GENETIC ANALYSIS OF THE APOSPORY-SPECIFIC GENOMIC REGION (ASGR) IN *PENNISETUM SQUAMULATUM*: FROM MAPPING TO CANDIDATE GENE

by

HEQIANG HUO

(Under the Direction of Peggy Ozias-Akins)

ABSTRACT

Pennisetum squamulatum reproduces by apospory, a type of apomictic reproduction where non-generative nucellar cells develop into an unreduced embryo sac. Apospory behaves as a dominant Mendelian trait in *P. squamulatum* and a genomic region from a single chromosome, called the apospory-specific genomic region (ASGR), is sufficient for inheritance of the trait. However, the ASGR is physically large (>50Mb), highly heterochromatic, hemizygous, and recombinationally suppressed. These characteristics have hindered high-resolution genetic mapping and map-based cloning of apomixis genes.

In order to aid physical map construction, additional molecular markers linked to the ASGR are needed. The first study focused on development of molecular markers based on the Long Terminal Repeats (LTRs) of retrotransposons which are abundant in the ASGR. Sequence-specific amplified polymorphisms (SSAP) were identified using LTR-specific primers paired with restriction site targeted primers. Over 60% of the SSAP markers generated were linked with apomixis and strongly clustered within 9 cM. Low recombination observed among SSAP markers was reinforced after converting five SSAP markers into Sequence Characterized Amplified Regions (SCARs).

In a second study, one candidate gene for apomixis identified through sample sequencing of ASGR-linked Bacterial Artificial Chromosome (BAC) clones, *ASGR-BABY-BOOM-like*, was characterized by gene silencing using RNA interference (RNAi). Fifty-nine transgenic tetraploid pearl millet plants were generated from 16 independent lines and crossed with *P. squamulatum* (male parent) to combine the silencing transgene with the *ASGR-BABY-BOOM-like* gene. Expression of *ASGR-BABY-BOOM-like* was reduced in three out of 14 F1plants with the genotype of ASGR/RNAi, and embryo development in these three reduced plants was significantly delayed. Over-expression of this gene in Arabidopsis also caused ectopic organ development on leaves. These combined phenotypes suggest that *ASGR-BABY-BOOM-like* plays a role in organ/embryo initiation and may be necessary for parthenogenetic development of the egg.

INDEX WORDS: *Pennisetum squamulatum*, LTR-retrotransposon, SSAP, molecular marker, genetic map, ASGR *BABY-BOOM-LIKE*, RNA interference, over-expression, Arabidopsis

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HEQIANG HUO

B. Ag., Qingdao Agriculture University, P.R.China, 1994

M. Ag., Huazhong Agriculture University, P.R.China, 1998

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HEQIANG HUO

Major Professor: Peggy Ozias-Akins

Committee: Wayne Hanna
Katrien Devos
Carol Robacker
Patrick Conner

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August, 2008

DEDICATION

To my late parents for their commitment and selfless love

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CHAPTER 1

INTRODUCTION

Apomixis is asexual reproduction through seeds (Nogler 1984), which is one of the most intriguing and enigmatic phenomena in the plant kingdom. The trait, first described by John Smith (1841) in *Alchornea ilicifolia,* a dioecious species of *Euphorbiaceae* (Gustafsson 1946), has been challenging and inspiring scientists since that time. Gregor Mendel's studies of the apomictic trait with various species of the genus *Hieracium* did not result in a solution to this puzzle (Nogler 1984). There are two basic forms of apomixis: sporophytic and gametophytic apomixis; the former of these is also called adventitious embryony. In sporophytic apomixis, the process of embryo sac development is circumvented, with an embryo forming directly from somatic cells of the nucellus or integument. Gametophytic apomixis can be subdivided into two types: diplospory and apospory. In diplospory, unreduced embryo sacs are formed from the megaspore mother cell (MMC), which avoids meiosis; in apospory, unreduced embryo sacs develop from aposporous initial cells which are somatic nucellar cells, although meiosis may initiate concurrently in the megaspore mother cell (Koltunow 1993; Ozias-Akins 2006). One of the key features of apomixis is that the progenies of apomicts are genetically identical to the maternal parent, thereby preserving hybrid vigor. The significance of apomixis from the agricultural and evolutionary perspectives has been discussed in many reviews (Bhat et al. 2005; Meeûs et al. 2007; Spillane et al. 2004; Spillane et al. 2001; van Dijk and van Damme 2000). Gametophytic apomixis has been described for over 400 species in more than 40 families, but most abundantly in the Poaceae, Rosaceae, and Asteraceae families; however, it is rarely found in major crop species (Bicknell and Koltunow 2004; Carman 1997; Ozias-Akins 2006). Attempts to transfer genetic loci controlling apomixis from wild relatives to cultivated crops by introgressive hybridizations have been unsuccessful because of certain factors such as polyploidy of apomicts, incompatibility of crossing, and the dosage effect of endosperm (Bicknell and

Koltunow 2004; Grimanelli et al. 1995; Morgan et al. 1998). Understanding the underlying molecular mechanism of apomixis in natural apomicts could advance the utilization of apomixis in crops.

We are working on a grass species, *Pennisetum squamulatum*, which reproduces by apospory. Apomixis in *P. squamulatum* behaves as a dominant trait with Mendelian segregation (Ozias-Akins et al. 2003). Extensive effort has been undertaken to investigate the molecular mechanism of apomixis in *P. squamulatum*. Molecular markers for apomixis first were identified from a BC3 line obtained during the introgression of apomixis from *P. squamulatum* to sexual pearl millet (*P. glaucum*) (Dujardin and Hanna 1989; Ozias-Akins et al. 1993). Two out of seven polymorphic markers were shown to be linked to apomixis (Ozias-Akins et al. 1993). Twelve PCR-based markers were further developed using an F1 population of *P. squamulatum* and tetraploid pearl millet (*P. glaucum*) (Ozias-Akins et al. 1998). Unfortunately, no recombination has been observed among these markers in a population containing 397 individuals (Ozias-Akins et al. 1998), which prevents high-resolution genetic mapping and map-based cloning. These markers clearly define one region on one chromosome, the Apospory Specific Genomic Region (ASGR). This region was shown to be conserved in other *Pennisetum* and *Cenchrus* species (Akiyama et al. 2005; Goel et al. 2006; Lubbers et al. 1994; Roche et al. 1999). Bacterial artificial chromosome (BAC) libraries subsequently were constructed with an apomictic polyhaploid genotype derived from a cross between *P. squamulatum* and pearl millet (*P. glaucum*) (Roche et al. 2002) and with apomictic *C. ciliaris*. BAC clones containing apomixis-linked markers have been used for physical cytogenetic mapping of the ASGR in *P. squamulatum* (Akiyama et al. 2004; Goel et al. 2003). Fluorescence in situ hybridization (FISH) of almost all tested clones produces signal on only a single chromosome in *P. squamulatum,*

suggesting that the ASGR is highly hemizygous (Akiyama et al. 2004; Goel et al. 2003). The physical size of this region has been estimated at more than 50 Mb and has been shown to contain abundant repetitive elements such as retrotransposons (Akiyama et al. 2004). In addition, partial sequencing of BACs from the ASGR revealed some potential candidate apomixis genes such as a *BABY-BOOM-LIKE* (*BBML*) gene (Conner et al. 2008). Given that the ASGR is physically large, heterochromatic, highly hemizygous and recombinationally suppressed, more molecular markers are required to expand the physical map of the ASGR and to attempt to better define its boundaries through genetic mapping. The candidate apomixis gene *BBML* in the ASGR needs to be tested for function. The two major objectives in this thesis focus on 1) identifying the Long Terminal Repeats (LTR) of retrotransposons which are abundant in the ASGR and using the data to develop retrotransposon-based molecular markers to facilitate genetic and physical mapping of the ASGR in *P. squamulatum*; and 2) characterizing the function of a *BABY-BOOM-LIKE* gene in *P. squamulatum* using RNA interference and over-expression.

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CHAPTER 2

LITERATURE REVIEW

Overview

Our planet is alive. It not only is moving but is supporting varieties of living organisms such as microbes, animals and plants which form the multitudes of ecosystems. In these systems, plants play a very crucial role because they are the largest group of organisms on earth that can convert solar energy into biological energy and provide food for animals, insects and even microbes. How the plants reproduce is an important factor for the sustenance of these ecosystems. The reproductive mode of flowering plants, the group of plants containing almost all major crops, may be classified into two major types: sexual and asexual. During sexual reproduction in flowering plants, the plants typically go through an alternation of gametophyte and sporophyte generations. The gametophytic stage starts with the haploid spores formed as a consequence of meiosis. For the female, a diploid somatic cell, the megaspore mother cell (MMC), undergoes meiosis to produce four reduced megaspores. Three of these megaspores will degenerate, while the chalazal megaspore undergoes three mitotic divisions to produce a seven-celled embryo sac with eight nuclei (Yadegari and Drews 2004). Three antipodal cells form at the chalazal end of the embryo sac and degenerate before or during embryonic development; the function of the antipodals remains unknown. A reduced egg cell is surrounded by two synergid cells which have been thought to play a role in emission of chemical signals for attracting pollen tubes to ovules (Yadegari and Drews 2004). Fertilization of the egg cell by one sperm from the male gametophyte (pollen) generates a diploid embryo, while the central cell containing two nuclei will form triploid endosperm after fusion with the second sperm. This process is referred to as double fertilization, and is the point at which the gametophytic stage terminates. Recombination of chromosomes during meiosis and the fertilization process are

responsible for genetic segregation from heterozygous parents. Genetic recombination also is the basis of genetic mapping.

In a broad sense, asexual reproduction includes vegetative reproduction and apomixis (Meeûs et al. 2007). Both types of asexual reproduction can result in the genotype of progenies remaining identical to that of maternal plants. Vegetative reproduction is one way by which the plants reproduce themselves through somatic tissues such as roots, bulbs, shoots, stolons, etc. Sometimes, this process is referred to as agametic reproduction, because it is independent from reproductive organs or gametes. Apomixis is another type of asexual reproduction, but one that involves the production of seeds (Nogler 1984a). In apomictic plants, the embryos of seeds develop from either somatic cells of ovules (sporophytic apomixis) or unreduced gametophytic cells (gametophytic apomixis). In sporophytic apomixis (also referred to as adventitious embryony), an embryo directly forms from somatic cells of the nucellus or integument instead of from an egg, while the sexually produced embryos may develop in parallel or may abort. One typical example for sporophytic apomixis is *Citrus*, in which multiple embryos develop from either nucellar cells or fertilized egg cells in the meiotic embryo sac (Koltunow and Grossniklaus 2003; Ozias-Akins 2006; Savidan 2000). Gametophytic apomixis has two different forms, apospory and diplospory, depending on the origin of the unreduced embryo sacs. In diplospory, unreduced embryo sacs are formed from the MMC by circumvention of meiosis. The initial cells for unreduced embryo sacs in aposporous plants are nucellar cells although meiosis in the MMC can still occur (Ozias-Akins 2006; Savidan 2000). In some aposporous species of *Pennisetum* and *Hieracium*, development of the meiotic embryo sac is arrested because of the initiation of aposporous embryo sacs (Ozias-Akins et al. 2003; Tucker et al. 2001), while in some species

such as *Commiphora wightii*, the sexual and aposporous embryo sacs may coexist (Gupta et al. 1996).

Thus, the major difference between sporophytic apomixis and gametophytic apomixis lies in whether unreduced embryo sacs form. Moreover, most, if not all, sporophytic apomicts are diploid while most gametophytic apomicts are polyploid (Asker and Jerling 1992; Bicknell and Koltunow 2004; Ozias-Akins 2006). Apomixis has been challenging and inspiring scientists for over a century because of its potential significance in agriculture and evolution (Bhat et al. 2005; Meeûs et al. 2007; Spillane et al. 2004; Spillane et al. 2001; van Dijk and van Damme 2000). Although apomixis is widespread among angiosperms, it is a quite rare phenomenon in major crops (Koltunow et al. 1995; Spillane et al. 2001).

