* mutant after expression of various *MCM2* genes
Panel A- non transformed mcm2 mutant, 30°C
Panel B- non transformed mcm2 mutant, 37°C
Panel C- mutant transformed with *ScMCM2*, 30°C
Panel D- mutant transformed with *ScMCM2*, 37°C
Panel E- mutant transformed with *VfMCM2*, 30°C
Panel F- mutant transformed with *VfMCM2*, 37°C
Panel G- mutant transformed with *TaMCM2*, 30°C
Panel H- mutant transformed with *TaMCM2*, 37°C

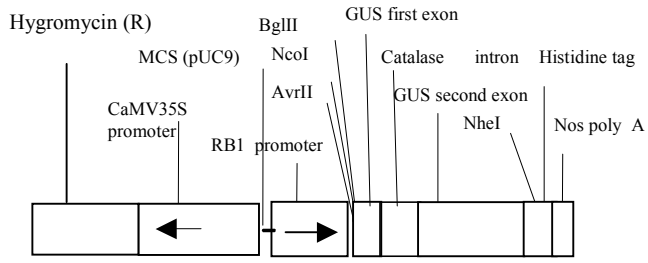
4.11. Expression of different plant MCM genes in Arabidopsis plants

The first attempt to introduce *TaMCM2* into plants was based on the seed specific USP promoter. The plasmid p30T containing the USP gene promoter was digested at the BglII site, located at the 3`end of the promoter. Blunt ends were generated by digestion with Klenow fragment. The *TaMCM2* cDNA was derived from the pGEM-T vector by digestion with Asp718/XbaI and was prepared for blunt end ligation. Both sense and antisense constructs were transformed into plants. About 80 *Arabidopsis* and 40 tobacco lines were obtained. However, all the selected plants turned out to be not transgenic, based on the absence of a predicted PCR product.

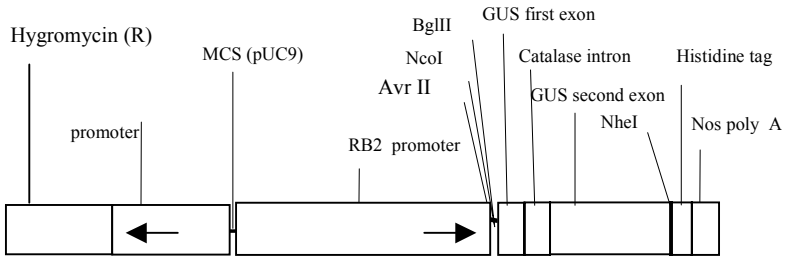
4.11.1. The use of an embryo sac specific promoter to control the expression of plant MCM genes in Arabidopsis

The embryo sac specific promoter was kindly provided by Prof. U. Grossniklaus, University Zurich. The promoter is part of the pCAMBIA plant expression vector and was provided both as a truncated promoter with ~650 bp (pRB1) and as a longer promoter of ~4 kb (pRB2). In both cases the promoter was fused to control the GUS reporter gene. The RB1 and RB2 vectors were modified to remove the GUS gene and to add the rare blunt cutter restriction site SrfI just behind the promoter. Using this restriction site it is possible to ligated blunt end fragments with high efficiency due to the addition of the enzyme during ligation.

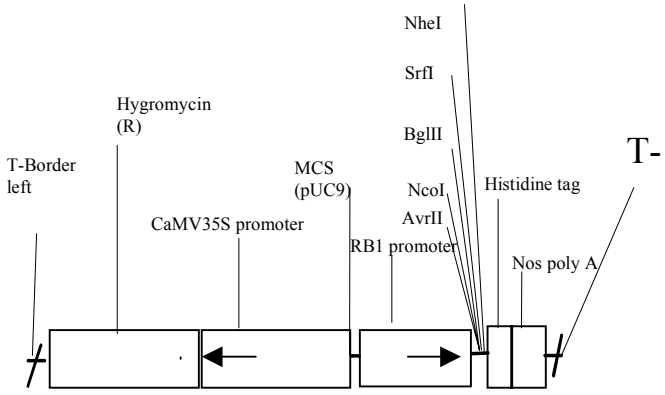
The removal of the *GUS* fragment was performed by the complete digestion of the vector with BglII and by partial digestion with NheI. The digested plasmid was gel purified, ligated and analysed by digestion with NheI. The addition of SrfI restriction site was done by the fusion of a SrfI site containing Nhe1/BglII linker to the BglII/NheI digested vector. The plant *MCM* genes (*TaMCM2*, *VfMCM2*, and *ZmMCM6*) were ligated as blunt end fragments into both vectors (Fig. 24).



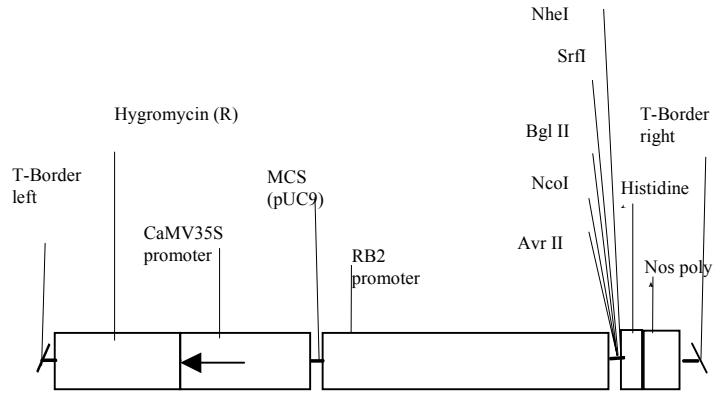
Structure of the CAMBIA vector with truncated promoter (RB1)



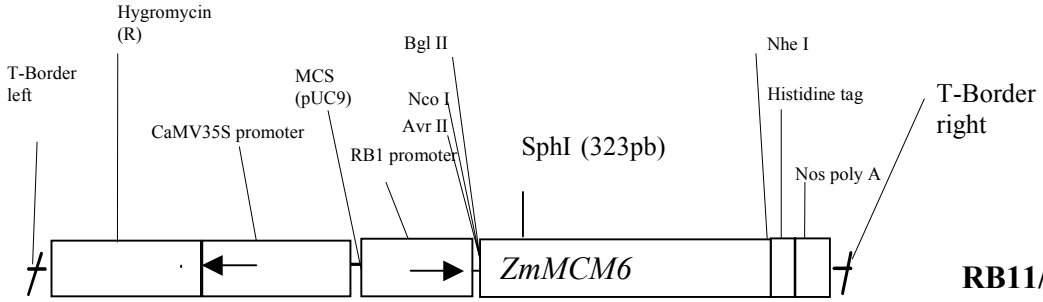
Structure of the CAMBIA vector with full length promoter (RB2)



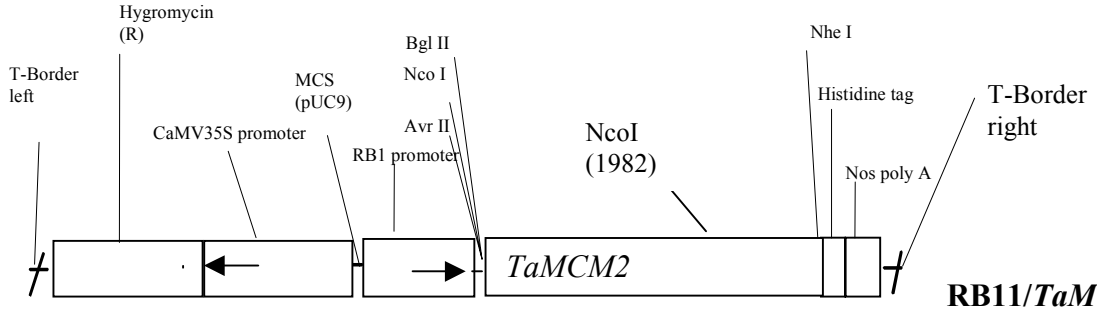
Structure of the CAMBIA vector with truncated promoter and SrfI site (RB11).



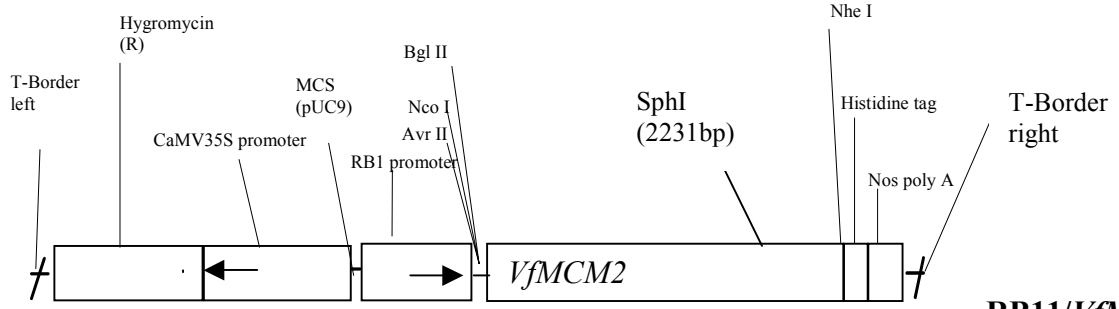
Structure of CAMBIA vector with full length promoter and SrfI site (RB21)



RB11/*ZmMCM6*



RB11/*TaMCM2*



RB11/*VfMCM2*

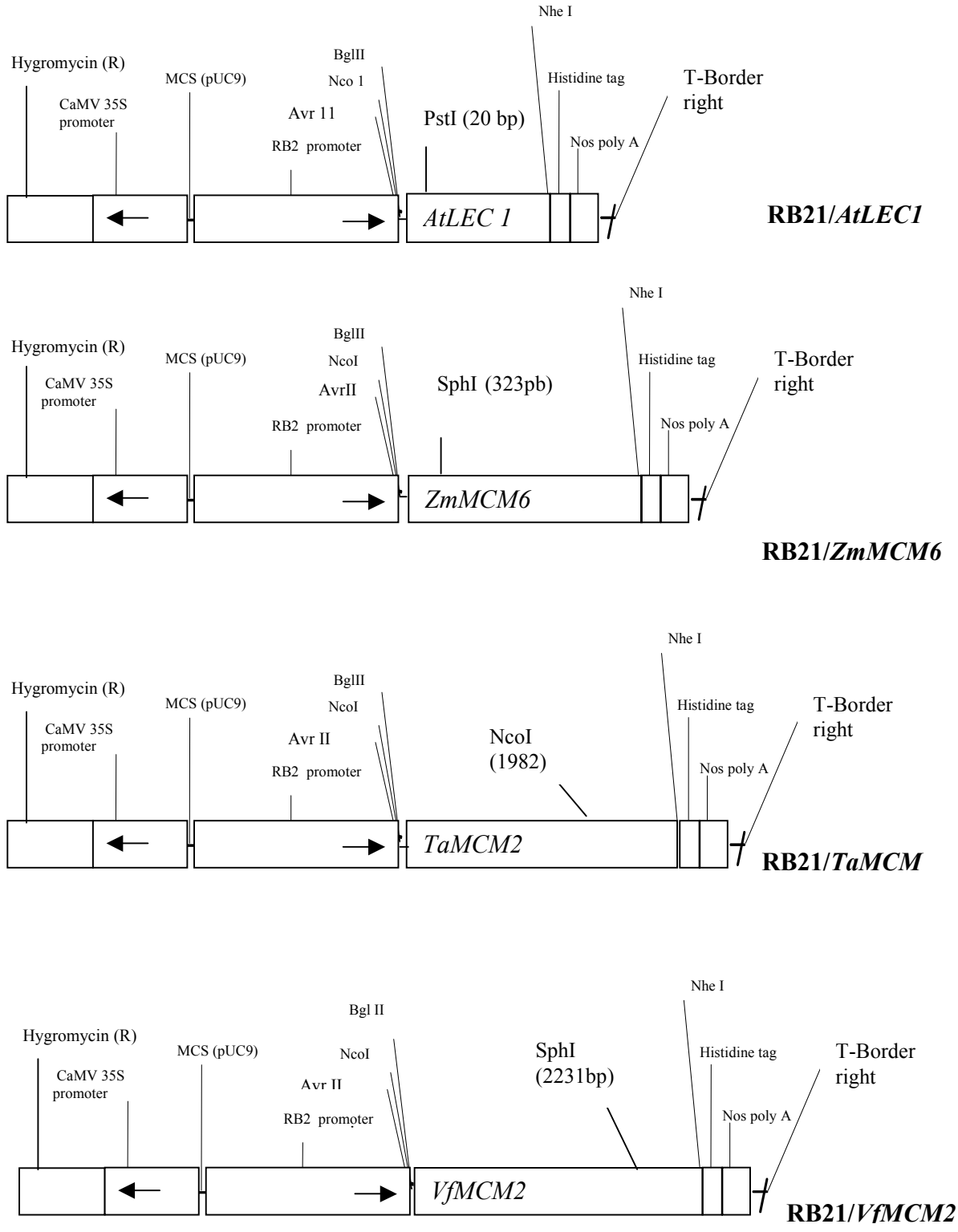


Figure 24

Structure of the constructs used for *Arabidopsis* transformation. RB1 and RB2 are the pCAMPIA vector which contains the truncated and complete promoter respectively in addition to the *GUS*. RB11 and RB21 are the

modified vectors of (RB1 and RB2 respectively) after removing of GUS gene and addition of Srf1 site.