Agricultural significance of apomixis

Current agriculture relies heavily on hybrid cultivars for maximum yields. In 2004, the world food prizes went to the Chinese professor Longping Yuan and Dr. Monty Jones of Sierra Leone due to their excellent work on rice breeding. The hybrid rice planted in these two countries resulted in a greater than 20% higher yield over previously grown varieties. A 20% increase in rice yield translates into enough food to feed an additional 60 million people per year in China (www.worldfoodprize.org/laureates/Past/2004.htm). As the global population increases rapidly, food shortage will be an ever increasing problem. However, major increases in the areas planted for rice and other major crops are unlikely to occur since most of the world's arable land already is under cultivation (Khush 1994). An alternative way to improve food production is to take advantage of advanced techniques to develop higher quality hybrid/engineered seeds with the genetic capacity to increase yields. Hybrid cultivars not only have more vigor than their inbred parents as a result of heterosis, but also a combination of different features from the

different genotypes necessary for a particular environment (Koltunow et al. 1995). For instance, hybrid seeds produced for arid farmlands may hold the genetic potential for high production as well as drought resistance. However, the heterosis of hybrids will be partially lost in subsequent generations due to genetic segregation, which requires that the F_1 hybrid seeds have to be made repeatedly and increases the cost to the farmer. Farmers must buy hybrid seeds every growing season, at a much higher price than that of conventional seeds (Koltunow et al. 1995). Cost and accessibility preclude most farmers in developing countries from growing hybrid cultivars. The solution to this problem may come from apomixis. Plants that reproduce by apomixis will form clonal seeds whose genotypes are identical to the maternal parent; in other words, apomixis can perpetuate heterosis through seeds. If the genetic control of apomixis can be introduced into hybrids, the time and cost of breeding will be dramatically reduced. Complications associated with sexual reproduction such as compatibility of gametes, accessibility/availability of pollen, outcross contamination, etc. will be avoided, and problems with virus transmission in vegetatively propagated crops such as potato would be eliminated (Grossniklaus et al. 1998a; Hanna 1995; Savidan 2000; van Dijk and van Damme 2000). However, in nature, only a few crops with agricultural value are found to reproduce apomictically even though apomixis has been described in over 400 species of angiosperms (Carman 1997; Ozias-Akins 2006). Much effort has been taken to introduce apomixis from wild relatives into related sexual crops using traditional breeding (Dujardin and Hanna 1989; Grimanelli et al. 1995; Hanna and Bashaw 1987). Unfortunately, few major crops have wild relatives which can be used for wide hybridization (Koltunow et al. 1995), and furthermore, the introgression of apomixis from apomictic relatives to sexual crops has proved to be slow and largely unsuccessful (Bicknell and Koltunow 2004; van Dijk and van Damme 2000). Alternatively, molecular biology is offering a potential way to

unveil the genetic mechanism of apomixis. A more detailed description of this research area will be given later in this chapter.

Evolutionary significance of apomixis

Compared to sexual reproduction, asexuality is relatively rare even though sexual reproduction is thought to have a twofold cost compared with asexuality because of the necessity for male gametes (Hurst and Peck 1996). There are many hypotheses as to why sexual reproduction predominates over asexual reproduction. One of the most popular hypotheses is that sexual reproduction can eliminate deleterious mutations and create more variation for adaptation through genetic recombination and natural selection. In asexual reproduction, the accumulation of deleterious mutations is assumed to reduce the fitness of the species, resulting in a phenomenon known as Muller's Ratchet, the evolutionary "dead end" (Otto and Lenormand 2002). Recombination rate and the level of epistatic interaction among loci are playing crucial roles in removal of deleterious mutations through natural selections (Otto and Lenormand 2002). When recombination and epistasis are allowed to co-evolve, however, computer simulation results have shown that asexual reproduction will out-compete sexual reproduction (MacCarthy and Bergman 2007). Assuming that this computer simulation is valid, the reason why most apomictic species, if not all, are facultative and not fully independent of sexual reproduction still remains unknown. Nevertheless, apomictic lineages often appear highly genetically variable in spite of the faithful transmission of the same genotype between generations (Adolfsson and Bengtsson 2007; Barcaccia et al. 2006; van Dijk 2003). Several processes such as hybridization among facultative genotypes and polyploidy may contribute to this variation.

Most apomictic species can produce fertile pollen through which dominant genes for apomixis can be transferred to related species or sexual genotypes within the same species. If a

mutation occurs in a sexual genotype before hybridization with an apomict, the mutation could be fixed due to apomixis, but other hybrids formed in a similar manner can lead to variation (Adolfsson and Bengtsson 2007). On the other hand, fertilization of unreduced egg cells can occasionally occur in some species, which enhances the diversity of apomicts (Matzk et al., 2001; Naumova et al., 2004). In addition, hybridization can lead to epigenetic changes in gene expression and phenotypic variability (Comai 2000; Salmon et al. 2005).

Gametophytic apomixis is strongly correlated with polyploidy. The relationship between polyploidy and apomixis has been extensively discussed (Koltunow and Grossniklaus 2003; Roche et al. 2001). One hypothesis is that a polyploid can tolerate more deleterious mutations than a diploid, which can buffer the effect of Muller's Ratchet in apomictic plants. However, recent research has shown that ancient polyploid plants have experienced massive gene loss and genomic rearrangement, in which duplicated genes have been removed through recombination, translocation, and deletion, to enforce diploidization (Wolfe 2001). The process of diploidization of polyploids has contributed to the present diversity of some plants (Paterson et al. 2004). The question of whether polyploid apomicts also experience diploidization is still unanswered, despite the fact that diploid apomictic *Boechera holboellii* is thought to be derived from a paleopolyploid (Grimanelli et al. 2001; Schranz et al. 2007). During the history of evolution, the accumulation of mutations in polyploids may be counterbalanced by the processes of gene conversion and genome reorganization (Adams and Wendel, 2005; Khakhlova and Bock, 2006). Given that hybridization can occur between apomictic and sexual genotypes, playing a role in population variation and evolution of some species (Dupont 2002), and that the variation/diversity in an agamic complex persists over evolutionary time scales (Richards 2003), research on apomixis contributes to both population biology as well as evolutionary biology.

As an indication of facultative apomixis, apomixis and sexual reproduction are not exclusive of one another. Sexual reproduction and apomixis can coexist in the same plant or even in the same ovule, resulting in a mixture of apomictic and sexual progenies. Many arguments on the relationship between apomixis and sexual reproduction have been elaborated (Grimanelli et al., 2001; Koltunow and Grossniklaus, 2003). One hypothesis is that apomixis is derived from deregulation of the sexual developmental pathway in space and/or time (Grimanelli et al. 2003). Meiosis in diplosporous *Tripsacum dactyloides* is precociously displaced by unreduced embryo sac formation. The MMC does not enter meiosis but directly differentiates into an unreduced embryo sac suggesting that sexual reproduction is short-circuited. Similarly, parthenogenesis in diplosporous *Tripsacum* is characterized by earlier embryogenesis than that observed in the sexual wild type (Grimanelli et al. 2003). Interestingly, the precociousness of embryo sac formation in sexual *T. floridanum* is similar to that observed in apomictic *Tripsacum*, which indicates that apomictic *Tripsacum* may have evolved from sexual *T. floridanum* or plants similar to *T. floridanum* through hybridization and polyploidization (Bradley et al. 2007). In *Paspalum notatum*, the simple doubling of chromosomes through colchicine treatment could induce facultative apomixis (Quarin et al. 2001). A study on the diversity of *Ranunculus* spp. showed that the apomictic hexaploid *R. carpaticola* is most likely derived from hybridization of two sexual species, diploid *R. carpaticola* and autotetraploid *R. cassubicifolius* followed by polyploidization (Paun et al. 2006). These examples suggest that genetic factors necessary for apomixis already existed in sexual counterparts and that polyploidization and hybridization could have been factors for altering gene expression.

That sexual and apomictic reproduction probably share similar pathways has been further supported by discoveries of genes involved in endosperm development in Arabidopsis. In

Arabidopsis, mutations in any of three members of the *FERTILIZATION-INDEPENDENT* SEED (*FIS*) class genes *MEA*/*FIS1, FIS2*, or *FIE/FIS3* can result in the initiation of fertilization–independent endosperm development (Chaudhury et al. 2001; Luo et al. 2000). After introducing these three *FIS*-class genes fused with *GUS* (β-glucuronidase) into sexual and apomictic *Hieracium*, the expression pattern of *AtFIS2::GUS* in aposporous initial cells of apomictic *Hieracium* was similar to that in the functional megaspore of sexual *Hieracium* (Tucker et al. 2003). More interestingly, one *Hieracium* mutant *loa1* (*loss of apomeiosis1*), an aneuploid somaclonal variant recovered from experiments with *Agrobacterium* transformation, was defective in forming functional aposporous initial cells, leading to the formation of progenies from this mutant mostly through sexual reproduction (Okada et al. 2007). The aposporous initials appeared to be partially reprogrammed for sexual reproduction, i.e., they showed deposition of callose in their cell walls similar to MMCs; however, they did not show expression of the meiosis-specific gene *HDMC1*. Mutations observed in Arabidopsis and maize have been shown to display apomeiosis, one component of apomixis (see the section on isolation of candidate apomixis genes in this chapter). Collectively, these data support that sexual and apomictic reproduction share genetic mechanisms. Studies on apomictic and sexual reproduction will be beneficial to each other for understanding the reproductive development of plants.

Genetics of apomixis

The genetic study of apomixis dates back to the era of Gregor Mendel. During investigations that led him to develop the laws of inheritance, this great geneticist was confused with the results of experiments on various species of genus *Hieracium* (hawkweed) (Nogler 1984a). Now we know that this species reproduces asexually through apomixis, producing clones of the maternal parent. With the help of molecular tools, the genetics of apomixis has

been extensively studied in last decade; however, the mechanism of apomixis is still not fully clear. So far, we know that three essential elements are required for apomixis. 1) Apomeiosis where the meiotic process can be bypassed or altered while a chromosomally unreduced cell (apomictic initials) forms either an embryo (sporophytic apomixis) or an unreduced embryo sac (gametophytic apomixis). 2) Parthenogenesis where embryos can spontaneously develop from an egg independently of fertilization. 3) Autonomous (fertilization independent) or pseudogamous (pollination/fertilization dependent) development of endosperm from the central cell (Grimanelli et al., 2001; Koltunow, 1993; Koltunow and Grossniklaus, 2003; Ozias-Akins, 2006; Spielman et al., 2003). The majority of published research has been focused on gametophytic apomixis since it is less confounded than sporophytic apomixis with regard to simultaneous occurrence of both sexual and asexual reproductive pathways.

Adventitous embryony

In sporophytic apomixis, the adventitious embryo directly develops from a somatic cell. No embryo sac formation and fertilization by male gametes are required for this type of embryo development, although the sexually produced endosperm must develop normally and becomes the major nutrient resource of adventitious embryos (Meeûs et al. 2007; Ozias-Akins 2006). However, the Indian fruit tree *Commiphora wightii* (‘Guggul’), a sporophytic apomict, not only produces zygotic and adventitious embryos, but can form autonomous endosperm (Gupta et al. 1996). The sporophytic apomixis initial cells are typically nucellar cells or integumentary cells (Ozias-Akins 2006). Nucellar embryos may out-compete the sexually derived embryo for nutrients in some cases (Batygina and Vinogradova 2007). Integumentary embryos are usually derived from internal epidermal cells of the inner integument, usually consisting of large cells

with dense cytoplasm. Unlike nucellar embryos, integumentary embryos usually degenerate at the early developmental stages (Batygina and Vinogradova 2007).

Little is known about the molecular control of sporophytic apomixis. In one population of polyembryonic *Citrus volkameriana* × monoembryonic (sexual) *Poncirus trifoliata*, apomixis was observed to be inherited simply as a quantitative trait. One complex model in which six quantitative loci are involved in spontaneous embryo development was proposed in the study (García et al. 1999). However, another model in which two dominant genes control apomixis in the genera *Citrus* and *Poncirus* was proposed in a more recent study (Hong et al. 2001).