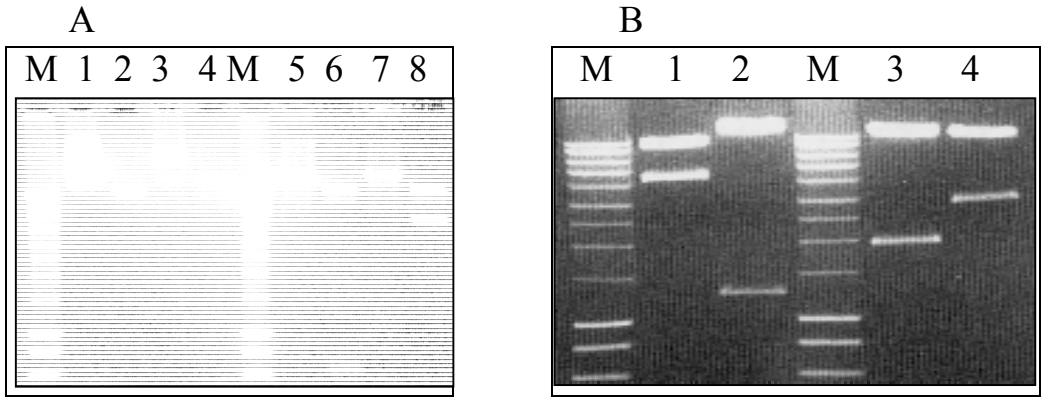


Figure 25
Restriction analysis of pCAMBIA plasmid derivatives.
(A) Analysis of the pCAMBIA derivatives after the deletion GUS reporter gene and introduction of SrfI site.
Lane M- size standard
Lane 1- pRB1, SrfI digestion
Lane 2- pRB11, SrfI digestion
Lane 3- pRB2, SrfI digestion
Lane 4- pRB21, SrfI digestion
Lane 5- pRB1, Srf I/EcoRI double digestion
Lane 6- pRB11, SrfI/EcoRI double digestion
Lane 7- pRB2, SrfI/EcoRI double digestion
Lane 8- pRB21, SrfI/EcoRI double digestion

(B) Analysis of various *MCM* gene constructs cloned in sense orientation into the Srf 1 site of pRB21
lanes M- size standard
Lane 1- construct pRB21/*AtLEC1*, PstI digestion (PstI digests at the pUC MCS)
Lane 2- construct pRB21/*VfMCM2*, SphI digestion (SphI digests in the pCAMBIA just behind the T-Border right
Lane 3- construct pRB21/*TaMCM2* , NcoI digestion
Lane 4- construct pRB21/*ZmMCM6*, SphI digestion

The transformed seeds were selected by growing on media containing hygromycin. Resistant plants were analysed by PCR using a promoter

specific primer located 200 bp upstream from SrfI the cloning site together with a gene specific primer (Fig. 26).

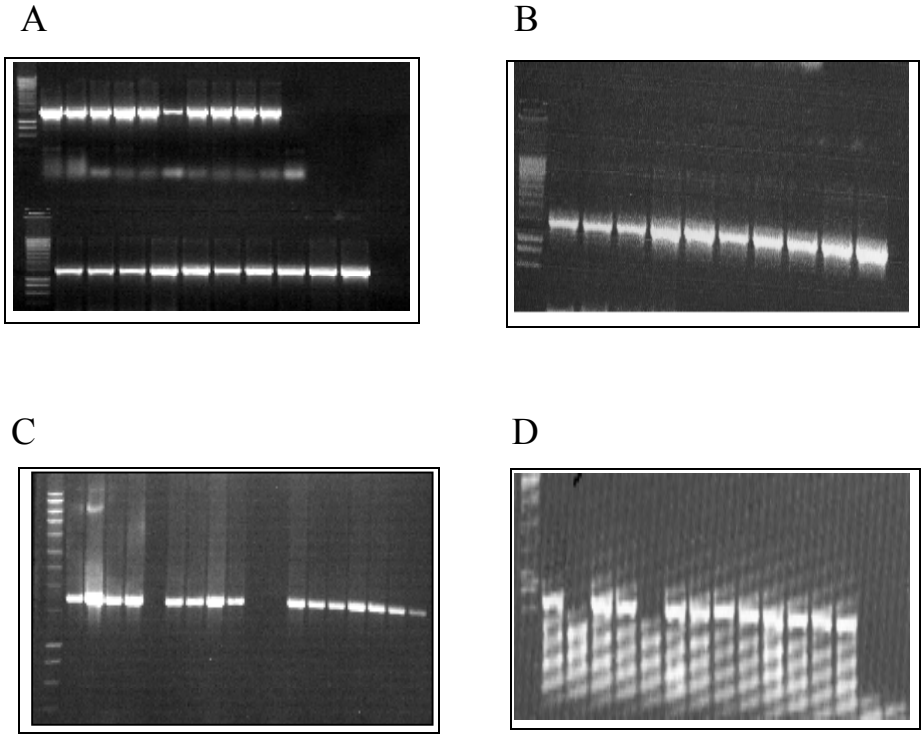


Figure 26.
The PCR products of the transgenic plants using primer specific for the embryo sac specific promoter at 200 bp upstream cloning site and gene specific primer.

Panel A- plants transformed with RB21/*TaMCM2* construct (using gene specific primer at 1233 bp).

Panel B - PCR product of plants transformed with RB21/*VfMCM2* construct(using gene specific primer at 1318 bp)

Panel C - PCR product of plants transformed with RB21/*ZmMCM6* construct (using gene specific primer at 1527 bp)

Panel D - PCR product of plants transformed with RB21/*AtLEC1* construct (using gene specific primer at 597 bp)

4.11.2. The effect of ectopic expression of plant MCM genes in transgenic Arabidopsis plants

The *Arabidopsis* plants transformed with pRB11/ *TaMCM2*, *VfMCM2* or *ZmMCM6* and pRB21/*TaMCM2*, *VfMCM2, ZmMCM6* or *AtLEC1* were selected on hygromycin and they show a high proportion of dead seeds and a high degree of sterility together with abnormal seed

structure as shown in (Fig. 27). The first generation of the *LEC1* gene transformed plants show a prominent defect in siliques. The longitudinal and transitional sections in the transgenic seeds show the seeds become more elongated compared to the seeds of the wild type. The transitional sections demonstrate the better development of cotyledons (Fig. 28).

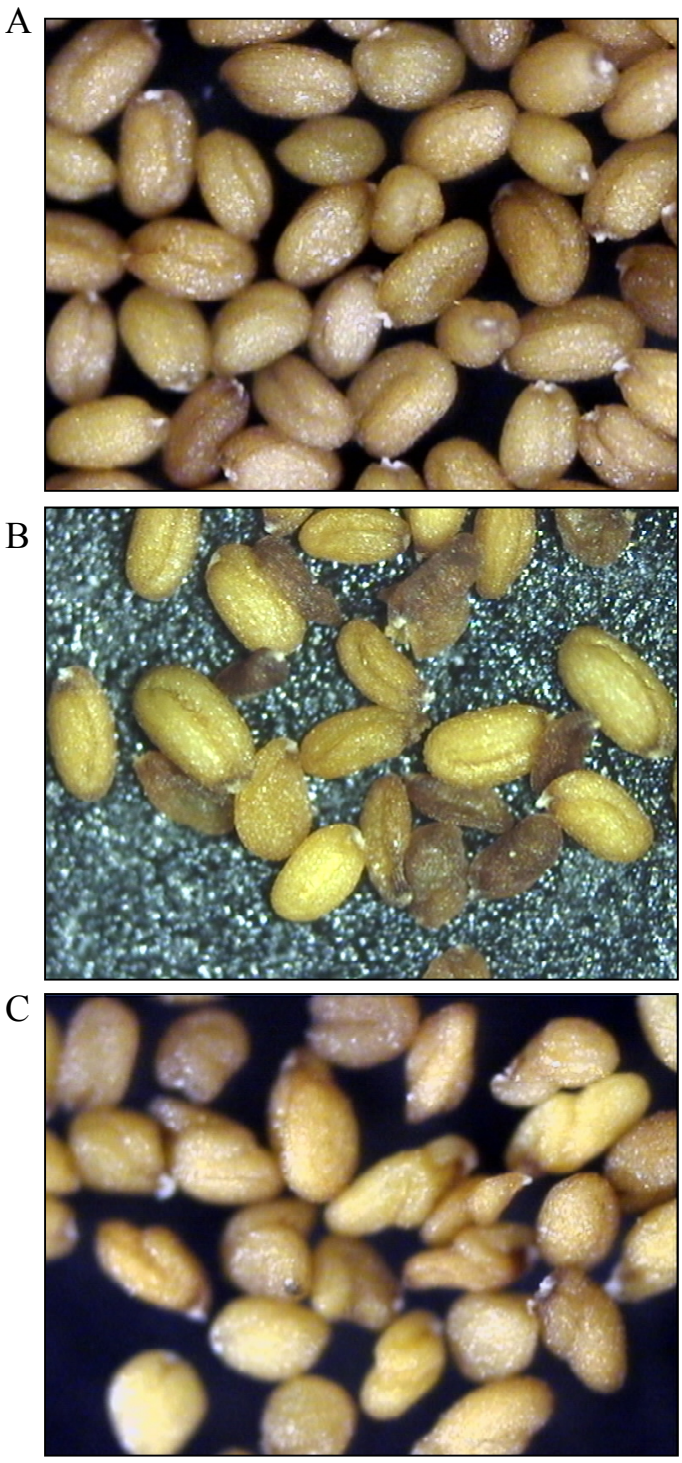


Figure 27.
Effects of the ectopic expression of plant *MCM2* genes as well as the *LEC1* gene in transgenic *Arabidopsis* plants.
(A) seeds of plants transformed with the RB2 promoter and GUS construct.
(B) seeds of the transgenic *Arabidopsis* plants transformed with RB21/*TaMCM2*
(C) seeds of the transgenic *Arabidopsis* plants transformed with RB21/*AtLEC1*

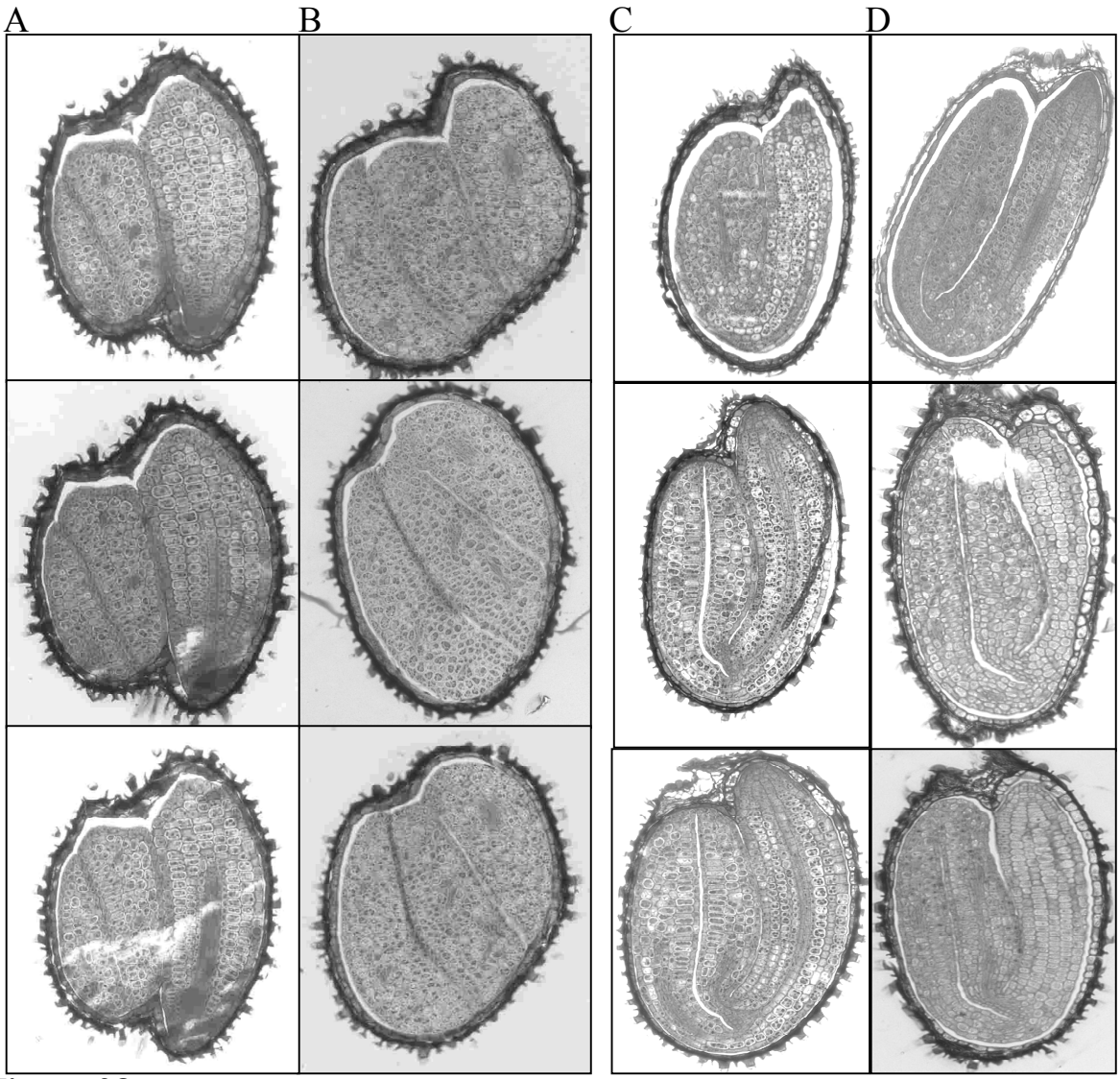


Figure 28.
Panel A- transitional sections of wild type of *Arabidopsis* seeds
Panel B- The of the transitional sections of seeds of *Arabidopsis* transformed with *AtLEC1*.
Panel C- longitudinal sections of wild type of *Arabidopsis* seeds
Panel D- The of the longitudinal sections of seeds of *Arabidopsis* transformed with *AtLEC1*.
(10x magnification)

4.11.3. Flow Cytometric assay of the transgenic seeds

The ectopic expression of *MCM* genes as well as the *LEC1* gene aimed to start the parthenogenesis program in the egg cell or/and the other cells present in the embryo sac of *Arabidopsis* plant. The detection of partheongeneticaly haploid seed became relatively easier with using the flow cytometer assay which can detect the DNA content of the seeds. The sexual seeds show a diploid and triploid peaks which represent the DNA contents of the embryo and endosperm. The parthenogenetic haploid seeds should contain the haploid and diploid DNA which represent the DNA contents of the parthenogenetic embryo and the autonomous endosperm. The screening of the transgenic seeds (of plants which were transformed with *TaMCM2*, *VfMCM2*, *ZmMCM6* and *AtLEC1* show no deviation about the normal DNA content pattern of the sexual seeds (Fig. 29).