Apospory

Genetic studies on gametophytic apomixis have been hampered because of low recombination, distorted segregation, polyploidy, and inherent difficulties in investigation of the tissues embedded in small-sized ovaries. Although crosses between apomicts and their sexual relatives have been used to study the inheritance of apomixis, the above mentioned factors as well as the frequent necessity to make interspecific crosses complicates such studies (Grossniklaus et al. 2001; Ozias-Akins et al. 2003). Two of the first genetic studies on inheritance of apomixis were with the aposporous species *Ranunculus auricomus* and *Panicum maximum*. Apospory in these two species was shown to segregate as a single dominant Mendelian factor. Apomeiosis and parthenogenesis also were shown to strictly co-segregate, suggesting that these two components rely on the same genetic control, i.e., that parthenogenesis is a pleiotropic consequence of apomeiosis (Nogler 1984b; Savidan 2000) or that multiple genes are clustered. Apospory also was observed to be inherited as a dominant Mendelian trait in *Pennisetum squamulatum* (Ozias-Akins et al. 1998), *Pennisetum ciliare* (Jessup et al. 2002; Sherwood et al. 1994), and *Paspalum notatum* (Martinez et al. 2001). In *Hieracium*, apospory

was found to be inherited as a single dominant trait in an earlier report (Bicknell et al. 2000), but in a recent report, after deletion mutagenesis of apomictic *H. caespitosum* through γ-irradiation, mutants with loss of apomeiosis only and mutants with loss of parthenogenesis only were obtained, suggesting that apomeiosis and parthenogenesis are controlled by different loci (Catanach et al. 2006). Seeds set by haploid parthenogenesis and by fertilization of aposporous egg cells were also detected in *Hypericum perforatum*, supporting that apospory and parthenogenesis in this species may be controlled by two distinct genetic factors (Barcaccia et al. 2006; Matzk et al. 2001). Albertini et al. (2001) first showed cytologically that two distinct genetic factors may control apomixis in *Poa pratensis*. However, the result was alternatively interpreted that the wide range of parthenogenesis (1.4% to 92.9%) and absence of embryos in plants could be caused by a difference in penetrance of the parthenogenesis gene (Noyes et al. 2007). Based on the flow cytometric seed screen analysis (FCSS) of segregating progenies from intercrossing and selfing between obligate sexual and facultatively apomictic parents, a more complicated model with five major genes required for apomixis was proposed by Matzk et al. (Matzk et al. 2005). The variation in reproductive mode in *P. pratensis* could be explained by the difference in expressivity and interactions of these five genes (Matzk et al. 2005). However, environmental factors and other modifier genes have to be considered before drawing a final conclusion (Ozias-Akins 2006; Quarin 1986).

Diplospory

Diplospory is another type of gametophytic apomixis. The unreduced embryo sac arises from a generative cell, either indirectly by modified meiosis (meiotic diplospory) or directly by mitosis (mitotic diplospory). In meiotic diplospory (also referred to as the *Taraxacum* type), meiotic prophase is initiated but normal chromosome pairing does not take place due to

asynapsis, resulting in a restitution of the somatic chromosome number at meiosis I (Bhat et al. 2005). Mitotic diplospory is the more common type, also called the *Antennaria* type of diplospory, where the megaspore mother cell directly undergoes mitosis (Bhat et al. 2005).

Meiotic diplospory

Richards (1973) suggested that diplospory and parthenogenesis were controlled by two dominant genes, while a single locus control model of apomixis in *Taraxacum* was proposed by Mogie, in which diplospory is recessive and parthenogenesis is a pleiotropic effect of diplospory (Mogie 1988). However, recent studies are supporting the independent genetic control of apomixis elements in *Taraxacum* (van Dijk, 2003; van Dijk and Bakx-Schotman, 2004; Van Dijk et al., 1999; Zavesky et al., 2007). The inheritance of diplospory in *Taraxacum* was shown to be controlled by a dominant genetic factor, *DIP* locus. This locus does not affect parthenogenesis or autonomous endosperm development, indicating that three elements of apomixis in *Taraxacum officinale* can be inherited independently (van Dijk 2003; van Dijk and Bakx-Schotman 2004). The latest study in intersectional crosses of various sexual genotypes with apomictic *T. paludosum* (sect. Palustria) as pollen donor further confirmed independent genetic control of all apomixis elements in *Taraxacum* (Zavesky et al. 2007).

Mitotic diplospory

Mitotic diplospory in *Tripsacum dactyloides* segregates as a dominant Mendelian trait, but likely under the control of a complex locus (Grimanelli et al. 2001). In *Erigeron annuus* (Asteraceae), diplospory and autonomous development of embryo and endosperm are regulated by two independently segregating loci (Noyes 2000, 2005; Noyes et al. 2007; Noyes and Rieseberg 2000). This conclusion came from genetic linkage analysis and observation of progenies from the cross between apomictic and sexual genotypes. Different AFLP markers

were found to be associated with parthenogenesis and diplospory, indicating that diplospory and parthenogenesis were unlinked and inherited independently (Noyes and Rieseberg 2000). Plants with diplospory but lacking autonomous development and meiotic plants with autonomous development produced from a cross between an apomictic tetraploid (2n=4x=36) and a sexual diploid (2n=2x=18) further support that apomixis in *E. annuus* is under the control of two independent genetic factors (Noyes et al. 2007).

Dosage effect on endosperm development in apomictic species

In sexual flowering plants, fertilization is required for endosperm development. Fusion of two polar nuclei in the central cell of the embryo sac with one sperm results in maternal and paternal genome contributions to endosperm at a relatively fixed ratio of 2m:1p. Deviation from this balance usually leads to abnormal development of the endosperm and abortion of seeds (Haig and Westoby 1991). A well studied example is maize where the *indeterminate gametophyte* (*ig*) mutant established that the ratio of 2m:1p is critical for successful endosperm development as well as seed viability (Lin 1984). After crossing with different ploidies of pollen, Lin found that only those seeds which had either a 2m:1p or a 4m:2p constitution of the endosperm were viable (Lin 1984). The maternal to paternal genomic ratio of 2:1 is usually not disturbed in sporophytic apomicts since the endosperm is derived from the fertilized central cell containing two reduced nuclei (2m+1p). However, the formation of unreduced polar nuclei in central cells of gametophytic apomicts disrupts this kind of balance irrespective of whether endosperm development is autonomous (without fertilization) or pseudogamous (fertilization required for endosperm development) (Ozias-Akins 2006). Because microsporogenesis and microgametogenesis in most apomicts occur normally, i.e., through the meiotic pathway where reduced pollen is generated, fertilization of unreduced central cells with reduced pollen in

pseudogamous apomicts results in imbalance of paternal and maternal composition of endosperm. If the 2m:1p ratio is essential for normal endosperm development, how do apomicts deal with genomic imbalance? There is no universal way for all apomicts to tackle this problem. In *Panicum* and *Pennisetum* species, there typically is only one unreduced polar nucleus in the central cell; therefore, its fusion with a reduced sperm retains the 2m:1p ratio (Nogler 1984a; Ozias-Akins et al. 2003). Fertilization of the binucleate central cell with two sperm is another strategy taken by *Dichanthium annulatum* (Scott 2007) and *Ranunculus auricomus* (Nogler 1984a), resulting in a 4m:2p ratio in the endosperm, while *T. dactyloides*, *P. notatum* and some species with autonomous endosperm such as *H. pilosella*, *Eulaliopsis binata*, and *E. annuus* can tolerate the variation in maternal:paternal genomic ratios (Grimanelli et al. 1997; Quarin 1999). Both tolerance of endosperm imbalance and two-sperm fertilization of the central cell were found in *Crataegus* species. The central cell of apomictic *Crataegus* is fertilized by either one or two sperm to generate 4m+4m+2p or 4m+4m+2p+2p endosperm (Talent and Dickinson 2007). However, the mechanism for tolerating or modifying this imbalance does not seem to be inherited from apomicts to sexual genotypes together with parthenogenesis and apomeiosis, since a high level of seed abortion was found during the introgression of apomixis into sexual crops from wild relatives (Morgan et al. 1998).

What underlies the mechanism in apomicts for bypassing the dosage effect of paternal and maternal genomic contribution to endosperms? Some results from Arabidopsis are informative. In Arabidopsis, mutations in maternal Polycomb group (PcG) genes (*fie*, *fis2*, or *mea)* can lead to the proliferation of abnormal (2n) endosperm independent of fertilization, which is similar to autonomous endosperm development in apomicts (Hsieh et al. 2003). Endosperm development is suppressed by the wild-type function of these genes during sexual

reproduction. Interestingly, paternal inheritance of these mutant genes has no effect on seed development. This phenomenon is referred to as genomic imprinting in which the expression of genes during seed development is dependent on their parent of origin. However, autonomous endosperm development in FIS family mutants is limited in scale, for example, endosperm development arrests in the free-nuclear stage in the unfertilized *fie* mutant (Chaudhury et al. 1997). In a recent study, Nowack and co-workers confirmed that Arabidopsis can bypass genomic imprinting to produce seed with a functional diploid endosperm using two types of mutants: *cdka;1* and *mea* mutants. In the *cdka;1* mutant, pollen with only one sperm is generated, and was found to preferentially fertilize the egg cell; moreover, egg cell fertilization promoted endosperm proliferation, indicating that a positive signal from the developing embryo overcomes the maternal repression within the central cell to initiate endosperm development (Nowack et al. 2007). By contrast, the FIS-class genes in wild type repress proliferation of the central cell. Using the *cdka;1* mutant as pollen donor, the FIS family mutant plants can produce viable seeds with a functional diploid endosperm (Nowack et al. 2007), which is reminiscent of autonomous apomicts such as *Hieracium*. The results from this study suggest that if genomic imprinting is broken down and a positive signal from the developing embryo is provided, the endosperm in natural sexual plants can develop autonomously.

Assuming that the positive signal from developing embryos is universally required for endosperm development, precocious embryos could be a common phenomenon in apomicts in order to provide this positive signal. Precocious embryo development in *P. pratensis* was found to start at one day before anthesis (Yudokova and Shakina 2007). In *P. squamulatum*, the embryo can initiate with the division of the egg cell as early as one or two days before pollination, at which time the polar nuclei in central cells are fusing and synergids are degraded

(Wen et al. 1990). Precocious embryo formation in *Eupatorium laevigatum* starts before the central cell divides to form the endosperm, resulting in production of embryo sacs with both an embryo and a central cell, and an undivided egg cell was observed in only about 10% of the ovules at anthesis (Bertasso-Borges and Coleman 2005). Precocious embryo development before pollination was also observed in *T. dactyloides* (Grimanelli et al. 2003), *H. perforatum* (Barcaccia et al. 2006), *P. maximum* (Naumova and Willemse 1995) and *Brachiaria brizantha* (Alves et al. 2001). If the precocious embryo in apomicts is a general phenomenon, some cross-talk between developing embryo and endosperm could exist; and the discovery of signaling components which coordinate the crosstalk would be very important for revealing the mechanism of apomixis.