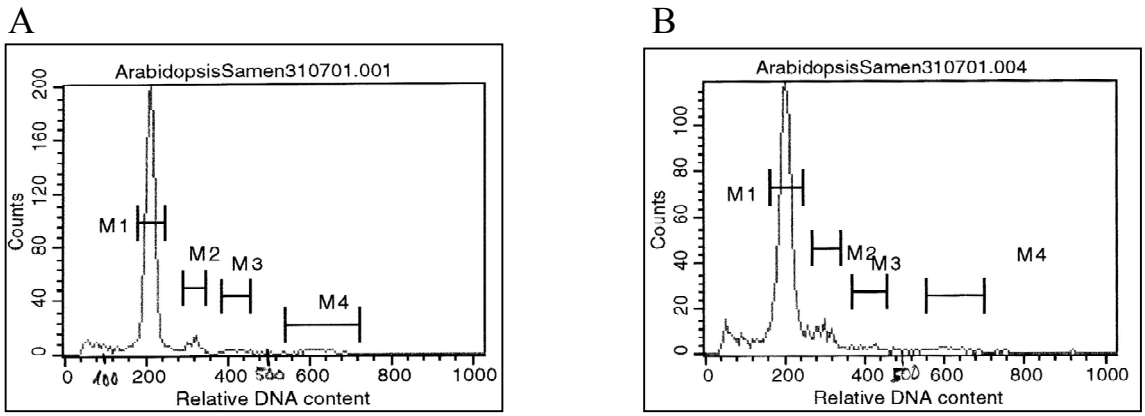


Figure 29.
The flow cytometer assay of the *Arabidopsis* seeds. (A) represents the pattern of wild type seeds of Colombia. (B) represent the common pattern founded in the transgenic plants.

4.12. Generation of subtracted cDNA bank for the isolation of embryo sac specific clones

The parthenogenetic line of the wheat `Salmon` system can produce haploid or diploid plants. The haploid plants lack any embryo sac structure within the ovule while ovaries of diploid plants contain a mature embryo sac (Matzk et al., 1997). Subtractive hybridisation of cDNA was used to identify candidate of genes with an embryo sac specific expression. Gynoecia of haploid and diploid cS plants were collected at an interval of 5 days before anthesis until the time of anthesis. Total RNA was isolated from the gynoecia and the quality of RNA was checked on a denaturing agarose gel (Fig. 30, A). No degraded RNA could be detected and the ratio between the 28S and 18S ribosomal bands is ~ 1:2.5 indicating a suitable RNA quality. The amount of RNA was determined and 35 mg of total RNA from each haploid and diploid plants were used to isolate mRNA and cDNA synthesis. The double stranded cDNA was digested with TaqI enzyme, which cuts eukaryotic DNA approximately every 150 bp. The TaqI fragments were ligated to the TaqI adapters and the efficiency of the process was tested by PCR amplification of the amplicons using the TaqI primers. The amplicon showed a smear ranging from 200 to 800 bp demonstrating the successful digestion and amplification of the TaqI fragments. The subtractive procedures was carried out as described above and the subtracted fragments were fractionated on a gel (Fig. 3o, D). The fragments were isolated and ligated into the PCR cloning vector. Test sequencing of 12 randomly chosen clones showed similarity with some known genes but also detects several unknown genes as showed in (Table 2).

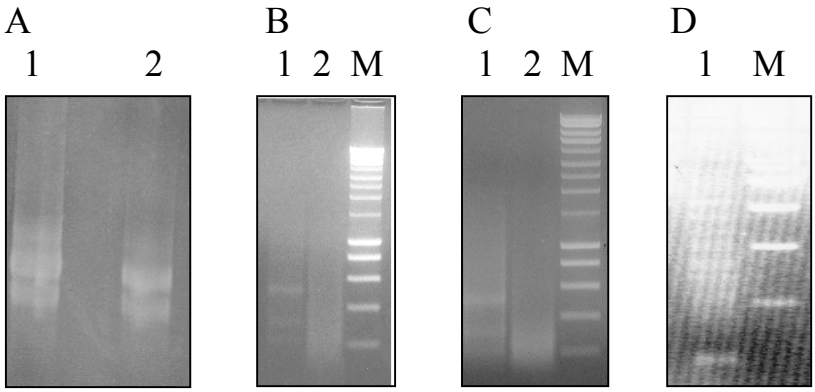


Figure 30.
Subtractive hybridisation of cDNA from embryo sac free haploid gynoecia and embryo sac diploid gynoecia of the cS-line.
(A)- Denaturing gel of Total RNA (1) extracted from haploid gynoecia and (2) extracted from diploid gynoecia
(B)- Amplicon amplification control of haploid the cDNA using a control primers (provided with the kit). (1) the amplification of control template
(provided with the kit). (2) the amplification of the haploid amplicon.
(C)- Amplicon amplification control of the diploid cDNA using a control primers. (1) the amplification of control template (2) the amplification of the diploid amplicon.
(D)- The amplification of the subtracted clones.

Clone	Size	Homology (over 90 %)
B1	400	*Arabidopsis* protein of elongation factor 2
B2	380	*Arabidopsis* SGS2 and SGS3 genes of post transcriptional silencing.
B3	500	Alpha tubulin protein of *Arabidopsis*
B4	300	Unknown *Arabidopsis* protein
B5	250	*V. faba* metallothionein like protein
B6	380	*H. vulgare* ribosomal protein
B7	150	Shows week homology with some resistance proteins in *Arabidopsis*
B8	300	*Arabidopsis* hypothetical protein
B9	550	*Arabidopsis* S-adenosylmethionine decarboxylase
B10	550	*Poa secunda* metallothionein-like protein
B11	250	*Oryza sativa* sircs1 protein
B12	1000	Unknown *Arabidopsis* protein

Table 2.
The homology search of some of cloned subtractive products

5. Discussion

5. 1. A differentially expressed MCM2 gene in parthenogenetic wheat gynoecia

The genetic mapping of apomixis locis did not succeed yet to isolate genes responsible for different elements of apomixis. The isolation of genes with differential expression patterns in sexual and apomictic accessions might provide an alternative approach for the molecular characterisation of genes relevant for apomixis. The isogenic sexual and parthenogenetic lines of the wheat Salmon system is a suitable system for the analysis of parthenogenesis as a single component of apomixis (Matzk et al., 1995; Bäumlein et al., 1996). Tsunewaki and Mukai (1990) demonstrated that the haploid parthenogenesis occurring in the Salmon lines is genetically controlled due to the absence of the parthenogenesis-suppression gene (*Spg*) which is located on the 1BS-chromosome of wheat and by the presence of both, a parthenogenesis-inducing gene (*Ptg*) located on the 1RS-chromosome of rye and a cytoplasmic factor of certain *Aegilops* species.

Comparative cytological studies of the sexual aS line and the parthenogenetic kS line revealed significant alterations interpreted as metabolic changes during the activation of the parthenogenetic kS egg cell 3 days before anthesis. This temporal differences in activation are characterised by changes in the fine structure of cell organelles responsible for synthesis, metabolism and transport of proteins as for instance the increase of the nucleolus and increasing numbers of ribosomes and Golgi apparatus. Further observations describe an increase in the size of the nucleus and the portion of the granular component within the nucleolus, the abundance of ribosomal subunits within the nucleoplasm and their concentration close to the nuclear envelope, the number of nucleoli and a higher frequency of pores in the nuclear envelope (Naumova and Matzk, 1998).

Here, the subtractive hybridisation technique between parthenogenetic and sexual lines was done using gynoecia samples collected from 5 days before anthesis until anthesis. The blast search of one differentially expressed sequence shows a high homologies to *MCM2* gene of human and yeast *MCM*2. The *TaMCM2* is preferentially expressed in the gynoecia of the parthenogenetic line. This could be confirmed at the protein level. Western blotting was used to trace the MCM2 protein in the gynoecial tissues at times points before, at, and after anthesis. Whereas the amount of MCM2 protein was similar in both lines before and after anthesis, the MCM2 protein disappeared in the sexual line at the time of anthesis but was still detectable in the parthenogenetic line. Since the *MCM* gene products are thought to be involved in the regulation of DNA replication, it was hypothesised that the observed changes in MCM2 concentration might be involved in the parthenogenetic process.

5. 2. Pleiotropic functions of MCM proteins

The MCM protein family has been suggested to function as regulators of DNA replication and the cell cycle. The cell cycle control is a complex process that is mediated mainly by protein kinases, which activate proteins with specific functions during the cell cycle. The MCM2 proteins belong to family of six MCM protein, including MCM 3, 4, 5, 6 and 7. All *mcm* mutants appear to affect the same set of autonomous replicating sequences (ARSs), leading to the suggestion that these proteins may function as a hexameric complex. However, in yeast only *mcm2* and *mcm3* mutants were conditionally lethal and exhibit a cell division cycle arrest in the S phase with a doubled DNA content (Gibson et al., 1990; Lei et al., 1997). There is evidence for additional functions -rather than DNA replication- of some members of the MCM protein family. Thus, MCM7 is the only MCM protein which was shown to interact with the important retinoblastoma

regulatory protein in human cells (Sterner et al., 1998). Other data suggest that it plays a key regulatory role despite its potentially passive role in nuclear transport **(**Tye, 1999**)**. Moreover, there are reports describing the interaction of MCM5 with the activation domain of the STAT 1α protein and that over-expression of MCM5 stimulates transcription (Zhang et al., 1998). The use of monoclonal antibodies directed against the MCM2 protein did specifically inhibit the transcription mechanism in microinjected *Xenopus* oocytes. Further results demonstrate that the MCM2, 3, 5, and 7 proteins can specifically bind to the C-terminal domain of the polymerase II (Yankulov, 1999).

The assumption that parthenogenesis is the result of a doses effect of gene products involved in normal sexual development, the known cytological changes in parthenogenetic egg cells (Naumova and Matzk, 1998), the suggested nature of MCM2 as a replication factor and putative licensing factor of cell division and the described observations concerning the MCM2 protein accumulation in sexual and parthenogenetic wheat gynoecia are at least consistent with the suggestion that the TaMCM2 gene product might be involved in the regulation of the parthenogenetic development of the kS and cS egg cells.

5. 3. Structural analysis of the TaMCM2 gene

The complete sequence of differentially expressed *TaMCM2* gene has been determined using RACE techniques. The cDNA sequence is 2923 bp in length. The derived amino acid sequence exhibits a zinc finger motif of the $CX_2CX_{19}CX_2C$-type in the N-terminal region of the protein. This motif seems to be characteristic for the MCM2 subfamily. The functional importance of this putative zinc finger motif in the MCM2 protein has been demonstrated by mutagenesis (Yan et al., 1991). In addition, protein sequence shows two more domains conserved among all MCM2 protein:

Domain II (position 583- 642) and domain III (position 643- 702). All members of the MCM protein family contain a conserved motif for nucleoside triphosphate binding. This motif shows moderate similarity with the NtrC family of bacterial transcription factors with a putative ATPase function involved in the facilitation of DNA melting in promoters (Koonin et al., 1993; Wedel and Kustu, 1995). This ATP binding domain was detected in the sequence of TaMCM2 between positions 532-582. MCM proteins are nuclear proteins. A bipartite nuclear localisation sequence, similar to that found in the SWI5 protein, has been identified in a non-conserved region of MCM3 (Young et al., 1997). The N-terminal domain of TaMCM2 contains one cluster of amino acids (KRHR) that may function as a nuclear localisation signal (NLS). A search of the SWISS PROT database revealed that this motif is present in a number of other nuclear proteins. Interestingly, several of these proteins, such as DNA polymerases and histones are also involved in DNA replication. Moreover, the TaMCM2 protein contains also four putative phosphorylation sites for tyrosine kinases (position 30: RGATDPSSY, position 82: RRMDEQDQY, position 272: KCSLEIDY, position 917: KAQEYEIY) as it is shown by scanning the SWISS-PROT database.

TaMCM2	148-	NFRRP**KRHR**ANFRQ	
ZmMCM3	33-	RDMVN**KRHR**LIIGM	*Q43704*
cdc10	256-	LEQRL**KRHR**IDVSD	*P01129*
DPB2	549-	LSGRF**KRHR**LEFBF	*P24482*
DPOA	1324-	VSPSA**KRHR**FSTWQ	*P26019*
H2B1	21-	ASGGK**KRHR**KRKES	*P02287*
H4	12-	GKGGA**KRHR**KILRD	*P02309*

Figure 32.

Putative nuclear localisation signal in TaMCM2 and other nuclear proteins. The other proteins are identified by the given accession numbers where ZmMCM3 (maize MCM3), cdc10 (transacting factor), DPB2 (DNA pol_E B subunit), DPOA (DNA pol α), H2B1 (histone H2B.1), H4 (histone H4).

5. 4. Gene and promoter structure of TaMCM2

Probing a wheat genomic library with the *TaMCM2* cDNA fragment resulted in the isolation of a 12 kb genomic fragment. Sequence comparison between the 2923 bp of the cDNA and the 8809 bp genomic fragment reveals the complex genomic structure of the gene which includes 18 exons (see appendix). The exon-intron border structure obeys without exception the GT/AG rule. Putative promoter elements are found in the 2400 bp sequence upstream of the translation start codon. Thus, the promoter region lacks a classical TATA box and this seem to be in agreement with the fact that many promoters of cell cycle genes, including human *MCM4* and maize *MCM3* are TATA-less (Sabelli et al., 1999; Korner et al., 1997; Connelly et al., 1998). However, instead of TATA box there is one typical GC box at (nt 544-549). The GC boxes appear to be of importance for recruitment of the basic transcription factors into start sites of housekeeping genes. In this connection, the presence of one GC box at a distant location from the transcription initiation site might regulate the transcription level. One G-box (nt 536-541), and four IIa-boxes (nt 378-382, 420-424, 937-941, 1047-1051). The G-box elements play an essential role in regulation in response to different stimuli in many plant promoters by the interaction with bZIP proteins (Menkens et al., 1995). The IIa-box element has originally been found in the promoter of the proliferating cell nuclear antigen (*PCNA*) from rice (Kosugi et al., 1995). PCNA is the auxiliary protein of DNA polymeraseone δ, and gene expression experiments using mutated promoter of *PCNA* have shown that the IIa element is involved in specifying PCNA mRNA expression in proliferating cells and tissues (Kosugi et al., 1995). This is raising the possibility that the IIa box elements may involved in the meristem-specific expression of the *TaMCM2*. In addition, there is also an ATF element (nt 1879-1884) .

5. 5. Expression pattern of the TaMCM2 gene during plant development

In several systems it was demonstrated that a functional MCM complex is especially required during embryogenesis. For instance, in *Drosophila* it was shown that the mid stage embryogenesis is greatly affected in *mcm2* and *mcm4* mutants (Treisman et al., 1995; Feger et al., 1995).

Another observation dealing with the role of MCM proteins during embryo development concerns the developmentally regulated *MCM6* gene expression during the *Xenopus laevis* zygotic development. A specific zygotic *MCM6* gene was found to be only expressed after gastrulation when the cell cycle is remodelled. The zygotic MCM6 protein is assembled into the MCM complex and differs from the maternal MCM6 in having a C-terminal extension containing a conserved cyclin-cdk phosphorylation site (Sible et al., 1998).

The firstly described member of plant *MCM* genes was the *PROLIFERA* (*PRL*) gene of *Arabidopsis* which was isolated by a gene trapping technique. The gene has been suggested to belong to the MCM7 subfamily (Springer et., 1995). Using a *GUS* reporter the expression of the gene was localised in early leaf primordia, but was eventually lost from the distal domain of immature leaves and in mature leaves. Moreover, staining was observed in root tips and lateral root primordia, but not in differentiated root tissue. The GUS reporter was also expressed in young flower buds, in carpels, and finally in the ovules in older flowers (Springer et al., 1995). The *PRL* gene is expressed from both paternal and maternal alleles in both embryo and endosperm. This rules out imprinting as an explanation for the maternal effect. The *PRL* gene is not expressed in the endoreplicating cells like those present in mature leaves or at later stages of endosperm development.

This is in contrast to what was found in *Drosophila*. Here, the MCM proteins have been detected also in endoreplicating cells, bound to chromatin during endocycles (Feger et al., 1995; Su and O`Farrell, 1998). These results demonstrate putative differences in MCM function between plants and animals and suggest that in plants the expression of MCM genes are necessary to connect the S phase with mitosis.

The isolation of a 2.4 kb fragment upstream of the start codon of the *TaMCM*2 gene was used to analyse the expression pattern of the wheat gene. The GUS staining shows the promoter activity of the *TaMCM2* gene is localised in parts of the cotyledons and in the hypocotyl region. The work of Springer et al. (2000) reports a missing cells in the central and apical region of the *prolifera* mutant embryo, a regions which contributes to the hypocotyl and cotyledons. The GUS staining of other plant parts shows no promoter activity in the apical meristem. The expression starts in the sepals of young flowers and become stronger before and at anthesis. In stamen, the GUS expression was initiated before meiosis and remained localised in the anther locules after anthesis. In the ovule the histological sections show no indications of GUS expression before or at anthesis. No GUS activity could be detected at early and late embryogenesis stages as shown in Fig. 5 (I and L). These results have similar aspects to what was described by Springer and Holdig (2000). These authors showed the accumulation of *PRL* to a high level in the initials of stamen primordia and later during the initiation of anther locules. After that the expression decreased but was maintained in the pollen mother cells and their subsequent meiotic products. In the experiments described here it seems that the *TaMCM2* gene expression reaches a higher level at a time when the *PRL* gene expression starts to decline. No detectable GUS activity was found during the different stages of embryogenesis or at later stages of seed maturation. The bases of siliques are the only region that shows GUS staining. These results indicate that the *TaMCM2* differs from the expression pattern of the *PRL/MCM7* gene of

Arabidopsis. The *TaMCM2* expression is more restricted to the plant gametophyte before and at anthesis, it seems not to be expressed in meristematic tissues and is most likely not involved in the embryo development. Part of these differences could be explained by the use of a the heterologous dicot *Arabidopsis* as transgenic host. Furthermore, the sensitivity of GUS detection might not be high enough to detect gene expression at the single cell level. Therefore, experiments were performed to detect the TaMCM2 protein at a cellular level using polyclonal antibodies.

5. 6. Production of polyclonal antibodies against TaMCM2 and the subcellular localisation of TaMCM2

The synthesis of sufficient amounts of TaMCM2 protein and its efficient purification is a prerequisite for the preparation of antibodies for histological staining. The MCM2 proteins have been expressed in *E. coli* and the product protein can accumulate without toxic effects (Sherman et al., 1998). The antibodies were raised in rabbits against the purified TaMCM2 protein. These antibodies gave a strong reaction with the purified protein as shown by ELISA. It also reacts specifically as shown by Western blot using a bacterial lysate, inclusion bodies and the purified antigen. The application of this antibodies against the plant proteins showed the preferentially expression of the TaMCM2 protein in the wheat gynoecium collected up to 5 days before anthesis. This result is in context with the observations of promoter activity in transgenic *Arabidopsis* where the promoter becomes active in sepals before and at anthesis. The subcellular localisation of the protein and its localisation in the ovule structure was necessary to understand its putative role in parthenogenesis.

The subcellular localisation of the MCM complex through the cell cycle shows a difference between yeast and mammals. In mammalian cells, the MCM complex remains in the nucleus throughout the cell cycle and is

firmly attached to chromatin during mitosis and G1. It is displaced from chromatin during replication, a process thought to require phosphorylation by cell division kinase (CDKs) and CDC6 (Kearsey and Labib, 1998). Reassociation with chromatin does not occur until the following mitosis, so that the cycle of association and disassociation regulates the number of times chromosomes are replicated during each nuclear division. However, the protein remains in the nucleus throughout cell division in animal cells, even after the envelope breaks down. In budding yeast, where the nuclear envelope does not break down, the CDC47 (MCM7) shuttles in and out of the nucleus during the G1 and S phase respectively (Sterner et al., 1998). The work on the PROLIFERA protein shows that it does not stay in the nucleus throughout the cell cycle, resembling the situation in yeast.

5. 7. Complementation assays of the yeast mcm2 mutant by TaMCM2

Due to the strong similarities between the MCM2 proteins, it was investigated whether plant *MCM*2 genes of wheat and broad bean (*TaMCM*2 and *VfMCM*2) could functionally complement the *mcm*2 yeast mutant. This yeast strain cannot grow normally when the temperature is elevated to 37 °C (Maine et al., 1984). The normal growth of the mutant yeast was retained when the yeast genomic fragment containing the *MCM*2 gene together with its promoter was integrated into the yeast genome. The expression dosage of different members of MCM genes are not equal, but they might be required in a precise ratio at the ARS (Lie et al., 1996). Nevertheless, the over-expression of MCM2 could complement the *mcm2* mutant and partially also the *mcm3* mutant (Yan et al., 1993; Tye, 1999).

The over-expression of the yeast MCM2 gene as well as plant MCM2 genes in the yeast mutant strain show the unstable accumulation of the proteins if compared to the protein accumulation in wild type. The week capability to accumulate MCM2 proteins was also demonstrated by Forsburg

et al. (1997). The low accumulation of plant MCM2 proteins in yeast cells may be due to the failure of the proteins to undergo post translational modifications necessary to prevent rapid proteolysis.

A first complementation test using the yeast and plant *MCM2* genes was based on the measurement of the optical density to the growth rate in liquid culture. The comparison of the growth curves did not show any significant differences between the mutant and those cells which were transformed with the yeast-, wheat- and broad bean-MCM2 genes (*ScMCM2, TaMCM2, VfMCM2*). This is in contrast to the results obtained by growth tests on solid media. Here, the *ScMCM2* gene exhibits the capability to complement the mutant phenotype at 37°C. Unfortunately, the two plant *MCM2* genes failed to complement the mutant phenotype. A similar result was also obtained using the flow cytometric analysis to determine the DNA content of the mutant. The majority of the mutant cells grown on 37°C have a 2n DNA content whereas the yeast *MCM*2 gene transformants shows a 1n DNA content. The plant MCM2 gene transformants show no significant difference. The appearance of the 2n DNA content is a characteristic feature of a prolonged S phase. The *mcm2* mutant fails to complete the DNA replication during the S phase as fast as the wild type. The prolonged S phase and 2n DNA content characterise many mutants of cell cycle regulators which control the G1 to S transition (Jallepalli et al., 1997; Zou and Stillman, 2000). The complementation test of the yeast *mcm2* mutant using plant *MCM*2 genes and the yeast *MCM*2 gene indicates the existence of specific mechanisms for the function of MCM proteins. Obviously, these mechanisms do not depend on the conserved regions of the MCM subfamilies, but may depend on specific structures.

5. 8. Ectopic expression of plant MCM genes and the LEC1 gene using an Arabidopsis embryo sac specific gene promoter

The preferential expression of the *TaMCM2* in the parthenogenetic line of the Salmon system, lead to the hypothesis that the ectopic expression of *MCM* genes within the embryo sac might lead to the induction of parthenogenesis. To test the induction of parthenogenesis, a *Arabidopsis* embryo sac specific promoter was used to direct the ectopic expression in the egg cell of *Arabidopsis*. The first gene used for ectopic expression was the parthenogenetically expressed wheat *MCM2* gene. To avoid possible differences between monocots and dicots, the *VfMCM2* gene of *Vicia faba* was used in addition. In addition, the *MCM6* gene of *Zea may*s (*ZmMCM6*) was used. This gene was chosen since this gene was found to be induced by *in vitro* fertilisation (Th. Dresselhaus, unpublished data). The gene was kindly provided by Dr. Thomas Dresselhaus, University of Hamburg. Finally, the *Arabidopsis* transcriptional factor encoding *LEC1* gene was used. The *LEC1* gene product is considered to be an inducer of the embryogenesis program in the vegetative cells (Harada et al., 1998).

The plants transformed with the *MCM* genes show a high ratio of infertility and plant inviability. In the case of MCM gene transformed plants seeds are dark coloured and shrunken. The flow cytometric analysis of the DNA content of the seeds did not show any indication for a parthenogenetic development of the egg cell.

The plants transformed with the *LEC1* gene show a normal vegetative growth but suffer from high infertility. These plants show an arrest in the silique development and low seed yields. In comparison to the control plants transformed with the promoter/*GUS* fusion, the seeds of *AtLEC1* gene transformed plants show an abnormal seed phenotype. The longitudinal and transitional sections of the seeds of the *AtLEC1* transformed plant show a better development of cotyledons comparing to the wild type seeds. The size increment of cotyledons may resulted from a prolongation of the early

embryogenesis phase (before globular stage) where the cotyledons start to develop. One suggested mechanism of *AtLEC1* ectopic expression is to be mediated by the delay in activating post embryonic program (de Vries, 1998). The seeds of the *AtLEC1* transformed plants have an elongated irregular shape and their size are usually bigger then the wild type seeds.