Embryological study of apomixis in *Pennisetum*

At least 17 species within the genus *Pennisetum* were reported to reproduce asexually (Ozias-Akins et al. 2003). In the ovule of sexual genotypes of *Pennisetum*, a single archesporial cell differentiates into a megaspore mother cell (MMC) and undergoes meiosis to give rise to four haploid cells, three of which degenerate and leave a single functional megaspore. This megaspore divides mitotically to form an eight-nucleated sexual embryo sac. The formation of an archesporial cell and development of a MMC is observed in apomictic genotypes of *Pennisetum* as well (Peel et al. 1997; Wen et al. 1998). The archesporial cell with its large nucleus and dense cytoplasm develops into a MMC which later divides meiotically to give rise to a triad or tetrad, but subsequently degenerates without further development (Peel et al. 1997; Wen et al. 1998). Simultaneously, one or more nucellar cells around the degenerated MMC enlarge and become aposporous initials which differ from other nucellar cells due to their large size, dense cytoplasm, and nucleus with clear profile (Wen et al. 1998). The aposporous initial

near the micropylar end of the ovule may develop into an aposporous embryo sac faster than other aposporous initials, which results in one four-nucleate embryo sac at the micropylar end and some one- or two-nucleate embryo sacs in proximity to the chalazal end (Wen et al. 1998). Multiple aposporous embryo sacs often overlap and interlock with each other (Chapman et al. 2000). Two synergids with one uninucleate central cell or one synergid with one binucleate central cell are two predominant phenotypes in aposporous embryo sacs of *P. squamulatum* and *C. ciliare* (Chapman and Busri 1994; Vielle et al. 1995). Wen et al. (1998) and Morgan et al. (1998) have observed similar phenomenon, i.e., central cells containing one or two polar nuclei in mature aposporous embryo sacs. Occasionally, three polar nuclei in the central cell were also observed (Vielle et al. 1995). Binucleate central cells were reported to be the major cause of poor seed set in an apomictic BC3 pearl millet because the central cell nuclei are unreduced (2n=4x) and when fused with a sperm from tetraploid pearl millet (n=2x), endosperm with a 4m:1p ratio is formed, which is an imbalanced maternal to paternal genome ratio (Morgan et al. 1998). Although the egg cell in aposporous embryo sacs is not fertilized for embryo development, there is no obvious difference between this egg cell and that from the reduced embryo sac in the ultrastructural study on *P. squamulatum* (Chapman and Busri 1994). Synergid degeneration was observed before pollination and fertilization in *C. ciliaris* (Vielle et al. 1995). The presence of an incomplete cell wall at the micropylar region of the egg cell has frequently been observed in sexual plants (Yadegari and Drews 2004), while a complete cell wall surrounding unreduced egg cells frequently was observed prior to fertilization in *P. ciliare*, suggesting that the initiation of parthenogenesis could happen before pollination (Vielle et al. 1995). In *P. squamulatum*, precocious embryos were even observed 1 to 2 days before anthesis (Wen et al. 1998). Precocious embryo development in *P. pratensis* was found to start 1 day

before flowering (Yudokova and Shakina 2007). In *Eupatorium laevigatum,* precocious embryo development was observed to start before the central cell divided to form the endosperm (Bertasso-Borges and Coleman 2005). Precocious embryo development before pollination was also observed in *T. dactyloides* (Grimanelli et al. 2003), *Hypericum perforatum* (Barcaccia et al. 2006), *P. maximum* (Naumova and Willemse 1995) and *B. brizantha* (Alves et al. 2001). Precocious embryos are thought to be a strong barrier to fertilization, probably a strategy for apomictic species to avoid the fertilization of unreduced egg cells (Chaudhury et al. 2001).

Marker development in apomixis and genetic mapping

Marker type

Genetic mapping is a crucial step for map-based gene cloning, and molecular markers are prerequisites for constructing such genetic maps. DNA markers are the most powerful molecular markers for genetic mapping since they have an advantage over phenotypic and biochemical (protein) markers in reproducibility, polymorphism, independence of environmental factors and epistatic interactions, and information content (even protein markers only reveal the difference in the gene products not the difference in genomic composition) (Kumar 1999). Basically, there are two types of DNA markers dependent on technique used for revealing the polymorphism: hybridization-based markers and PCR-based markers. RFLP (Restriction Fragment Length Polymorphism) is the most widely used hybridization-based marker (Botstein et al. 1980). Polymorphism of restriction fragments occurs if two plants have differently sized bands which can be caused by a point mutation in the recognition site, insertion/deletion, translocation, inversion and duplication. The advantage of RFLP is that it is co-dominant, allowing heterozygous individuals to be differentiated from homozygous dominant individuals. However, the application of RFLP is limited because of its technical demand, low level of polymorphism,

requirement of extensive time and labor, high quantity and quality of DNA required, and use of radioactive/toxic reagents (Agarwal et al. 2008).

PCR-based markers usually require the design of two genome specific oligonucleotides for each amplification, although random amplification with single short oligonucleotides also is possible. SSR (Simple Sequence Repeat /microsatellite)(Litt and Luty 1989), SCAR/STS [(Sequence Characterized Amplified Region (SCAR) or Sequence-Tagged-Site (STS)](Paran and Michelmore 1993), and allele specific amplification (Okayama et al. 1989) need the genomic sequence information for designing specific primers; while RAPD (Randomly Amplified Polymorphic DNA)(Williams et al. 1990), AP-PCR (Arbitrarily Primed PCR)(Welsh and McClelland 1990), and DAF (DNA Amplification Fingerprinting)(Caetano-Anolles and Bassam 1993) are developed by using random primer PCR methods, in which a single arbitrary oligonucleotide primer is used to amplify template DNA without prior knowledge of the target sequence. AFLP (Amplified Fragment Length Polymorphism) is another type of marker which is carrying elements of both RFLP and RAPD, because, whereas the method is PCR-based, its polymorphism is derived from variations in restriction sites (Vos et al. 1995). The primers used for AFLP are basically developed from the adapters ligated to restriction enzyme digested DNA fragments, so AFLP does not require genomic sequence information, but it can produce reproducible and high levels of polymorphism (Vos et al. 1995).

Sequence specific amplification polymorphisms

In recent years, polymorphism caused by transposable elements has become an increasingly popular source of molecular markers (Feschotte et al. 2002; Kumar and Hirochika 2001). There are two types of transposable elements: DNA transposons and retrotransposons. Retrotransposons, also called type I transposable elements, are the most abundant mobile genetic

elements in eukaryotes. Depending on the presence or absence of long terminal repeats (LTR), retrotransposons fall into two groups, LTR and non-LTR retrotransposons. LTR retrotransposons appear to be the most abundant and the most transcriptionally active in plants (Schulman 2007). According to the *Drosophila* type *copia* or *gypsy* retrotransposons, LTR retrotransposons of other organisms have been further divided into two major groups: *Ty1-copia*- or *Ty3-gypsy*-like (Kumar and Bennetzen 1999; Xiong and Eickbush 1990). The major difference between the two types of elements lies in the order of the integrase gene in the *pol* polyprotein region. Typically, LTR-retrotransposons are flanked by direct long terminal repeats which do not encode any known proteins, but contain the transcriptional promoter and terminator. Each LTR contains two short inverted repeats, usually TG/CA, at either end. A PBS (Primer Binding Site) and PPT (Polypurine Tract) are adjacent to the 5' and 3' LTR region, respectively. The PBS is responsible for priming synthesis of the (-)-strand of the cDNA, while the PPT primes the (+)-strand (Schulman 2004). The PBS sequence of retrotransposon mRNA is complementary to a cellular RNA, usually the 3'end of a host tRNA, which can be used for identifying the PBS of a retrotransposon. The core of a retrotransposon is the region encoding for proteins and necessary for the retrotransposon life cycle. This encoding region is relatively conserved, which is useful for isolation of retrotransposons. The structure of an LTR-retrotransposon is illustrated in Fig.2.1.

Unlike type II transposable elements, transposons such as *Ac*/*Ds* and *En/Spm* which excise as DNA and reinsert elsewhere in the host genome, retrotransposons utilize a mechanism called "copy and paste" to reproduce themselves and increase their copy number in the host genome. Normally, an autonomous retrotransposon contains encoding regions for enzymes and other proteins that are essential for transposition, using RNA as an intermediate to generate

extrachromosomal DNA and inserting it into the host genome by integrases. Because of this replicative transposition mechanism, retrotransposons have become highly repetitive and ubiquitous in plants and are widely distributed across the host chromosomes. Therefore, they can dramatically alter the host genome by increasing its size, disrupting genes, mediating chromosomal rearrangement, and contributing regulatory regions to genes (Kumar and Bennetzen 1999; Kumar and Hirochika 2001; Schulman 2007; Schulman 2004). These features make retrotransposons an ideal source for development of molecular markers.

SSAP (Sequence Specific Amplified Polymorphisms) is the first retrotransposon-based molecular marker developed (Waugh et al. 1997). It was actually modified from AFLP, but incorporates specific sequence information from LTR retrotransposons (Waugh et al. 1997). SSAP amplifies fragments between a retrotransposon integration site and a restriction site to which an adapter has been ligated. Three major steps are involved in the SSAP procedure (Fig. 2.2). Step1: digestion of genomic DNA with two enzymes (usually one is a four- base cutter and the second is a six-base cutter) and ligation of adapters to the digested DNA. Step 2: pre-amplification with adapter primers; the first two steps are the same as in the AFLP protocol. Step 3: selective amplification with one adapter primer extended with one or two selective nucleotides and one radioactive or fluorescence-labeled primer that is usually designed based on the LTR sequence of a retrotransposon. In some cases, SSAP has been shown to be more informative than other marker systems such as AFLP and SSR. Waugh et al. (1997) found SSAP to reveal 25% more polymorphism than AFLP in barley. In *Pisum*, SSAP exhibited a three-fold improvement over AFLP in discriminating among accessions (Ellis et al. 1998). SSAP has also been shown to have the higher assay efficiency index and marker index than AFLP and SAMPL (Selective Amplification of Microsatellite Polymorphic loci) (Porceddu et al. 2002b). When

SSAP was applied to assess the genetic diversity of tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*), SSAP revealed the highest number of polymorphic bands per assay and was the most informative system when compared to AFLP and SSR (Tam et al. 2005). In cucumber (*Cucumis sativus* L.), more polymorphisms also were displayed in SSAP than AFLP (Lou and Chen 2007). Fay et al. (2005) pointed out that AFLP is not optimal for some organisms containing large or highly repetitive genomes. For example, in *Iris missouriensis*, which contains large repetitive elements, standard AFLP did not produce useful markers, even after the restriction enzyme *Mse*I was replaced with *Hin*d III, while the SSAP markers were highly informative for population genetic analysis (Cornman and Arnold 2008).

Since it was innovated in 1997, SSAP has been used for detection of new insertions in tobacco (Melayah et al. 2001) and oat (Yu and Wise 2000); for genetic linkage analysis and mapping in pea (Ellis et al. 1998; Schneider et al. 1999), barley (Waugh et al. 1997), oat (Yu and Wise 2000), lettuce (Syed et al. 2006), and *Louisiana irises* (Bouck et al. 2005); for diversity study and phylogenetic analysis in *Triticeae* species (Gribbon et al. 1999), chickpea (Sant et al. 2000), pea (Pearce et al. 2000), alfalfa (Porceddu et al. 2002b), wheat (Queen et al. 2004), lotus (Madsen et al. 2005), tomato and pepper (Tam et al. 2005), apple (Venturi et al. 2006), potato (Lightbourn et al. 2007), cucumber (Lou and Chen 2007), and blue agave (Bousios et al. 2007). However, development of retrotransposon-based markers requires sequence information for the retrotransposon, usually the LTR part, for designing primers. One rapid method for isolation of retrotransposon terminal regions is to take advantage of the highly conserved retrotransposon domains for amplification between these conserved regions and a restriction site to which an adapter has been ligated (Pearce et al. 1999). Alternatively, the LTR terminal sequence

information could be retrieved by searching the increasing sequence information available in genomic databases.