5.9. A candidate approach for wheat embryo sac specific genes

The subtractive hybridisation between the haploid and the diploid plants of cS line at the time five days before until anthesis produce a DNA fragments which range from 150 bp to 550 bp. The searching homology in the known data bases show that they belong to different genes which are generally active in the actively dividing cells. The clones B1 shows 91% homology at the protein level with the elongation factor EF2 of *Beta vulgaris*. It localises in the cytoplasm and promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the p-site of the ribosome. The phosphorylation with EF-2 kinase cause a complete inactivation of the EF-2. the B3 clone shows 100 % homology with *Arabidopsis thaliana* tubulin alpha-6 chain (TUA6) . A specific alpha tubulin was proofed to be associated with the initiation of parthenogenesis in salmon wheat lines (Matzk, et al., 1997). The clone B6 shows 94 % homology with the ribosomal protein gene family of *H. vulgare*. These genes are highly expressed in meristematic and young leaves and in root tips (Madsen, et al., 1991). The clone B11 shows 85% homology with the *Oryza sativa* protein phosphatase 2A regulatory subunit B (B 56 family). The protein phosphatase 2A (PP2A) is a major intracellular protein phosphatase that regulates multiple aspects of cell growth and metabolism. The ability of this widely distributed heterotrimeric enzyme to act on a diverse array of substrates is largely controlled by the nature of its regulatory B subunit. There are multiple families of B subunits. The clones B5, B9, and B10 show

a strong homologies to *V. faba* metalothionin like protein, *Arabidopsis thaliana* S-adenosylmethionine, and *Poa secunda* metalothionin like protein respectively. These genes are involved in the pathway of DNA methylation and they may regulate the gene expression during the embryo sac formation.

Conclusions

The *TaMCM2* gene of wheat has been cloned and the genomic structure has been determined.

A *TaMCM2* promoter GUS construct was transformed into *Arabidopsis* and shows preferential activity in flowers at anthesis.

Polyclonal antibodies against *E. coli* expressed TaMCM2 protein have been generated used for detection and cellular localisation.

Although MCM2 proteins are highly conserved, plant *MCM2* genes do not complement a corresponding *mcm2* yeast mutant.

Plant *MCM* genes as well as the TF gene *LEC1* have been expressed in *Arabidopsis* under the control of two versions of the embryo sac-specific promoter RB1/2. A more detailed embryological analysis is in progress. Close to one hundred transgenic *Arabidopsis* lines need a further detailed molecular and embryological examination. A new egg cell specific gene promoter (pRB22) will be manipulated as a suitable binary vector for the analysis of egg cell specific genes which were isolated for instance from egg cell specific libraries of sexual and parthenogenetic lines.

Zusammenfassung

Die Kontrolle und Nutzung apomiktischer Samenbildung wird als eines der herausragenden Ziele der Pflanzenzüchtung und Produktion eingestuft. Gleichzeitig stellt die Aufklärung der zellulären und molekularen Vorgänge beim Übergang zwischen Sporophyt und Gametophyt und umgekehrt eine Herausforderung für die Grundlagenforschung dar.

Apomixis umfasst in Wesentlichen drei Komponenten: Vermeidung der Meiose (Apomeiose, Diplosporie), befruchtungstunabhängige Initiation der Endospermbildung (Parthenkgenese) sowie die teilweise befruchtungstunabhängige Endospermbildung.

Das aus drei isogenen Weizenlinien bestehende `Salmon-System` repräsentiert unikales Ausgangsmaterial zum Studium der Parthenkgenese als eine Komponente der Apomixis. Während eine Linien sich rein sexuell vermehrt, erfolget die Initiation der embryogenesen in den beiden anderen Linien befruchtungstunabhängig durch Parthenkgenese. Verbuche an isolierten, *in vitro* kultivierten Eizellen zeigen, dass dies eine Eizelle-inhärente Eigenschaft ist.

Arbeiten zur Isolierung von Genen mit differentieller Expression in den Gynoecien der sexuellen *versus* der parthenkgenetischen Linien führten zur Identifikation von Genen der *MCM*-Familie. MCM stht für mini chromosome mainentance. Entsprechende Gene wurden zunächst in Hefe beschrieben. Homologe mit hochkonservierter Struktur finden sich aber in allen untersuchten Organismen. MCM-proteine bilden einen hexameren Komplex und sind im Zellzyklus währed des G1/S Übergangs an der Initiation der DAN-Replikation beteiligt.

Die Charakterisierung eines in parthenogenetischen Weizen-Gynocien bevorzugt exprämierten *MCM2*-gen *(TaMCM2)* betrifft zunächst die Aufklärung der komplexen Exon-Intron-Struktur sowie die Isolierung

der Promotorregion. Promotor-Reortergen-Konstruktionen wurden in *Arabidopsis* transformiert und zeigen komplex Expressionsmuster. Für eine weiterführende Charakterisierung, insbesondere durch die Erzeugung von spezifischen Antikörpen, wurde das *TaMCM2*-Gen in verschiedenen bakteriellen Expressionssystemen exprämiert sowie Antikörper erzeugt und zur Detektion des Proteins eingesetzt.für eine funktionelle Analyse des *TaMCM2*-Gens wurden Versuche zur Komplementration der mcm2-Hefemutantte durchgeführt. Offfensichtlich kann das Pflanzen-Gen die Mutante nicht komplementieren. Darüber hinaus wurden durch subtraktive Hybridvisierungsverfahren Kandidaten für Embryosack-spezifischen Gene isoliert. Schließlich wurden unter Nutung eines Embryosack-spezifischen Genpromoters verschiedene MCM-Gene aus Weizen, Mais und Ackerbohne (*TaMCM2, VfMCM2, ZmMCM6*) in transgenen *Arabidopsis*-Pflanzen untersucht. Parallel dazu wurde das Embryogenese-induzierende Leafy Cotyledon (LEC1)-Gen für diese Versuche eingesetzt. Eine eingehende embryologische Auswertung dieser Pflanzen ist noch in Arbeit.

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Appendix

Appendix 1.

The alignment between the *TaMCM2* cDNA and the genomic fragment shows the exon intron structure of the wheat *MCM*2.

```
GenoS
     TCGACGATCAGGAGTAGTTAGGAGGCTCCTAGGCAGGAGGCCTTGCCTTTTCAATCGTAG 60
     ATGCTTTTGTGCTGGCCTTCTTAAGGCAAACTTGTCTAACTTATGTCTGTACTCAGATAT 120
     TGTTGCTTCCGCTGACTCTTGTGTATTCGAGCCCTCGAGGCCCATGGCTTGTAATACAAA 180
     ACTTGTATTATTTTTATTTGTGTCTAGAGTTGTGTTGTGATATCTTTCCACGAGTCCTTGA 240
     TCTTGATCGTACATATTTGCATGTATGATTAGTGTACGATTGAATCGAGGGCGACACAAC 300
      TTGCCCAATGATCGACAAGCCATTGAGAATTAATGGATCTTCAAGAGGAAGACGGACGCT 360
      GATGGTAGTGTTACTATCTAAAAAAAGAAAACATGTTTTTTCATGCAATTTTATTTGATC 420
      TAAAAGATAAGAAAGACCGATGAAAAACCGAAACGTCGGAAAACTCCAAAAAAACCCGTT 480
      TAAAAAGCCGAAAAAGTTTGCAGAAAAAAATCCAGAGGAAGCGCCCAGAGCGCGACCACGT 540
      GGCGGGCGGCTGAGAGCGCCAAGTGACGGGAGCTCCTATCTGCCGCTTATTCCAGCAGAC 600
      TGTCGGGCAAACGCACAAGCGATCCGACTGGACCGGCCCACTGTCGAACGAGGGATCTGT 660
      GTATCGAGAAGCGATCGAACCAGAGACCTCCAGCATCACGGAATGTTCTGCTAGCCAGAA 720
      GAAACAACCACCCTGAGAGATTAAATACCAGCGCAACTCTATAAGAACAAAAAAGCAACG 780
      CAGATCCAACATTTTTGTTTTTCTTTTTTCTTCCGTTTTTGATTTTTTTGTTCGAAATTT 840
      GAAAACATGCTCTCCACACTGACGTTACTTCACAAATTAGAAAAAAATGTTATGTTTTCA 900
      AAAATAATTCGTATATTTCAGAAAATGTTCAGGATTCTAAAAATGGTTCATGTTTAAAGA 960
      AATCCGATTTGTTTTAAAAAAGAATGTTTTATGAAACATGATCGCTTTTCAAAAAAAATA 1020
      ACAAATTTTGACAATACAAGCATTTTCTAAAAATGCGAACAATTTTTGAAAATCACCAAA 1080
      AATATGTGAACATATGGACTTTTATTTGAAAACAGGAAATCTAATTTTCAACTCTTAACA 1140
      AAACTATTAGACTTGAACATTTGTTAAAACTGCCAAAAATAAAATAAAATATGACATTTT 1200
      TTGAAAGCAGGAACAATTTTTCGAAACACAAACATTTTTTAACTGTGGAACAAATTTGGA 1260
      AACATGAAGAATTTTTGTAAACACGAACATTTTTTTAGAACGAGAACATTTTTTGGAAAC 1320
      ACAAAAGGTTTTTGAAAATATGATTTTTTTTAAACTGCAGAACTAATTTGGAAATATGAA 1380
      CAAATTTTGGAAACAAGAACATTTTTTGAAAATGAGATTTTTTTTTCGAAATTTGCAAACA 1440
      ATTTTTTAAAACTGGAACAATTTTTGGAAAAACAAAAGTTTCTGAAAAGACGAACATTTT 1500
      TTAAAACCGCAGAATCAATTTGAAAACCTGAACAATGTTTGGAAACATGAACATATTTTG 1560
      AAACTCCCATTTGTTTTGTAAAATGCGGATTGTTTTATTAATAATGAAAGATTTTCAAAA 1620
      AACATGATTTTTAAAATAAATATGAAAAAGGAAACAAAAAGAAACAGAAAAAAGAAAATA 1680
      AATTAAATAGAAAATAGTTCAGGAACCTCCTAGGAGGTTCTCAAAACCAGAAAATACCAG 1740
      CTTGGAACGTCCTGGAAGGTTCTCAAAATTGGGTTGGCTGGAGCGCTCGACGGGCCGGCC 1800
      CACATCATCCCTCGCTTGCGAACCTTGTGTGAAAGCTCGACAACCTGCGCAGTGTGTGTC 1860
      CTATATTTTTTTTTTCAAGTGACGCTGATCGTTGTGAGGCTGCTGAAAGAGCGCTCGTCA 1920
      ACTTTTTTTTTGAGACAAAGAGCGCTCGTCAACTAGTTGCTCACGTTGGATGGGTCACAC 1980
      CTTAGGCTTCTCCCAACTCAGTCTCAGCCCACGATGTATGAAGGGCCTGCAGTGTTGTAA 2040
      TGTACAATTGGCACAAGGCTTTCGGCCCATTTGTAGCATCATAACCAGCCCGTGCCGCCT 2100
      CCCCCTTCCCGCCATCACCCACCCCACTTCCCGCCGAAACCCTCTCTCCCTCTCCCCCAA 2160
      TATATCCCCTTCCCGCCGAAACACAGTCTCTCCCCACCATTCCCCCCTTCCCGCCGAAAC 2220

      GenoS CCTAGCCTCGGCGCACAGCCACACGCGTCCTCCACAGGAGCCTCCTCCGACCCCGCGGAT 2280
cDNAS       ..................CCACACGCGTCCTCCACAGGAGCCTCCTCCGACCC.GCGGAT

     GenoS  GGTAAGCGGCCCGTCTCCCCCAGCCTCCCCTCTCTGCCCGAACCACGCCGCCGCCGCGCG 2340
cDNAS       G.....................................................

     GenoS  CGGTCGTCGCCGCCTCCGGATCTAACCTTCTCGCCGGCTGATTTGCTTTTCCTAGGACGA 2400
cDNAS       ..................................................GACGA

     GenoS  CTCGGAGAACAACGCGCCGTCGACCCCGGGCTCGCCGGGCTTCAGCACCGACCGGCTGCC 2460
cDNAS       CTCGGAGAACAACGCGCCGTCGACCCCGGGCTCGCCGGGCTTCAGCACCGACCGGCTGCC


     GenoS  GCCCAACACCACCACCAGCCGCGGCGCCACCGACCCGTCCTCCTACTCGGACGACGATGG 2520
cDNAS       GCCCAACACCACCACCAGCCGCGGCGCCACCGACCCGTCCTCCTACTCGGACGACGATGG
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     GenoS  CGAGGCGGAGGTCGACCCCAATGTGCTCCCCGAGGACGACGGCGCCACCGTTATCCGCGA 2580
cDNAS       CGAGGCGGAGGTCGACCCCAATGTGCTCCCCGAGGACGACGGCGCCACCGTTATCCGCGA

     GenoS  CGAGGAGGAAGACGATGGGGAGGACCTCTTCAACGACGACTACCTCAAGTAAGACAGAAA 2640
cDNAS       CGAGGAGGAAGACGATGGGGAGGACCTCTTCAACGACGACTACCTCAA...........

     GenoS  CGTCCGAGCTGGAGATTTTCCGGATCTAGTGTTATTTGGGACAGCGTTTAGGTGTTTCTA 2700
     GenoS  CTAGTAGTTGACAGGTTTAGTCCGTTGGATCTGGCAGTAAATTGGTTATTCACCGACTTT 2760
     GenoS  TGGGGTTCGAGAATCTAGGGATTGCCGCCTAGATTTACTTCATAAATAGTCAGATTTGTG 2820
     GenoS  GCACAAACAAGGGAATAGTCAAACACTTCACCGGATTGTGTCTTTTTTGTTACCTTTAAT 2880
     GenoS  GTTTTGTTAGTTCCTTGAACTGTACAATTTAGAAATGTCTGCTATCCTCCTGTATTGAAT 2940
     GenoS  ATTAAAACCTGATGTTTGTGCTCGGTAATTTGTTGGGGTACATTTCAGTCTACCAATTTC 3000
     GenoS  AGTGGCCTATATGAACTATTACCTGTCATGTCCTATCCCTTTTTGATGAATTAGTTGTCT 3060
     GenoS  GAATAGGATTTGTTCATCAGTTGTCTGCTGTCCAAATGCATTGTATTGTTCAGTCATGTA 3120
     GenoS  CACAAATTAACTGATCCTCTCTGTTGTCCTGCAGTTTTGCCAATGTGTTTTTTTTACTCT 3180
     GenoS  GGATTCATTGATGTTCTGAAAACTGCATTGTTGAGTAATAATTCATTTTGCTATACAGTT 3240
     GenoS  TGGTATTTGGTTTCAAGATAGTTCAATTAACTAGAGGATTGCCTTTTTAGTGTATGAAAT 3300
     GenoS  AAGCCATACCTCAAACAGACACAAACATGTTTGAAATTGTGAAGTTCTTGCTAGCCCTCT 3360
     GenoS  TATTTTTTTCAACCTTTTTGCAGTGATTTACCGAAGAATGGATGAGCAGGATCAGTATGA 3420
cDNAS       ..................... TGATTTACCGAAGAATGGATGAGCAGGATCAGTATGA

     GenoS  GTCGGTTGGGTTAGACGACTCGATAGAGGATGAGAGGAACCTGGATGAGATCATGGCTGA 3480
cDNAS       GTCGGTTGGGTTAGACGACTCGATAGAGGATGAGAGGAACCTGGATGAGATCATGGCTGA

     GenoS  TCGAAGGGCTGCAGAAGCGGAACTTCATGCAAGGGATGTGAGGACTGGTGCAACAGCTGA 3540
cDNAS       TCGAAGGGCTGCAGAAGCGGAACTTCATGCAAGGGATGTGAGGACTGGTGCAACAGCTGA

     GenoS  TCGAAAATTACCTCGTATGCTTCATGATCAGGGTAATGGCATCACACACAGTTCTTATTG 3600
cDNAS       TCGAAAATTACCTCGTATGCTTCATGATCAGG............................

     GenoS  TCTATGGAGTATTAGGACAAACCTTACCTTTTTCCTTTGCACTTATATTCTGTTTGTTTG 3660
     GenoS  AATTGCTGCTTGAGTATTTGGGTTATGTTTATTTTTCATATTGTTAGTTGTGGTGAAATG 3720
     GenoS  TTCTTTCTTTAGTATGTTTGAAATGAGCATATTTTTTCCCTTGTGGTTTCAAGTTTGATT 3780
     GenoS  GAAATGGCACATGGCTAGGAACTCTGAAGGCCATATAATAACACTAATTTTTTCTTAAAA 3840
     GenoS  TATGAATAACTAGTATCATATTTACATTGTGTACTTTTCTGACCGTCTCCATTTATCAAA 3900
     GenoS  AGACATTTCCCAAAAGATGTGATGTATCTTCAAAAATTAGAATCACTATTTAATGATTTA 3960
     GenoS  TACAAAAATAAGTAAGGTTGGATTTGCTTGAGGACCTTAGTTATAGTATCCTCATGGATG 4020
     GenoS  GTTATGACTCGTGGAGTCTTCCTCAGTAGTTGGTATCCCTGATTGATAACATAAAAAGCA 4080
     GenoS  AATGTTTGGATGTCAGTCATTGTAAATTAAGGACAACATTTCTTCGATTGTGCAGATACA 4140
     GenoS  GATGAGGATATGAACTTCAGGCGTCCTAAAAGGCACAGGGCTAATTTTAGACAACCAAGT 4200
cDNAS       .ATGAGGATATGAACTTCAGGCGTCCTAAAAGGCACAGGGCTAATTTTAGACAACCAAGT

     GenoS  GGAGGACCAAGAACACCCAGAAGTGATGATGATGGTGATGGTCTCACTCCTAGTTCACCT 4260
cDNAS       GGAGGACCAAGAACACCCAGAAGTGATGATGATGGTGATGGTCTCACTCCTAGTTCACCT

     GenoS  GGAAGATCTCAGCCATATTCTGGCGGTGATGTGCCTATGACTGATCAGACCGATGATGAT 4320
cDNAS       GGAAGATCTCAGCCATATTCTGGCGGTGATGTGCCTATGACTGATCAGACCGATGATGAT

     GenoS  GGATATGAGGTATTTTCATTTTGAACGAAAATTAATCATGATTTTGTTTAGCATTTGCTG 4380
cDNAS       GGATATGAG...................................................

     GenoS  TTGAATCTGTTCCTATCTTCTAGATATTTCAGTTGAAATAGGCAACTCATGTTTGAAGGG 4440
     GenoS  CCTATATGGTTGTCCAGTTTTCAAATGGTTGTATTTCTGCAGGATGAATTTGATGAAGAA 4500
cDNAS       .........................................GATGAATTTGATGAAGAA

     GenoS  GATGAGATGAACATGTATCGTGTGCAAGGAACACT.CGAGAGTGGGTCACAAGAGATGAA 4560
cDNAS       GATGAGATGAACATGTATCGTGTGCAAGGAACACTTCGAGAGTGGGTCACAAGAGATGAA

     GenoS  GTCCGGCGCTTCATCGCAAAGAAATTTAAAGAATTTCTTCTTACATATGTAAACCCTAAG 4620
cDNAS       GTCCGGCGCTTCATCGCAAAGAAATTTAAAGAATTTCTTCTTACATATGTAAACCCTAAG

     GenoS  AATGAACAAGGAGAGTTTGAATATGTCAGACTCATTAATGAGATGGTTTTAGGTACTGTT 4680
cDNAS       AATGAACAAGGAGAGTTTGAATATGTCAGACTCATTAATGAGATGGTTTTAG........

     GenoS  TTTTTCCACTTCCCCAGTTTACATGAAAGAGGCTGCACTATACTGCTTATAACAATTGGC 4740
     GenoS  ATCTTGTAACCTTTACTTGCATTGTTTTGCAGCTAACAAGTGTAGTTTGGAGATAGACTA 4800
cDNAS       ............................CTAACAAGTGTAGTTTGGAGATAGACTA

     GenoS  CAAGCAATTTATTTATATACACCCAAACATTGCCATCTGGTTGGCCGATGCACCTCAATC 4860
cDNAS       CAAGCAATTTATTTATATACACCCAAACATTGCCATCTGGTTGGCTGATGCACCTCAATC

     GenoS  GGTGCTGGAAGTTATGGAGGAAGTGGGCAAAAATGTTGTTTTTTGATCTCCACAAGAATTA 4920
cDNAS       AGTGCTGGAGGTTATGGAGGAAGTGGGCAAAAATGTTGTTTTTTGATCTCCACAAGAATTA
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      GenoS  CAGAAACATTCATCAAAAAATATATGTGCGAATAACCAACCTTCCTGTCTATGATCAAAT 4980
cDNAS        CAGAAACATCCATCAGAAAATATATGTGCGAATCACCAACCTTCCTGTCTATGATCAAAT

      GenoS  ACGCAATATCAGGTAAAGAACATACCCTTTTACCATGTTTTCTCTTTGGTCCCAAATTAA 5040
cDNAS        ACGCAATATCAG...........................................

      GenoS  ATCATGTCCTTAATCCTACTTGTCATTGTTCAGGCAAATTCATCTGAACACAATGATTCG 5100
cDNAS        ............................. GCAAATTCATCTGAACACAATGATTCG

      GenoS  AATTGGGGGTGTTGTTACTCGAAGGTCAGGTGTGTTCCCTCAGCTGCAGCAGGTCAAGTA 5160
cDNAS        AATTGGGGGTGTTGTTACTCGAAGGTCAGGTGTGTTCCCTCAGCTGCAGCAGGTCAAGTA

      GenoS  TGACTGTAGCAAATGTGGAACTGTCCTGGGTCCTTTCTTCCAGAACTCTTACACTGAAGT 5220
cDNAS        TGACTGTAGCAAATGTGGAACTGTCCTGGGTCCTTTCTTCCAGAACTCTTACACTGAAGT

      GenoS  AAGGGTTGGGTCTTGCCCTGAATGCCAATCCAAAGGTCCATTTACTGTCAACATTGAGCA 5280
cDNAS        AAAGGTTGGGTCCTGCCCTGAATGCCAATCCAAAGGTCCATTTACTGTCAACATTGAGCA

      GenoS  AGTAAGTAGCATTAAACAGTTAAGCTTAAGTATTATTTTTCTCTTTGCATGATATTGATC 5340
cDNAS        A.........................................................

     GenoS  TTTCTGCATTTGGTATGCATGAACTATTTACCTTGGAACTATATACAGGAACTATCAG 5400
cDNAS        .....................................ACTATATACAGGAACTATCAG

     GenoS  AAACTCACTCTTCAGGAAAGCCCAGGGATTGTTCCTGCTGGCAGGCTTCCCAGGTACAAG 5460
cDNAS        AAACTCACTCTTCAGGAAAGCCCAGGGATTGTTCCTGCTGGCAGGCTTCCCAGGTACAAG

     GenoS  GAAGTGATACTTCTGAATGATCTGATCGACTGTGCTCGTCCAGGAGAGGAAATTGTATGT 5520
cDNAS        GAAGTGATACTTCTGAATGATCTGATCGACTGCGCTCGTCCAGGAGAAGAAATT.....

    GenoS  TCTCCAGACTCCTCTTATTGGTTCGTATATATTACTGTAGAAAGCACACATTGATTAGTG 5580
    GenoS  CTGCTTATCCATTTCAGGAGGTTACAGGGATATACACAAACAATTTTGACCTGTCTTTAA 5640
cDNAS        ................GAGGTTACAGGGATATACACAAACAATTTTGACCTGTCTTTAA

    GenoS  ATACAAAGAATGGTTTCCCAGTTTTTGCCACAGTGGTGGAGGCAAACTATGTATCAAAGA 5700
cDNAS        ATACAAAGAATGGTTTCCCAGTTTTTGCCACAGTGGTGGAGGCAAACTATGTATCAAAGA

    GenoS  AGCAGGACCTGTTCTCTGCATACAAATTAACAGATGAGGACAAGGCAGAGATTGAGAAGT 5760
cDNAS        AGCAGGATCTGTTCTCTGCATACAAATTAACAGATGAGGACAAGGCTGAGATTGAGAAGT

    GenoS  TGTCAAAGGATCCTCGTATCAGTGAAAGGGTTTGTCCCTGAATCCTTAAATCTCTTCCAG 5820
cDNAS        TGTCGAAGGATCCTCGTATCAGTGAAAGG..............................

    GenoS  CAACTATGATACATAGCCAACCTTATTTTACAACCAGTACTTTGGATTTACAGATTGTCA 5880
cDNAS        ..................................................ATTGTCA

    GenoS  AATCAATTGCACCATCCATTTATGGTCATGAAGATATCAAGACTGCCATTGCACTAGCTA 5940
cDNAS        AATCAATTGCACCATCCATTTATGGTCATGAAGATATCAAGACTGCCATTGCACTAGCTA

    GenoS  TGTTTGGGGGGCAAGAAAAGAACGTGAAGGGAAAACATCGCCTAAGAGGTGATATTAATT 6000
cDNAS        TGTTTGGCGGGCAAGAAAAGAACGTGAAGGGAAAGCCTCGCCTAAGAGGTGATATTAACT


    GenoS  GTCTCCTCTTAGGTGACCCAGGCACTGCAAAATCCCAATTTCTCAAGTAAGGATGAAGTA 6060