Marker development for apomixis

The first molecular markers linked to apomixis were developed in *P. squamulatum* by using RFLP (Ozias-Akins et al. 1993). Various markers for mapping of apomixis have been developed since then: SSR and AFLP in *T. officinale* (Falque et al. 1998; Vijverberg et al. 2004), AFLP in *E. annuus* (Noyes and Rieseberg 2000), RFLP in *T. dactyloides* (Grimanelli et al. 1998b; Leblanc et al. 1995), RFLP, RAPD and AFLP in *B. brizantha* (Pessino et al. 1998; Pessino et al. 1997), AFLP in *P. simplex* (Labombarda et al. 2002), AFLP and SAMPL in *P. pratensis* (Barcaccia et al. 1998; Porceddu et al. 2002a), RAPD and RFLP in *P. squamulatum* (Ozias-Akins et al. 1993; Ozias-Akins et al. 1998), RFLP and RAPD in *C. ciliaris* (Dwivedi et al. 2007; Gustine et al. 1997; Roche et al. 1999), RAPD, RFLP and AFLP in *P. notatum* (Martínez et al. 2003; Stein et al. 2007). Whatever markers are used for genetic mapping of apomixis, one crucial step is the determination of marker dosage. A single dose marker refers to a DNA marker representing a single allele that segregates in the progeny in a ratio of 1:1. Since most apomicts are polyploid, only single dose markers are not affected by the ploidy level and genome constitution and are expected to segregate in a manner similar to diploid species. Markers in two or three copies per locus vary in their expected segregation ratios according to the genome constitution (Wu et al. 1992). Although double-dose markers have been applied to genetic mapping of apomixis (Noyes and Rieseberg 2000), single-dose markers can greatly simplify the mapping procedure (Stein et al. 2007).

Genetic mapping of apomixis

Genetic mapping of apomixis has been hindered due to inherent features of apomicts such as maternal inheritance, polyploidy and crossing barriers between species. Apomicts can produce progenies without fertilization of an egg cell, which means that most crosses must use the apomict as male parent. Although the pollen of apomicts is fertile and can be used for generating F1 populations from the crossing with related species, introgression of apomixis has been difficult because of factors such as incompatibility or polyploidy (Bicknell and Koltunow 2004). In addition, segregation distortion in F1 populations is a very common phenomenon, which may result from tight linkage of apomixis genes to deleterious genes and heterochromatin (Roche et al. 2001). Heterochromatin expansion and its interaction with centromeric proteins can lead to segregation distortion (Henikoff et al. 2001). Another reason for why the construction of genetic maps of apomictic species has lagged behind that of sexual species comes from differences in ploidy levels. Most apomicts are polyploid, while most sexual species are diploid. For polyploid species, large segregating populations and complicated statistical approaches are required for reliable estimation of genetic distance because of the high number of possible genotypes. The greatest source of frustration encountered for mapping of apomixis is the suppression of recombination that has been observed at the apomixis "locus" of many species. The first observation of a recombinationally suppressed region came from the study on *P. squamulatum*. Twelve PCR-based molecular markers co-segregated with apospory and showed no recombination in a population of 397 individual plants (Ozias-Akins et al. 1998). Further studies using FISH showed that the region defined by these markers is hemizygous, heterochromatic and physically large (Akiyama et al. 2004; Goel et al. 2003; Ozias-Akins et al. 1998). The strong suppression of recombination around the apomixis locus in *P. squamulatum* is

not an exceptional event; this phenomenon is also found in other species such as diplosporous *E. annuus* (Noyes and Rieseberg 2000), *T. dactyloides* (Grimanelli et al. 1998a), aposporous *P. simplex* (Labombarda et al. 2002), *C. ciliaris* (Akiyama et al. 2005; Goel et al. 2006; Jessup et al. 2002), all of which demonstrated that a cluster of molecular markers is strictly linked to the apomeiosis trait. However, in the mapping study on *T. officinale,* recombination of markers was observed in a relatively large number of plants (11 out of 73) and no markers were found to completely co-segregate with diplospory, indicating that there is no evidence for suppression of recombination at the *DIP* locus (Vijverberg et al. 2004). Several factors such as heterochromatic nature, hemizygosity, and translocation/inversion may account for the lack of recombination around the apomixis locus at least in *P. squamulatum* (Akiyama et al. 2004; Akiyama et al. 2005; Calderini et al. 2006; Goel et al. 2006; Ozias-Akins et al. 2003; Roche et al. 2001).

Lack of recombination is particularly problematic because it markedly reduces the power of the map-based cloning strategies. Alternatively, researchers have turned to comparative genomics for improving the resolution of genetic mapping of apomixis. As genome sequencing projects on Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) have been completed and sequences of some other crop species such as maize are on the horizon, comparative mapping of apomixis could be of use because previous studies showed that among related species, especially within the grass family, chromosomal regions and even colinearity at the DNA-sequence level are well conserved during evolution (Devos 2005). However, comparative mapping in some apomictic species with rice or maize markers found that these markers are again clustered together at the apospory locus indicating that the power of comparative mapping with markers from the model species to improve the resolution of genetic mapping of apomixis is not as strong as expected (Grimanelli et al. 1998a; Pessino et al. 1997; Pupilli et al. 2001; Pupilli et al. 2004).

However, partial sequencing of BAC clones in *Pennisetum* and *Paspalum* revealed that mobile elements are abundant in the region regulating apomixis, which raises the possibility for developing retrotransposon-based markers for fine mapping of apomixis (Akiyama et al. 2004; Calderini et al. 2006; Gualtieri et al. 2006).

Isolation and characterization of candidate genes for apomixis

Isolation of candidate genes

Comparative gene expression methods such as differential display and subtractive hybridization techniques were applied to isolate differentially expressed genes in ovules of sexual and apomictic plants. Because apomictic reproduction and the characteristics associated with it make it difficult to produce near-isogenic materials for studying the mode of reproduction, pooled mRNA from unpollinated mature ovaries of five sexual or facultatively apomictic *Brachiaria* hybrids were used for differential display to avoid the problem caused by polymorphism due to heterozygosity instead of expression differences. Two fragments were detected only in apomicts, but northern blotting did not validate the specificity (Leblanc et al. 1997). A similar strategy was also applied to *P. notatum* (Pessino et al. 2001). Three transcripts were found to express in apomictic F1 individuals at a level around 30 times higher than in sexual F1 individuals (Pessino et al. 2001). In *P. maximum*, one apomixis specific gene (*ASG-1*) was identified, and *in situ* hybridization showed that ASG-1 was detected in ovules of the apomictic plant only after the aposporous initial cell(s) appeared (Chen et al. 2005; Chen et al. 1999). In buffelgrass (*C. ciliaris*), differential display and suppression subtractive hybridization were used to compare gene expression in ovaries of sexual and apomictic accessions (Singh et al. 2007; Vielle-Calzada et al. 1996). Although two cDNAs in each study were found to be specific to apomicts, only the two genes identified by Singh et al. (2007) (*Pca 21* and *Pca 24*) were

further confirmed by *in situ* hybridization to specifically express in developing embryo sacs of apomictic ovaries. In another recent study on *Brachiaria*, ovaries at earlier stages (megasporogenesis and megagametogenesis) of sexual and apomictic accessions of *B. brizantha* were used for gene expression comparison, and six fragments were isolated that were preferentially expressed in the apomicts (Rodrigues et al. 2003). Two out of six were further confirmed by *in situ* hybridization to specifically express in apomictic plants – one in cells of the embryo sac and integument, another in different cells of the ovule, notably in synergids. In *P. pratensis*, three different types of F1, apomictic (aposporic and parthenogenetic), sexual (non-aposporic and non-parthenogenetic) and recombinant (aposporic but non-parthenogenetic), were used to compare differential gene expression by using the cDNA-AFLP transcript profiling technique. Nearly 179 transcript-derived fragments showed quantitative or qualitative expression differences between apomictic and sexual genotypes (Albertini et al. 2004). The use of a recombinant F1 could be one reason for the high efficiency of this study. Two genes, namely *PpSERK* (somatic embryogenesis receptor kinase) and *APOSTART*, were further characterized by RT-PCR and *in situ* hybridization. However, neither one was found specifically expressed in apomictic or sexual types; only quantitative differences in expression between apomictic and sexual genotypes were observed (Albertini et al. 2005).

Mutants with phenotypes corresponding to elements of apomixis have been found in Arabidopsis, maize, barley and carrot (Spillane et al. 2001). *Genes involved in meiosis:* Mutations have been recovered that affect megaspore mother cell development and meiosis. If a mutation affects early megasporogenesis, cell fate may be changed and more cells with the potential for developing into embryo sacs could appear, which is similar to the phenotype of aposporous plants. Such a phenotype has been found in the *mac1* mutant of maize where up to

21 MMCs were observed in a single ovule (Sheridan et al. 1996). The mis-regulation of meiosis as observed in some female gametophytic mutants could result in diplospory (Spillane et al. 2001; Yadegari and Drews 2004). Mutations in *SWI1, NZZ/SPL,* or *DYAD* can cause defects in meiosis and result in unreduced embryo sacs in Arabidopsis (Caryl et al. 2003; Ravi et al. 2008). Similar mutants have been found in maize and barley (Spillane et al. 2001). *Genes involved in embryo development:* The *Indeterminate gametophyte1* (*ig1*) mutant of maize shows increased haploid progenies (Evans 2007), while over-expression of some genes such as *LEAFY COTYLEDON* (*LEC1*) (Lotan et al. 1998), *BABY BOOM* (*BBM*)(Boutilier et al. 2002), *WUSCHEL* (*WUS*) (Zuo et al. 2002) and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*AtSERK1*)(Hecht et al. 2001) have been shown to induce embryogenic cell fates in Arabidopsis. Homologous genes also have been described in carrot (*Daucus carota*), corn (*Zea mays*), rice (*Oryza sativa*), sunflower (*Helianthus annuus*), Brassica (*Brassica napus*) and *P. pratensis* (Hu and Wang 2008). *Genes involved in endosperm development:* After screening Arabidopsis mutants for seed development in the absence of fertilization, three loss-of-function mutations were identified: *fertilization-independent endosperm* (*fie*) (Ohad et al. 1996), *medea* (*mea*) (Grossniklaus et al. 1998b), and *fertilization independent seed2* (*fis2*) (Chaudhury et al. 1997). In these three mutants, autonomous endosperm development can occur whereas embryo development is arrested in the absence of egg cell fertilization. Similar to the FIS class gene, loss of MSI1 function also can initiate the endosperm development in the absence of fertilization. Upon pollination, fertilization of the central cell in *msi 1* mutant could be avoided due to its division prior to fertilization and only the egg cell was fertilized, resulting in the formation of seeds containing embryos surrounded by diploid endosperm (Kohler et al. 2003). In one recent report that takes advantage of an Arabidopsis mutant lacking female gametophytes, Steffen et al. (2007)

isolated forty-three genes expressed in female gametophytes by using microarray. Among these expressed genes, 11 are exclusive to the antipodal cells, 11 exclusively or predominantly in the central cell, 17 exclusively or predominantly in the synergid cells, one exclusive to the egg cell, and three strongly expressed in multiple cells of the female gametophyte.

No mutant described above, however, shows all three essential components of apomixis, i.e., apomeiosis, parthenogenesis and autonomous endosperm development, which indicates that despite the progress in understanding apomictic reproduction that can be made from studying mutations in sexual reproduction, understanding the function of a gene or genes involved in controlling apomixis that occurs naturally in some plant species is essential and crucial for harnessing its potential for plant breeding .

Characterization of candidate genes

When map-based gene discovery or comparison of transcript profiles between sexual and apomictic lines to detect differentially regulated genes is the major approach to identify gene(s) from apomictic plants, gene characterization will be essential. To date, one of the most efficient methods is RNA interference (RNAi).

The RNAi phenomenon was serendipitously observed in the study of petunia floral pigmentation. In an attempt to alter floral color in petunia, pigment-producing genes were introduced to evoke over-expression. Surprisingly, the transgenic plants showed a reduction in pigments rather than an increase as predicted. The phenomenon originally was termed cosuppression because RNAs from both the transgene and homologous endogenous gene were degraded (Napoli et al. 1990). Much more recently we learned that cosuppression shares a common underlying mechanism with RNAi (Tijsterman et al. 2002). The study of *Caenorhabditis elegans* provided the first evidence that dsRNA could result in gene silencing.