cDNAS        GTCTCCTCTTGGGTGACCCAGGCACTGCGAAATCCCAATTTCTCAA...........

    GenoS  AACAAATATTTTACAGCAGTTCCTTGTAAGACAATTCTCCACTAACTCTTGTTCTGTAGG 6120
cDNAS        .................................                       G

    GenoS  TATGTCGAGAAAACAGGACACAGGGCTGTATACACAACTGGGAAAGGAGCTTCTGCTGTT 6180
cDNAS        TATGTTGAGAAAACAGGACACAGGGCTGTATACACAACTGGGAAAGGAGCTTCTGCTGTT

    GenoS  GGACTCACCGCAGCAGTTCACAAGGATCCAGTAACACGGGAGTGGACACTTGAGGGAGGT 6240
cDNAS        GGACTCACAGCAGCAGTTCACAAGGATCCAGTAACACGGGAGTGGACCCTTGAGGGAGGT

    GenoS  GCACTGGTTCTTGCTGATAGAGGCATATGCCTTATTGATGAATTTGATAAGATGAATGAT 6300
cDNAS        GCACTGGTTCTTGCTGATAGAGGCATATGCCTTATTGATGAATTTGATAAGATGAATGAT

    GenoS  CAAGACAGGTATGAAATACCCTTTTGATGTTGCATGATTAGCATTTAATGTTTCACAGTT 6360
cDNAS        CAAGATAG.....................................................

    GenoS  TATTGAATATTAACTTATTTCCTACTTATTGTTGCAGGGTAAGTATTCATGAAGCCATGG 6420
cDNAS        .....................................GGTAAGTATTCATGAAGCCATGG

    GenoS  AGCAACAAAGTATCAGCATATCAAAGGCAGGAATTGTCACATCCCTTCAAGCTCGATGCA 6480
cDNAS        AGCAACAAAGTATCAGCATATCAAAGGCAGGGATTGTCACATCCCTTCAAGCTCGATGCA

    GenoS  GTGTTATTGCTGCAGCAAACCCAGTTGGAGGAAGGTAAGCTGAATTATGTGCAATGCTAG 6540
cDNAS        GTGTTATTGCTGCAGCAAACCCAGTTGGAGGAAG..........................
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     GenoS  TTATACTGGCCCTCCCTATTAAGCAACATCTTGATTCTCAACATGTTGACTTTGCAGATA 6600
cDNAS       ...........................................................ATA

     GenoS  TGATTCTTCAAAGACATTCACCCAAAATGTTGAGCTAACAGATCCAATTATTTCACGTTT 6660
cDNAS       TGATTCTTCAAAGACATTCACCCAAAATGTTGAGCTAACAGATCCAATTATTTCACGTTT

     GenoS  TGATGTCCTCTGTGTTGTGAAGGTTTGTAGTTCTCCATGTAATATATTTTGTCCAGTACT 6720
cDNAS       TGATGTCCTCTGTGTTGTGAAG.....................................

     GenoS  TGGTTGCCTATCAATACTGAGTTTTCTTACCGCGATGCAGGATATTGTTGACCCATTTAC 6780
cDNAS       ...................................GATATTGTTGACCCATTTAC

     GenoS  AGATGAAATGCTTGCAAGGTTTGTTGTTGATAGCCATGCCAGATCTCAGCCCAAGGGTGG 6840
cDNAS       AGATGAAATGCTTGCAAGGTTTGTTGTTGATAGCCATGCCAGATCTCAGCCCAAGGGTGG

     GenoS  TAACCTTGAAGATAGAGTTGTAGCTGACGAGGAGGATGATCCATTGACTGTGGCCCGAAA 6900
cDNAS       TAACCTTGAAGATAGAGTTGTAGCTGACGAGGAGGATGATCCATTGACTGTTGCCCGAAA

     GenoS  TGCTGACCCAGATGTACTTCCCTTGTACTTGTCTGTAAGAAGCTAATATTTTACTTAAAA 6960
cDNAS       TGCTGACCCAGAT.............................................

     GenoS  GTTGCACCTCTAACAAAATCCTCCTTTGCCAGATCCTTTCTCAAGACATGCTGAAGAAGT 7020
cDNAS       ...............................ATCCTTTCTCAAGACATGCTGAAGAAGT

     GenoS  ATATCACATATGCTAAGTTGAATGTATTCCCCAAAATACATGATGCTGACCTGGACAAGA 7080
cDNAS       ATATCACATATGCTAAGTTGAATGTATTCCCCAAAATACATGATGCTGACCTGGACAAGA

     GenoS  TTAGCCATGTCTATGCTGAACTTCGACGTGAATCATCTGTAAGCCATCACTTTTTCCTAG 7140
cDNAS       TTAGCCATGTCTATGCTGAACTTCGACGTGAATCATCT.....................

     GenoS  CCTTGTTGACTGATAAGGTCAAATATTGAAAATTGATATCCTTGAAAACCATGAAGCAAT 7200
     GenoS  TGAGTTTGCAACTGTTTTTGCAGCACGGTCAAGGAGTCCCCATTGCAGTAAGGCATATCG 7260
cDNAS       ......................CACGGTCAAGGAGTCCCCATTGCAGTAAGGCATATCG

     GenoS  AATCAATCATGCGAATGTCTGAGGCACATGCAAAGATGCATCTGAGAAGCTATGTGTCTC 7320
cDNAS       AATCAATCATTCGAATGTCTGAGGCACATGCAAAGATGCATCTGCGAAGCTATGTGTCTC


     GenoS  AAGAAGATGTTGACATGGCCATTCGTGTGCTGCTTGACTCATTCATCTCAACCCAGAAAT 7380
cDNAS       AGGAAGATGTGGACATGGCCATTCGTGTGCTGCTTGACTCGTTCATCTCAACCCAGAAAT

     GenoS  TTGGTGTCCAGAAGGCACTTCAGAAGGTAACTTTTGAACAGTTGAACTTGAACTGCTGTT 7440
cDNAS       TTGGTGTCCAGAAAGCACTTCAGAAG.................................

     GenoS  TTGTTTCCCACCAGTGCCTAAAGTATATTTTGGTTCGAAGTGGGCTACATAATGCAGTTC 7500
     GenoS  TGTTAGTGAATATGTCATTCATTTGCATAGCTTTAGGAGAAGTCTTGATACTCAGTGATC 7560
     GenoS  TTATATTCAGAGTCAAAATCTTACATCTCTTTTTGCCTAGTTGGTGGTTTGTAACAAACT 7620

     GenoS  TCTTTTGTCTGCAGAATTTCCGGAAATACATGACTTACAAGAAGGATTACAATGAACTGC 7680
cDNAS       ..............AATTTCCGGAAGTACATGACGTACAAGAAGGATTACAATGAACTGC

     GenoS  TTCTGCTCCTTCTGCGCACCCTGGTCAAGGAGGCGCTGCACTTTGAAGAAATCGTGTTGG 7740
cDNAS       TTCTGCTCCTTCTGCGCACCCTGGTCAAGGAGGCGCTGCACTTTGAAGAAATCGTGTCTG

     GenoS  GATCAACCGCACGCCTGACTCATGTCGAGGTTAAAGTGGATGACCTGAAGAACAAGGTAA 7800
cDNAS       GATCAACCACACGCCTGACTCACGTTGAGGTTAAAGTGGATGACCTGAAGAACAAG...

     GenoS  GAGCTATGAACACCTCTGTCCATGATTCTTCCTTGCCTGAGCAATACTGCTGCATGTCTG 7860
     GenoS  TCCATTGGTGCCTGTCGTGTGCTGATGTTCTTGTCTGCTAATCCAGGCCCAAGAGTACGA 7920
cDNAS       .............................................GCCCAAGAATATGA

     GenoS  GATCTACGACCTGAGGCCGTTCTTCTCCAGCTCTCACTTCAGCGACAACAGCTTCGTCCT 7980
cDNAS       GATCTACGACCTGAGGCCGTTCTTCTCCAGCTCTCACTTCAGTGACAACAGCTTTGTCCT

     GenoS  GGACGAAGGGCGTGGGATCATCAGGCATCCAGTTGCAGCATAACAACTCAATCAACATCA 8040
cDNAS       CGATGAAGGGCGTGGGATCATCAGGCATCCAGTTGCGGCATAACAACTCAATC........
     GenoS  GCCGATGACCCCTGTGTGATGAACAGCTACCTCTTTGTTCAAGAGCTAGAAATGTCCACT 8100
     GenoS  TGTAGTGTAAGTCGGCAAAATGACTGCTTGTGTCTGTTTTGTGAGAGTTCTGATATTGGT 8160
     GenoS  GTACTGTTTGCTATCTGAAACCCTTGTGCCTACAGCGGCATTTCGAGATGTCTGTACCTT 8220
     GenoS  CTTGATATCATAATTCAGTGTTTATGGAACTGACGCCATCATCATACTTTTATCATAATT 8280
     GenoS  CAGTGTTGCAACTGACGCCATCCTCCTATCTTGGAACGCCAGCAACCAATTTTTTTGACT 8340
     GenoS  TTGAGGGCGTCCATGGCATCCAATTGGTTGTCTTCATGGAGGTGTGAGTAAGGCTCAGAA 8400
     GenoS  ACTGACATGATAATCGGAGGCAGTGAAATAATGCCCATCTGAAGTGAATTTGTAGACAAT 8460
     GenoS  GTCGTCCTCAATGTCATCCCGCAAAGGGGTGTTATGTAGGCACGCCCAAAGGGTAACAAA 8520
     GenoS  TTCCATGATGAAGCCCAAAGTCCAAGTGTGAGGTGTCTTAATTTTCTCCACCCAAGCATC 8580
     GenoS  GTCTTTCATTGCCTTCCACCGCTTCCTCTTCAAGGCCTCAAAGATGATTGGTGCAATCTC 8640
     GenoS  TTTTGGCTAGCTCCCATTAACCCATGGCGAGTCCCAGAATTGTGTTGGTGCTCCATTGCC 8700
     GenoS  CACGATGATATTCGTGGAGGCTTAGAAGACCTTTGAGGTGCAATCTCTTTTGACTTTKGG 8760
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      CDNAS       GAACAGCTGAGGAGAAACTCCTGCGCCTTCGAGCTGACTTCTGGGGGTCG
8810
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Appendix 2.
The following amino acid sequences belong to the wheat MCM2 (TA MCM2), V. faba MCM2 (VF MCM2), Z. maize MCM6 (ZM MCM6), yeast MCM2 (SC MCM2), and A. thaliana LEC1 (AT LEC1).

TaMCM2

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  1 MDDSENNAPS TPGSPGFSTD RLPPNTTTSR GATDPSSYSD DDGEAEVDPN VLPEDDGATV
 61 IRDEEEDDGE DLFNDDYLND YRRMDEQDQY ESVGLDDSIE DERNLDEIMA DRRAAEAELH
121 ARDVRTGATA DRKLPRMLHD QDTDEDMNFR RPKRHRANFR QPSGGPRTPR SDDDGDGLTP
181 SSPGRSQPYS GGDVPMTDQT DDDGYEDEFD EEDEMNMYRV QGTLREWVTR DEVRRFIAKK
241 FKEFLLTYVN PKNEQGEFEY VRLINEMVLA NKCSLEIDYK QFIYIHPNIA IWLADAPQSV
301 LEVMEEVGKN VVFDLHKNYR NIHQKIYVRI TNLPVYDQIR NIRQIHLNTM IRIGGVVTRR
361 SGVFPQLQQV KYDCSKCGTV LGPFFQNSYT EVKVGSCPEC QSKGPFTVNI EQTIYRNYQK
421 LTLQESPGIV PAGRLPRYKE VILLNDLIDC ARPGEEIEVT GIYTNNFDLS LNTKNGFPVF
481 ATVVEANYVS KKQDLFSAYK LTDEDKAEIE KLSKDPRISE RIVKSIAPSI YGHEDIKTAI
541 ALAMFGGQEK NVKGKPRLRG DINCLLLGDP GTAKSQFLKY VEKTGHRAVY TTGKGASAVG
601 LTAAVHKDPV TREWTLEGGA LVLADRGICL IDEFDKMNDQ DRVSIHEAME QQSISISKAG
661 IVTSLQARCS VIAAANPVGG RYDSSKTFTQ NVELTDPIIS RFDVLCVVKD IVDPFTDEML
721 ARFVVDSHAR SQPKGGNLED RVVADEEDDP LTVARNADPD ILSQDMLKKY ITYAKLNVFP
781 KIHDADLDKI SHVYAELRRE SSHGQGVPIA VRHIESIIRM SEAHAKMHLR SYVSQEDVDM
841 AIRVLLDSFI STQKFGVQKA LQKNFRKYMT YKKDYNELLL LLLRTLVKEA LHFEEIVSGS
901 TTRLTHVEVK VDDLKNKAQE YEIYDLRPFF SSSHFSDNSF VLDEGRGIIR HPVAAP
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VfMCM2

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  1 MEPGIPPSTP DSPTSPSIGF NTDQLPHTHT SRASDDEASV DPDIIRDEPE PEEDEDGEDL
 61 YNDNFLEDYR RMDEADQFES VGLDDSVEDE RDFDQIMEDR RAAEVELDTR DGRASNRTKL
121 PQLLHDQDTD DDGYRPSKRA RADHRSSIPP SDDDLDGMNS SPGRSQRGQH SRDDNPTTDQ
181 NEDDQYEDDF DDEAGYEMYR VQGTLREWVT RDEVRRFIAR KFKDFLLTYV NPKNEHGDFE
241 YVRLINEMVS ANKCSLEIDY KQFIYVHPNI AIWLADAPHS VLEVMEDVAK SVVFQLHPNY
301 KHIHQKIYVR ITNLPVYDQI RNIRQIHLNT MIRIGGVVTR RSGVFPQLQQ VKYDCSKCGA
361 ILGPFFQNSY SEVKVGSCPE CQSKGPFTVN IEQTIYRNFQ KLTLQESPGI VPAGRLPRYK
421 EVILLNDLID CARPGEEIEV TGVYTNNFDL SLNTKNGFPV FSTVVEANHV TKKQDLFSAY
481 KLTQEDKEEI ENLGKDPRIG ERIVKSIAPS IYGHDDIKTG IALAMFGGRE KNVEGKHRLR
541 GDINVLLLGD PGTAKSQFLK YVEKTGQRAV YTTGKGASAV GLTAAVHKDP VTREWTLEGG
601 ALVLADRGIC LIDEFDKMND QDRVSIHEAM EQQSISISKA GIVTSLQARC SVIAAANPIG
661 GRYDSSKLFT QNVELTDPII SRFDILCVVK DVVDPVTDEM LAKFVVDSHF KSQPKGVNND
721 DKSVSESQDA SGMPTDPEIL PQDLLKKYIT YAKLNVFPRF NDADLDKLTH VYAELRRESS
781 HGQGVPIAVR HIESMIRMSE AHARMHLRQH VTPEDVDMAI RVLLDSFIST QKFGVQKALQ
841 KSFRKYITFK KDYNDVLLYI LGLLVKGAID FEEIVAGSTS SLTHVEVKVD DLSMKAQEHD
901 IYDLKPFFNS SQFSRSNYVL DEERSMIIHH LR
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ZmMCM6

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  1 MEAFGGFFVD EKAARVENIF LEFLKRFKES DGAGEPFYEA EMEVMRSRES TTMYVDFAHV
 61 MRFNDVLQKA ISEEYLRFEP YLRNACKRFA LEHRAGENRA PLISDDSPNK DINIAFYNIP
121 MLKKLRELGT AEIGKLTSVM GVVTRTSEVR PELLQGTFKC LDCGNVVKNV EQQFKYTEPI
181 ICVNATCQNR TKWALLRQES KFTDWQRVRM QETSKEIPAG SLPRSLDVIL RHEIVEKARA
241 GDTVIFTGTV VAVPDVMALT SPGERAECRR EAPQRKNGGV QEGVKGLKSL GVRDLSYRLA
301 FVANSVQVAD GRREVDIRER DTDGDDSERQ KFTEEEEDEV VRMRNTPDFF NKIVDSICPT
361 VFGHQEIKRA VLLMLLGGVH KITHEGINLR GDINVCIVGD PSCAKSQFLK YTAGIVPRSV
421 YTSGKSSSAA GLTATVAKEP ETGEFCIEAG ALMLADNGVC CIDEFDKMDI KDQVAIHEAM
481 EQQTISITKA GIQATLNART SILAAANPTG GRYDKSKPLK YNVALPPAIL SRFDLVYIMI
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541 DEPDENTDYH IAHHIVRVHQ KREEALAPAF STAQLKRYIS FAKSLKPQLS SEAKKVLVES
601 YVTLRRGDST PGTRVAYRMT VRQLEALIRL SEAIARSHLE RVVLPAHVRL AVKLLKTSII
661 SVESSEVDLS DFQDAEDGTN VPSESDAGQP AEEDAAPQQQ GAENDQAADN GKKKLVITEE
721 HFQRVTQALV MRLRQHEESV KKDGDGLAGM KQGDLIIWYV EQQNAKGAYS STAEVKEEVK
781 CIKAIIERLI QREGHLIVID EGTAAAAEDG SGARRTSESR ILAVNPNYVI D
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ScMCM2

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  1 MSDNRRRRRE EDDSDSENEL PPSSPQQHFR GGMNPVSSPI GSPDMINPEG DDNEVDDVPD
 61 IDEVEEQMNE VDLMDDNMYE DYAADHNRDR YDPDQVDDRE QQELSLSERR RIDAQLNERD
121 RLLRNVAYID DEDEEQEGAA QLDEMGLPVQ RRRRRRQYED LENSDDDLLS DMDIDPLREE
181 LTLESLSNVK ANSYSEWITQ PNVSRTIARE LKSFLLEYTD ETGRSVYGAR IRTLGEMNSE
241 SLEVNYRHLA ESKAILALFL AKCPEEMLKI FDLVAMEATE LHYPDYARIH SEIHVRISDF
301 PTIYSLRELR ESNLSSLVRV TGVVTRRTGV FPQLKYVKFN CLKCGSILGP FFQDSNEEIR
361 ISFCTNCKSK GPFRVNGEKT VYRNYQRVTL QEAPGTVPPG RLPRHREVIL LADLVDVSKP
421 GEEVEVTGIY KNNYDGNLNA KNGFPVFATI IEANSIKRRE GNTANEGEEG LDVFSWTEEE
481 EREFRKISRD RGIIDKIISS MAPSIYGHRD IKTAVACSLF GGVPKNVNGK HSIRGDINVL
541 LLGDPGTAKS QILKYVEKTA HRAVFATGQG ASAVGLTASV RKDPITKEWT LEGGALVLAD
601 KGVCLIDEFD KMNDQDRTSI HEAMEQQSIS ISKAGIVTTL QARCSIIAAA NPNGGRYNST
661 LPLAQNVSLT EPILSRFDIL CVVRDLVDEE ADERLATFVV DSHVRSHPEN DEDREGEELK
721 NNGESAIEQG EDEINEQLNA RQRRLQRQRK KEEEISPIPQ ELLMKYIHYA RTKIYPKLHQ
781 MDMDKVSRVY ADLRRESIST GSFPITVRHL ESILRIAESF AKMRLSEFVS SYDLDRAIKV
841 VVDSFVDAQK VSVRRQLRRS FAIYTLGH.
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AtLEC1

```
  1 MTSSVIVAGA GDKNNGIVVQ QQPPCVAREQ DQYMPIANVI RIMRKTLPSH AKISDDAKET
 61 IQECVSEYIS FVTGEANERC QREQRKTITA EDILWAMSKL GFDNYVDPLT VFINRYREIE
121 TDRGSALRGE PPSLRQTYGG NGIGFHGPSH GLPPPGPYGY GMLDQSMVMG GGRYYQNGSS
181 GQDESSVGGG SSSSINGMPA FDHYGQYK
```

Appendix 3.

The following sequences represent partial sequence of the subtracted fragment (B1 to B).

B1

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AGTCCTGACCGACCACTGGGACATCATGTCTTCTGATCCTTTGGAGGCCGGCTCCCAGTCTGCCACCCTTGT
CACGGAGATCCGCAAGCGCAAGGGTCTTAAGGAACAGATGACCCCTCTGTCTGATTTTGAGGACAAGCTCTA
AACTTTTGGTTCTGTTATCTCCTATATG
```

B2

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AGTCCTGACCGAGGCATGGGCAACCAAGAACTTCTTGAGTATTTCAGTGATTATGCTGCGACCAAAGCACGC
CATGCACATGGTCCAGGTGGGCACCGTGGCATGAGTGTGCTAATATTTGAAAGCTCTGCTGTGGGCTATATG
GAGGCAGAACGTCTTCATAAGCATTTTATTGATCAAAGAACAGACAGGGACACTTGGCAGAATCGCAGGGTT
CCTTTCTTACCTGGTGGGAAGAGACAA
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B3

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GAGTCCTGACCGAGCATGGCATTCAGCCTGATGGACAGATGCCCGGTGACAAGACTGTTGGGGGAGGTGATG
ATGCTTTCAACACCTTCTTCAGTGAGACTGGTGCTGGGAAGCATGTCCCCCGTGCTGTCTTTGTTGATCTTG
AGCCCACTGTGATTGATGAGGTGAGGACTGGCACTTATCGCCAGCTCTTCCACCCTGAGCAGCTTATCAGTG
GCAAGGAGGATGCAGCCAACAACTTTGCCCGTGGTCACTACACCATTGGCAAGGAGATTGTTGACCTATGCC
TGGACCGTATCAGGAAGCTTGCAGACAACTGCACTGTTTT
```

B5

GAGTCCTGACCGAGGCCGCCGCCGAGAACGGAGGATGCAAGTGCGGCCCCGACTGCTCCTGCAACCCGTGCA
CCTGCAAGTGATCCATCAAATCAAGACAAGGAGACGCCATGGACGGACCTGATGAAGGAGCACTAGTGGTTA
TGCATGGTGTGTGTTTCGTTTTTAATTGCCACCGAGAATAAGATGAGATCTATCG

B6

AGTCCTGACCGACGTCCAAGCCCTTAACCTCAACGTTACTCTCAGCATTCTTCAGCAAATCCAACACGAACT
GGGCCGACTTTGCAGGCCAGCGACCCTGCCCATTTGGCTGGCGGTTCTTTACTTGCGCAGTACGACCCACAC
CTCTGNAGTATCTCCGGAAGGGAATCGCTTGNTTGTGGGCGAGAACATCCTCAAGGTACCTNTTAGCCTTGC
CCAAAGGCATCCTGCGAAGAGCAAACGCTGTCTCGCGTGTGTTCTTGAAGTGGACCTTGAGATCCTTGCCAC
AGNCCTTGGCCGACTTGGTC

B7

CTGATTGTAATACCGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTG
ATGGATATCTGCAGAATTCGGCTTAGCGTGGTCGCGGCCGAGGTAGCGATGAGTCCTGACCGAGTGCCGTAT
GCGTGAACCGAATCTACTAGTAGTGATTGTATGGAGTATGTTAGTTTG

B8

TCGTAGACTGCGTACCCGGGGAGCTCCTCGCCGACATCCAGGCGGGGACCTTCGCCGTCCAGGAAGACGGCT
CCGGCGGGGTGTTCCTCAGCACAACAATTCCCTACACCTTCCTCCAAGAGGCCGCGTACAAGGTTCTGAAGC
AGGAGCTCGCGAGCAGGATTCGGTCACAGGGCGTAGCTCCGATGAACGCCACAGCCGACGACCTGTGCTTCC
TCAAGGAGAACTTTCGCAGCGTCAAGGTTCCGAGGCTAGCGCTGGTGTTCGGTCAGGACTCATCGCTACCTG
CC

B9

GTCCTGACCGAGCCTGTTCATCTACTCTCAGAAGANTGTGATCAAGACCTGTGGGACTACCATGCTCCTGCT
CACCATTCCTAGGATTCTCGAGCTTGCTGAAGAGCTGTGCATGCCGCTTGCTGCCGTGAAGTACTCTCGTGG
GATGTTCATCTTCCCCGGCGCACAGCCTGCTCCCCACAGGAGCTTCTCTGAGGAGGTTGATGTCCTGAACCG
CTACTTCGGCCACTTGAAGTCTGGCGGCAATGCTTATGTGATCGGAGACCCAGCGAAGCCTGGCCAGAAGTG
GCACATNTACTATGCCACCGAGCAACCTGAGCAGCCCATGGTCACCCTGGAGATGTGCATGACTGGGCTGGA
CAAGAAGAAGGCCTCTGTCT

B10

AGTCCTGACCGAACGACGGGGTGCCCTTGGAGGGGGCCACGCCCATGATGAGGGTCTGGGAGGCGGTGTTCA
CTCCCTCGTCCATCTCAGGGTACATCTTGCACCCTCCGCAGCCGTTGCCGCACTTGCAGCCGGATCCGCACC
CGCAGTTGCCTCCGCAGCACGACATGGTTGGTGATG

B11

TCCTGACCGATGGAGGAGAAGCGGAGGCTGAAGTGGGCGAAACTAGAGTCCGCCGCAGCTCTCCAGCCAGTG
ACTGGCCACACCGCCGTCCTGGTGGGGTAGGTCACCAACCGTCGGCGATAATCTTGTCGCCGCCACCATCGT
TTAGCGTGTGTTTGGACGGTGGAGGTGCATACCAATGGTGTTCTTCCCCTGTTCGGTGAAGCGCGCCCAAGT
GATGATCGGGTACGCAGTCTACGAGACCTGTAGC

B12

GACTCATCGCTATGGAGAAAAATCTGGGCAAGGAAGCAGTGCCTCTCCAGCAAGCATGATAAAGAAGAGTAC
TCTGGTGACACCGAAGAAGATGTCGCAATCTGGCCTTACATTTCAACCTCAAGATGACGACTCCTGTTCAGT
GACTTCTTCAACCACAGCTTCAGCACGAGCTGGCAGAACTAAGACAACCGTTGCCTTTGCTCCTACATTTGT
ATGTGACGACCGTGCTGACAAGAGGAAAGAGTTCTACACGAAATTAGAAGAGAAGCACAAAGCTTTGGAAGC
TGAGAAGAATGAGGCTGAAACAAGGAAAAAGGATGAGCAAGAAGCGGCTTTAAAACAACTAAGGGAGTCCTT
GGTCATCAGAGCAAAACCCATGCCAAACTTCTACCAAGAAGGGCCCCCTCCAAAGGTTGAGCTTAAGAAGGT
GCCTCCAACCCGTGCCAAGTCACCAAAGTTTACAAGGAGAAAAGAGCACCGGC

Declaration

Hereby I declare that all the work presented in this manuscript is my own, carried out solely with the help of the literature and aid cited.

Gatersleben, November, 2001

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