When Guo and Kemphues attempted to determine the function of *par-1* in *C. elegans*, they injected the antisense RNA into the worm, while the sense RNA was injected as a control. Surprisingly, identical phenotypes (embryonic lethality) were found in both antisense- and control-injected worms, which led to the conclusion that sense RNA was as effective as antisense RNA for suppressing gene expression in *C. elegans* (Guo and Kemphues 1995). Fire et al (1998) further demonstrated that dsRNA is the actual trigger of RNAi because the injection of both antisense and sense RNA of a target gene into the worm can cause gene silencing ten-fold more efficiently than either strand alone. Simultaneously, Waterhouse et al. (1998) also found that concurrent expression of RNA in both orientations induced more efficient gene silencing in plants than either orientation alone. Thereafter, RNAi refers to the biological process in which gene expression is inhibited in a sequence specific manner caused by dsRNA (Hammond et al. 2001). There are three different pathways for RNA silencing. 1) Cytoplasmic RNA silencing also known as post-transcriptional gene silencing (PTGS). Virus-induced RNA silencing and gene silencing caused by transgenes such as the example described above belong to this type. This type of gene silencing is the basis of present experimental RNA interference (Baulcombe 2004; Mansoor et al. 2006). 2) RNA silencing caused by micro-RNAs (miRNAs). miRNA is a type of small RNA, which negatively regulates gene expression in plants and animals. miRNA can cause PTGS through RNA cleavage or arrest of protein translation (He and Hannon 2004). miRNAs are encoded by specific genes and have a characteristic stem-loop structure. 3) heterochromatin silencing. This type of RNA silencing is associated with DNA methylation and results in transcriptional gene silencing (TGS) (Lippman and Martienssen 2004).

However, these three types of silencing share similar mechanisms in that double-stranded RNA (dsRNA) is a common trigger. These dsRNAs are recognized by Dicer, a member of the

RNase III endonucleases, and rapidly processed into short RNA duplexes of 21-24 nucleotides in length (Fig. 2.3). Twenty-one nt siRNAs are believed to guide mRNA cleavage, while 24-nt siRNAs are believed to exclusively mediate chromatin modifications (Brodersen and Voinnet 2006; Hamilton et al. 2002; Lippman and Martienssen 2004). The antisense strand of the 21-nt duplex will preferentially incorporate into the RNA-induced silencing complex (RISC) with a specific protein *AGO-1*, where sequence-dependent degradation of complementary target mRNAs occurs (Baulcombe 2004; Mello and Conte 2004).

Theoretically, RNAi has numerous advantages for functional characterization of a gene over chemical/physical or insertional mutagenesis. 1) Mutagenesis is random, and large collections of mutant lines are necessary to obtain good coverage of whole genomes; therefore, this type of mutagenesis is labor-intensive and time consuming. RNAi is sequence-specific, and only a few transformants are required for each target gene (Matthew 2004; Small 2007). 2) RNAi is dominant, so phenotypes can be observed in the T1 generation. Most mutations are recessive and will only manifest their phenotypes in segregating M2 populations which complicates large-scale analysis of mutants (Small 2007). 3) RNAi most often leads to a knockdown in gene expression rather than total elimination and allows selection of lines with different degrees of silencing. This facilitates the study of essential genes whose inactivation would lead to lethality or extremely severe pleiotropic phenotypes (Wesley et al. 2001). 4) RNAi can be controlled in a tissue-specific or time-dependent manner. Gene regulatory regions can be selected for RNAi construct that would enforce gene silencing only in specific tissues as has been shown with Arabidopsis (Chen et al. 2003; Huanca-Mamani et al. 2005). 5) With the increasing number of genome and EST sequencing projects, RNAi can be quickly and easily used in a wide range of genotypes or even species, whereas insertional mutant collections are limited to just a few due to

the effort involved (Small 2007). 6) RNAi allows the silencing of several related genes in parallel by targeting conserved regions of the genes, which make it an ideal research tool for the study of redundant gene functions, especially in polyploids (Lawrence and Pikaard 2003; Miki et al. 2005; Travella et al. 2006).

Because dsRNA is one major inducer for RNAi, various approaches have been used to produce dsRNA in plants. Initially, transgenic plants with constructs to produce sense or anti-sense RNA were achieved separately, and the dsRNA can be subsequently produced by crossing the two types of transgenic plants (Waterhouse et al. 1998). A more efficient method has been developed in which both sense and antisense sequences are cloned into a single construct with an intron as a spacer. RNA produced with this kind of construct can easily form dsRNA which is processed by DICER into siRNA for RNAi (Smith et al. 2000). Hairpin RNA (hpRNA) constructs containing sense/anti-sense arms ranging from 98 to 853 nt were shown to elicit efficient silencing in a wide range of plant species, and inclusion of an intron in these constructs had a consistently enhancing effect (Wesley et al. 2001). The construct for producing hpRNA has been optimized and is now the most widely used system for silencing genes in plants (Helliwell and Waterhouse 2005). To date, RNAi has been applied to various disciplines of plant research including metabolic engineering, disease resistance and plant development, which has been recently reviewed by Mansoor et al. (2006).

Reproduction in plants is an area of intense investigation in plant developmental biology where RNAi has contributed to the functional characterization of genes. In one Arabidopsis study, *AGP18* (a gene encoding an arabinogalactan protein) was silenced through RNAi using a 740 bp fragment of the gene containing the first exon and 5' untranslated region in both sense and antisense orientations separated by a chalcone synthase intron and under the control of the

CaMV35S promoter. The reduction of *AGP18* in female reproductive organs resulted in failure of the functional megaspore to enlarge and divide. Although at the DNA level, *AGP17* and *AGP19* share 55 and 52% homology with *AGP18* in the 740-bp cDNA fragment that was used to generate the RNAi construct, none of the T2 *AGP18*-RNAi lines analyzed showed a significant decrease in either *AGP17* or *AGP19* expression, indicating that post-transcriptional gene silencing caused by RNAi was specific to *AGP18* (Acosta-Garcia and Vielle-Calzada 2004). In another study, both CaMV35S and a promoter from a gene expressed specifically in the functional megaspore and megametophyte (pFM1) were used to develop RNAi constructs to knock down *CHR11*, a gene encoding chromatin-remodeling protein 11. Unlike *AGP18*, *CHR11* is not specifically, although it is predominantly, expressed in female organs during the sporophytic and gametophytic phases, and the constitutive reduction of *CHR11* caused by CaMV35S–RNAi did not show defects during gametophytic development or early seed formation but resulted in reduced plant height and embryo size. However, the specific silencing caused by pFM1-RNAi at the onset of female gametogenesis resulted in defective female gametophytes arrested before the completion of mitotic haploid nuclear divisions (Huanca-Mamani et al. 2005). One good example for using RNAi to simultaneously silence multiple members of a gene family came from the study of late pollen actins in Arabidopsis (Pawloski et al. 2006). *Arabidopsis thaliana* has eight actively expressed actin genes, four of which encode the late pollen actins (LPAs) that are highly expressed in mature pollen, pollen tubes, and a few cells within the ovule. The authors assembled the first 90-nucleotides from the 3'UTR of each member into one RNAi construct; the antisense and sense assembly of 3'UTR sequences was separated by a GUS linker. The results showed that all four actins were silenced simultaneously with only one RNAi construct (Pawloski et al. 2006). In addition, RNAi has also been widely

used in petunia (Kapoor et al. 2002), oilseed rape (Byzova et al. 2004), tobacco (Nawaz-ul-Rehman et al. 2007), maize (Cigan et al. 2005) and rice (Moritoh et al. 2005; Prasad and Vijayraghavan 2003) for reproductive developmental study.

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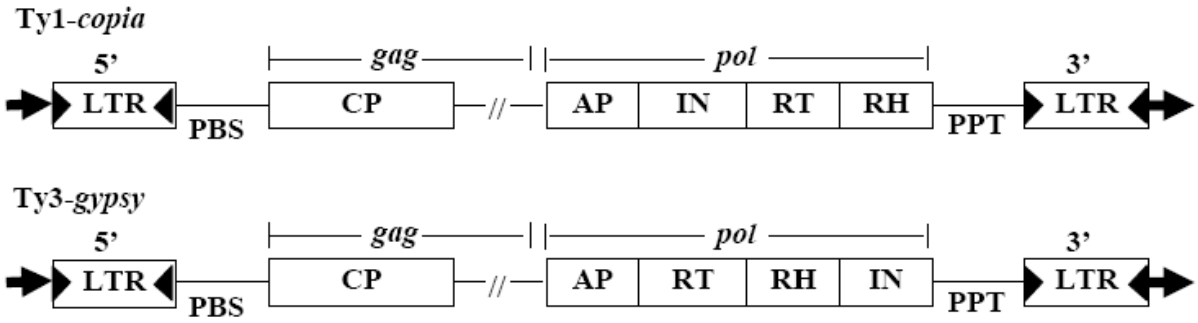

Fig. 2.1 Organization of LTR retrotransposons. LTR retrotransposons can be classified into two major groups, Ty1-*copia* and Ty3-*gypsy*. The elements are flanked by 5-bp direct repeats in the host DNA (arrows). There are two long terminal repeats (LTR) at each end of the elements. Within the LTR, a pair of short inverted repeats, usually 5'TA/CG3'(arrow heads) are located at each end. Downstream to the 5' and upstream to 3' LTRs are the primer binding site (PBS) and the polypurine tract (PPT), respectively. The genes within the LTR retrotransposons encode capsid-like protein (CP), aspartic proteinase (AP), integrase (IN), transcriptase (RT) and RNase H (RH). The protein coding region is generally synthesized as a *gag-pol* polyprotein which is subsequently cleaved by AP. RT and RH are responsible for synthesis of cDNA from the RNA transcript, while the integrase inserts the cDNA copy into the genome. There is a nucleic acid binding moiety between CP and AP. The figure is not drawn to scale.

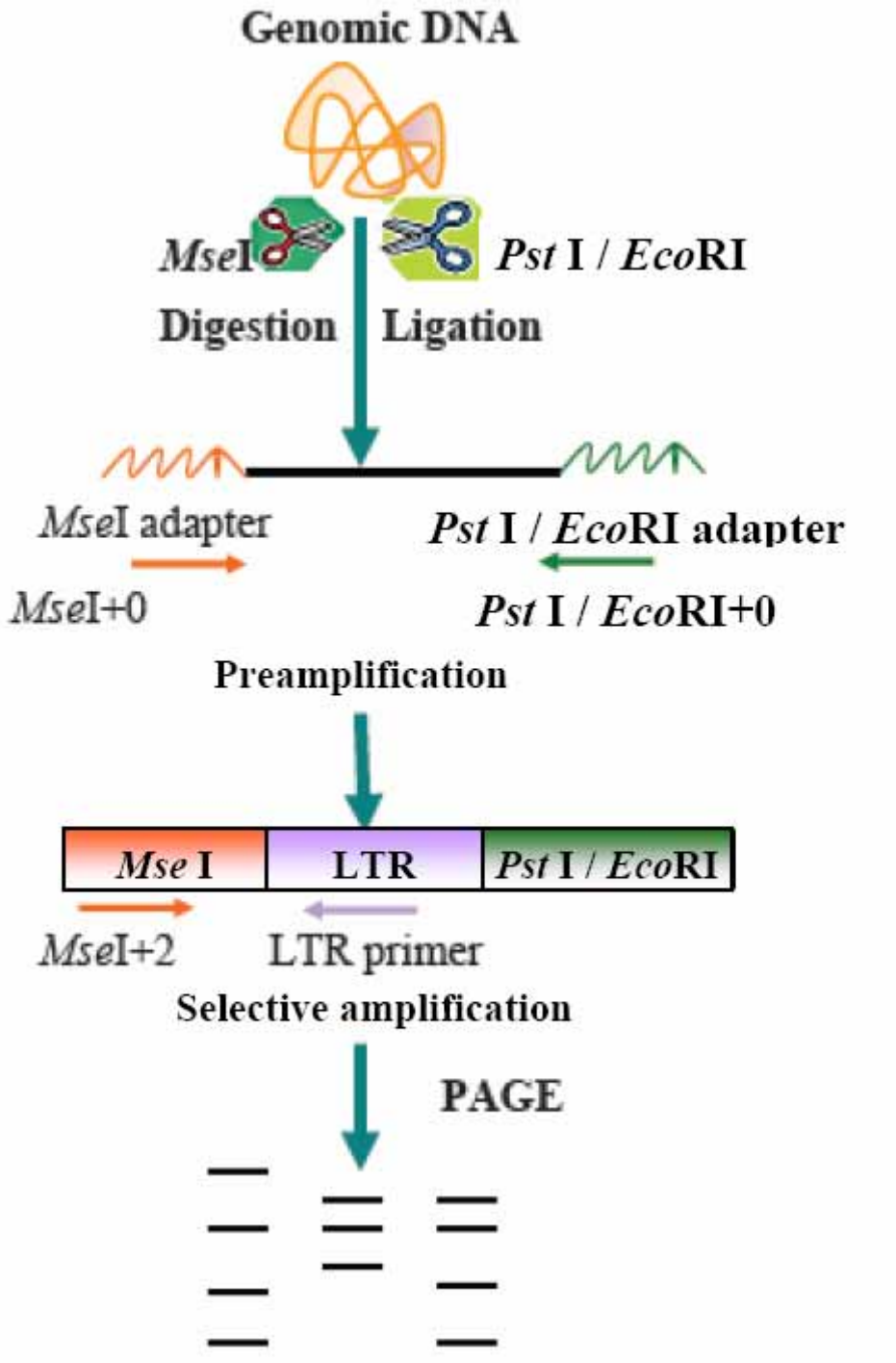

Fig.2.2 Schematic representation of the ASGR- specific LTR Sequence-Specific Amplification Polymorphism (SSAP) protocol.

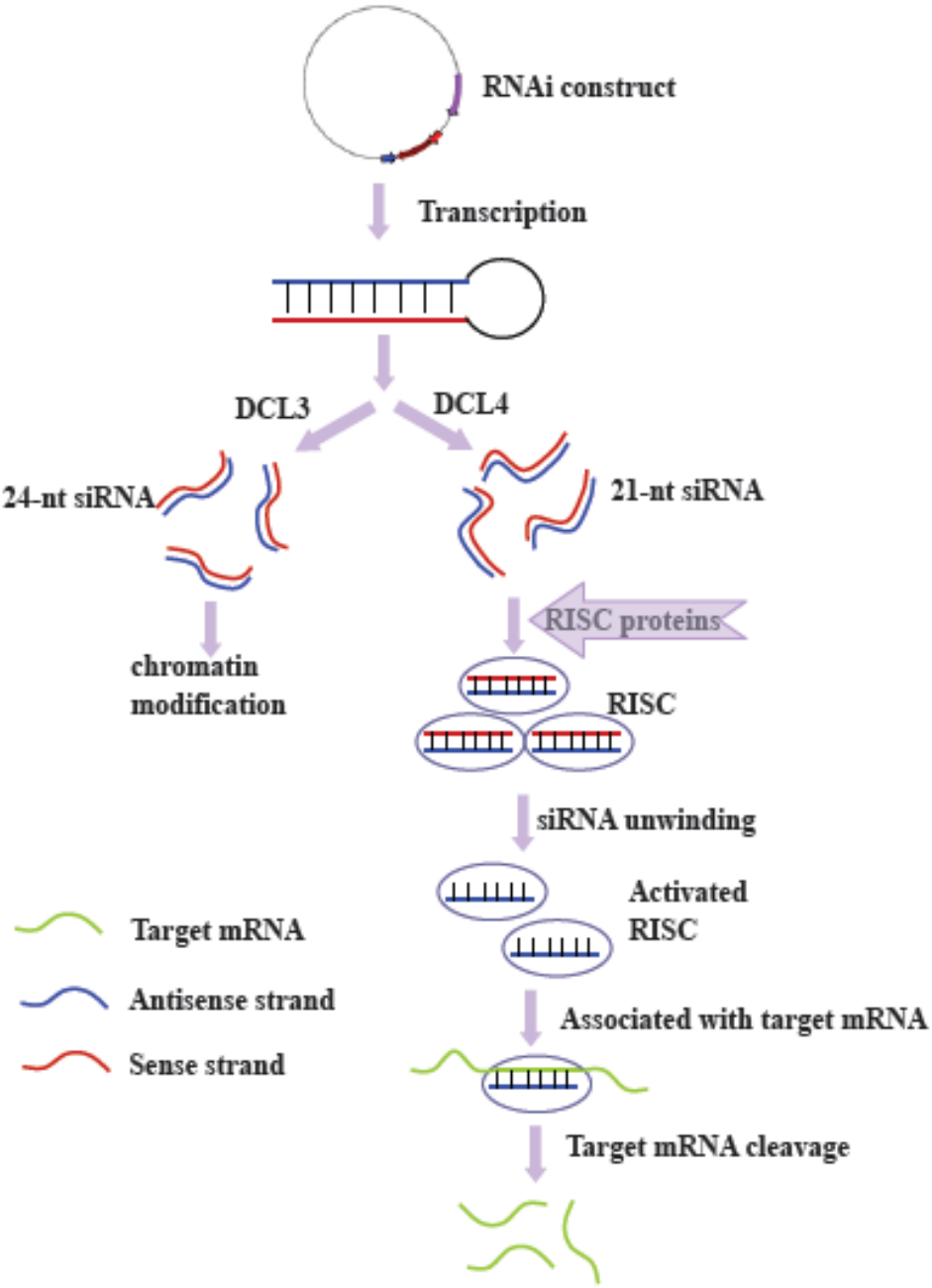

Fig.2.3 Basic RNAi mechanism in plants. After a typical RNAi construct containing a promoter (lavender), an antisense strand target fragment insert (blue), a spacer (dark red) and a sense strand target fragment insert (red) is transcribed, hairpin RNA is produced. The dsRNA will be cleaved into either 24-nt siRNA by Dicer-like-3 (DCL3) or 21-nt siRNA by Dicer-like-4 (DCL4). siRNAs of the 24 nt size class can direct DNA or histone modification at homologous loci; while siRNAs of 21 nt size will combine with RISC proteins (*AGO-1*, is the major component) to form a RNA-induced silencing complex. In the activated RISC, only one siRNA strand (preferentially, the antisense strand) incorporates into *AGO1*-loaded RISC to guide endonucleolytic cleavage of homologous target RNA, leading to its degradation.

CHAPTER 3

GENETIC MAPPING OF THE APOSPORY SPECIFIC GENOMIC REGION (ASGR) IN *PENNISETUM SQUAMULATUM* USING RETROTRANSPOSON-BASED MOLECULAR MARKERS[1]

[1] Huo, H., Conner, J.A., and Ozias-Akins, P. To be submitted to Theor Appl Genet

Abstract

Pennisetum squamulatum reproduces by apospory, a type of apomictic reproduction where non-generative nucellar cells develop into unreduced embryo sacs. Previous studies have shown that apospory in *P. squamulatum* is transmitted as a dominant Mendelian trait and that one genomic region from a single chromosome is sufficient for inheritance of the trait. This region previously was designated as the apospory-specific genomic region (ASGR). Further characterization of the ASGR showed that it is hemizygous, heterochromatic, recombinationally suppressed with co-segregation of numerous molecular markers, and physically large (> 50Mb), making high-resolution genetic mapping and map-based cloning difficult. Partial sequencing of BAC clones mapped to the ASGR revealed that *Opie-2*-like retrotransposons are abundant in this region. In this study, the long terminal repeat (LTR) regions of ASGR-abundant retrotransposons in the genome of *P. squamulatum* were identified and sequenced. LTR-specific primers for sequence-specific amplification polymorphism (SSAP) were designed based on alignment of all isolated LTRs plus one type of LTR retrotransposon from the sexual relative *P. glaucum*. Two different enzyme combinations were tested in three different types of SSAP to generate 290 single-dose markers from 38 primer combinations. The SSAP markers combined with one previous AFLP marker and one SCAR marker were used for genetic linkage analysis and construction of a genetic map that resulted in the formation of 27 linkage groups at LOD 10, one of which contained the previously mapped ASGR markers and >60% of the SSAP markers. After removing identical markers (identical band scoring) on the largest linkage group, forty-six markers were finally used for genetic mapping at a LOD score of 10 where they were observed to distribute across 10 different loci covering 19 cM, although 45/46 were distributed within 9 cM, indicating that these retrotransposon-based markers are strongly clustered. Six markers

were recovered and sequenced; five of them were successfully converted into SCARs (Sequence Characterized Amplified Region). One SSAP marker (E86-1104-522) showed recombination in three F1 plants, but only two F1 plants showed recombination with this SCAR. Expression of the ASGR retrotransposon was detected with one SCAR marker (Pst 56-1205-400) in root, anther, leaf and ovary of *P. squamulatum*.

Introduction

Although sexual reproduction is ubiquitous in the plant kingdom, asexual reproduction through seeds (apomixis) predominates in some angiosperm species (Asker and Jerling 1992; Nogler 1984). Apomictic plants can produce progenies that are genetically identical to the maternal parent. This feature brings huge potential value to apomixis in agricultural and evolutionary research (Meeûs et al. 2007; Spillane et al. 2004; Spillane et al. 2001; van Dijk and van Damme 2000). For example, apomixis preserves heterosis into the next generation of a hybrid, a feature that could dramatically reduce the cost of hybrid seed production. However, apomixis is rarely found in crop species although it has been demonstrated to be relatively prevalent in angiosperms (Bicknell and Koltunow 2004; Carman 1997; Ozias-Akins 2006). Extensive efforts have been undertaken to introduce the apomixis trait to crop species from their wild relatives (Dujardin and Hanna 1989; Grimanelli et al. 1995; Spillane et al. 2001). The most successful crossing was accomplished between pearl millet (*Pennisetum glaucum*) and *P. squamulatum*, an aposporous relative (Dujardin and Hanna 1989). However, the introgression of apomixis into related crop species is hindered by a variety of factors such as incompatibility between pollen donor and recipient or hybrid pollen sterility (Ozias-Akins et al. 2003) and endosperm imbalance in the new hybrids (Scott 2007; Spillane et al. 2004). An alternative approach to producing apomictic crops might be to transfer to a sexual plant one or more well-

characterized genes known to confer the trait of apomixis. The recent exploration of molecular mechanisms underlying apomixis might eventually enable the application of apomixis to crop breeding (Grimanelli et al. 2001; Grossniklaus et al. 2001; Koltunow et al. 1995; Ozias-Akins 2006). In *P. squamulatum*, unlike some species such as *Taraxacum officinale* (van Dijk et al. 1999), *Poa pratensis* (Albertini et al. 2001), *Erigeron annuus* (Noyes and Rieseberg 2000) and *Hieracium caespitosum* (Catanach et al. 2006), where the formation of unreduced embryo sacs and parthenogenesis were confirmed to be controlled by independent loci, apospory has been shown to be transmitted as a single locus, but this "locus" actually is a large chromosomal block which we named the apospory-specific genomic region (ASGR) (Akiyama et al. 2004; Goel et al. 2003; Ozias-Akins et al. 1998). Further characterization of the ASGR showed that it is hemizygous, physically large, highly heterochromatic and abundant in repetitive elements such as *Opie-2*-like retrotransposons (Akiyama et al. 2004; Goel et al. 2003). Maternal apomeiosis and suppressed recombination in the ASGR hamper marker development, genetic mapping, and map-based cloning. Although recombination-based high-resolution mapping is unlikely to be successful, physical mapping could be accomplished if the region could be saturated with molecular markers. We sought to test the ability of the long terminal repeat (LTR) retrotransposons abundant in the ASGR to provide a unique resource for molecular marker development.

Retrotransposons, also called type I mobile genetic elements, are ubiquitous in plants and constitute a major portion of their genome (Flavell et al. 1992; Kumar and Bennetzen 1999; Suoniemi et al. 1998; Voytas et al. 1992). Depending on the presence or absence of long terminal repeats (LTR), retrotransposons are classified into two groups, LTR and non-LTR retrotransposons, respectively (Kumar and Bennetzen 1999). In plants, LTR retrotransposons

appear to be the most abundant and the most transcriptionally active (Arabidopsis Genome Initiative 2000; The Rice Chromosome 10 Sequencing Consortium 2003). Their ubiquity in plants, wide dispersion on all chromosomes and activity in creating genomic diversity make them ideal for use as molecular markers (Kumar and Hirochika 2001; Schulman 2004). The SSAP (Sequence Specific Amplified Polymorphism) technique was modified from AFLP (Amplified Fragment Length Polymorphism) (Vos et al. 1995; Waugh et al. 1997), in which one AFLP adapter primer for selective amplification was replaced with a LTR-specific primer. SSAP has been shown to be more efficient than other marker systems such as AFLP, SAMPL (Selective Amplification of Microsatellite Polymorphic loci) and SSR (Simple Short Repeats) (Ellis et al. 1998; Lou and Chen 2007; Porceddu et al. 2002; Tam et al. 2005; Waugh et al. 1997). To date, SSAP has been used for genetic linkage and diversity analysis in barley (Waugh et al. 1997), pea (Ellis et al. 1998; Pearce et al. 2000; Schneider et al. 1999), chickpea (Sant et al. 2000), oat (Yu and Wise 2000), alfalfa (Porceddu et al. 2002), wheat (Queen et al. 2004), lotus (Madsen et al. 2005), iris (Bouck et al. 2005), lettuce (Syed et al. 2006), apple (Venturi et al. 2006), potato (Lightbourn et al. 2007), cucurbit (Lou and Chen 2007), blue agave (Bousios et al. 2007), Narbon vetch (*Vicia narbonensis*) (Sanz et al. 2007), and tomato and pepper (Tam et al. 2005). In the present study, we characterized the LTR region of a particularly abundant retrotransposon in the ASGR, and used the sequence information to develop numerous apomixis-linked markers that will augment the toolbox for construction of a physical map of the ASGR in *P. squamulatum*.

Materials and Methods

Plant materials and trait screening

Eighty-nine individuals from one separate populations of a cross between induced tetraploid pearl millet (*P. glaucum*) (2*n*=4x=28) and *P. squamulatum* (Ps26, PI 319196, 2*n*=56) (Goel et al. 2006) were used for molecular mapping. An apomictic individual (line 58) from the fourth back cross between BC3 (Dujardin and Hanna 1989) and tetraploid pearl millet was also included in this study. Plants (52 apomicts and 37 sexuals) were characterized for mode of reproduction by clearing of ovules in methyl salicylate (Young et al. 1979) and for marker phenotype using two ASGR-linked sequence characterized amplified region (SCAR) markers (UGT197 and Q8M) (Ozias-Akins et al. 1998). A sexual diploid Tift23BE, sexual tetraploid hybrid of Tift239DB and Tift23BE, and tetraploid IA4X pearl millet were also used in this study.

BAC clones

Nineteen ASGR-linked BAC clones containing retrotransposon-related sequence (P601, P602, P701, P702, P703, P704, P706, P707, P708, P709, P800, P801, P802, P803, P804, P900, P901, P902, P903) previously had been isolated and fingerprinted (Akiyama et al. 2004; Goel et al. 2006; Roche et al. 2002).

Nucleic acid extraction and purification

Genomic DNA was extracted as previously described (Ozias-Akins et al. 1993). Plasmid DNA was extracted and purified with the QIAGEN plasmid mini kit following the manufacturer's instructions (QIAGEN, Valencia, CA). PCR products for TA cloning were directly purified with QIAquick PCR purification kit or gel-purified with QIAquick gel extraction kit (QIAGEN). Anther, root, leaf, and ovary RNAs were isolated with the QIAGEN RNeasy mini kit (QIAGEN). Ovaries were collected under a microscope from heads of Ps26 at

D0 (anthers beginning to exsert but prior to pollen shed) and D1 (1 day after pollination). DNA quantification was conducted with the Fluorocount microplate fluorometer using Hoechst 33258 (Packard, Meriden, CT, USA) following the instruction of Molecular Cloning Protocols (Sambrook and Russell 2001), while RNA was quantified with RiboGreen® RNA Quantitation Kit following the manufacturer's protocol (Molecular Probes, Eugene, OR, USA).

Generation of additional LTR sequence from the ASGR

BlastX and BlastN at the NCBI (www.ncbi.nlm.nih.gov/blast/Blast.cgi) were conducted with sequence contigs from BAC-skim sequences of the ASGR-mapped BACs (Conner et al. 2008) against the non-redundant protein sequence database (BlastX) and nucleotide collection database (BlastN). Only the contigs showing similarity to the LTR-retrotransposons were selected for further analysis. A LTR-retrotransposon called Ofovin from a pearl millet BAC clone (AF488414) has been shown to have similarity to some contigs of P602 and P800 when BlastN was performed. All contigs hitting Ofovin were manually assembled into artificial P602 and P800 contigs which were used for comparison to one complete copy of the Ofovin retrotransposon using Artemis Comparison Tools (ACT, www.sanger.ac.uk/ Software/ACT). One complete copy of Ofovin was subsequently tested by using Dot Matrix analysis (Vector NTI, Invitrogen, Carlsbad, CA), and LTR regions were confirmed with a web-based program, LTR_FINDER v1.03 (http://tlife.fudan.edu.cn/ltr_ finder). The LTR of Ofovin was used for alignment with contigs of P800 and P602 using Bl2seq to search for the contigs which have similarity to Ofovin LTRs. ClustalX was used to align one contig from P800, CTG35-2-33, with the four copies of Ofovin LTRs from the pearl millet BAC clone.

Two sets of primers based on either the conserved or non-homologous regions between CTG35-2-33 and Ofovin LTRs were designed for isolation of additional LTR sequences from

the genome of Ps26 and ASGR-linked BACs (Table 3.1). Products amplified from Ps26 genomic DNA with primers based on the conserved region (882/883) were ligated with the pGEM-Easy TA cloning vector (Promega, Madison, WI) and the ligated plasmids were transformed into *E. coli* JM109 competent cells (Promega, Madison, WI) via the heat-shock method. Based on insert sizes and *Alu*I digestion patterns of inserts with similar size, 47 colonies were initially chosen for sequencing. Seven fragments amplified with the same primers from 5 BACs were gel purified and directly sequenced. Sequencing of inserts and PCR products was carried out with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Alignment was conducted with all generated LTR sequences plus CTG35-2-33 and one Ofovin LTR using ClustalX. Six LTR-specific primers were generated from the alignment (Table 3.1). The dendrogram and alignment results were viewed with TREEVIEW 2.0. LTR-specific primers were designed and labeled with fluorescent IRDye 700 **(**MWG-BIOTECH**,** High Point, NC, USA) for SSAP.

Southern Blotting

Genomic DNA (8 µg) was digested overnight with 50 units of *Hin*dIII restriction enzyme (New England Biolabs, Beverly, MA) in a 30 µl reaction at 37°C. Fragments were separated on a 0.8% agarose gel (Invitrogen, USA) in 1× TBE buffer overnight at 2V/cm and transferred to Genescreen Plus nylon membranes (NEN Life Sciences, Boston, MA) using the alkaline transfer method (Sambrook and Russell 2001). Products amplified from P800 with primers 898/901 were purified with QIAquick gel extraction kit (QIAGEN, Valencia, CA) and labeled with α-^{32}P-dCTP using a random priming DNA labeling kit (Roche Applied Science, Indianapolis, IN, USA). The membrane was pre-hybridized in 30 ml hybridization buffer (6×SSC, 1% SDS, 100 µg/ml salmon sperm DNA) at 65°C overnight. Labeled probe was denatured at 95°C for 10 min

and placed immediately on ice then added into the hybridization buffer. Hybridization was conducted at 65°C overnight followed by four washes at the same temperature for 15 min each with the following buffers: 1) 2×SSC, 0.1% SDS; 2) 1× SSC, 0.1% SDS; 3) 0.5× SSC, 0.1% SDS; and 4) 0.1 ×SSC, 0.1% SDS. The membrane was exposed with the Cyclone Imaging System (Packard, Meriden, CT).

Development of Sequence Specific Amplified Polymorphism

EcoR I/*Mse* I-LTR/ *Mse* I SSAP

The procedure used in this type of SSAP has been described by Porceddu et al (2002). Briefly, digestion and ligation of genomic DNA (500 ng) was conducted for 4 h at 37°C in a reaction volume of 50 µl, including 5 U of each restriction enzyme (*Eco*R I and *Mse* I; New England Biolabs, Beverly, MA, USA), 1 U of T4 DNA ligase (Promega, Madison, WI, USA), 10 mM ATP, 50 pmol *Mse* I adaptor, and 5 pmol *EcoR* I adaptor (Table 3.1) in RL buffer (20 mM Tris-acetate, 20 mM magnesium acetate, 100 mM potassium acetate, 5 mM DTT, 0.05 µg/µl BSA). Pre-amplification was performed in a 20 µl PCR reaction containing 5 µl of ten-fold diluted (digested and ligated) DNA, 30 ng of the *EcoR* I and *Mse* I adaptor primers (Table 3.1), 10 mM dNTPs, 0.5 U *Taq* DNA polymerase (New England Biolabs, Beverly, MA), in 1x PCR buffer (10 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$). The pre-amplified PCR product was diluted ten-fold and 5 µl of the diluted template was used for a 20 µl selective amplification. The reaction mixture was the same as the pre-amplification reaction except that an IRDye-labeled LTR-specific primer and *Mse*I+2 primers (30 ng each) were used (Tables 3.1 and 3.2). The cycling conditions for pre-amplifications and selective amplifications were initiated with one cycle of 45 s at 94°C, 30 s at 65°C, 1 min at 72°C, followed by 13 cycles of a touch-down profile in which the annealing temperature was decreased at a rate of 0.7°C /cycle, and 20 cycles at a

constant annealing temperature of 55.9 °C. A final extension step at 72°C for 7 min was carried out. After selective amplifications, 10 µl of blue stop solution (*LI-COR* Bioscience, Lincoln, Nebraska, USA) was added to each sample. Samples were denatured at 94°C for 5 min and then immediately placed on ice. A 6.5% polyacrylamide gel was pre-run at 1500V for 20 min on a *LI-COR* 4300 DNA Analyzer (*LI-COR* Bioscience, Lincoln, Nebraska). Samples (0.5 µl each) were loaded onto the gel and then run for 3.5 h under the same parameters.

Initially, 10 F1 plants (5 apomicts and 5 sexual plants) and the two parents were used to test 96 primer pairs (16 *Mse* I +2 × 6 LTR primers). Thirty-two primer combinations that produced the largest number of informative fragments were used for screening 89 individuals from the F1 cross plus two parents.

Pst I/*Mse* I-LTR/*Mse* I SSAP

Another enzyme combination *Pst* I/*Mse* I was also tested for digestion with the same procedure described above except that *Pst* I/*Mse* I instead of *Eco*R I/*Mse* I adapter primers were used for pre-amplification (Tables 3.1 and 3.2). Primer combinations used in *Eco*R I/*Mse* I-LTR/*Mse* I SSAP were applied to this type of SSAP except the noted ones in Table 3.2.

Pst I/*Mse* I-LTR/*Pst* I SSAP

LTR/*Pst* I primer combinations were applied to selective amplifications in this type of SSAP instead of LTR/*Mse* I primer combinations as described in the other two types of SSAP above (Tables 3.1 and 3.3). Ten F1 plants (6 apomicts and 4 sexual plants) and the two parents were used to test 24 primer pairs. Six out of eight informative primer combinations were finally used to screen the F1 population plus two parents.

Linkage analysis and map integration

Because *P. squamulatum* is a heterozygous polyploid with segregating alleles in progeny of the cross with pearl millet, dosage of each marker had to be determined. A practical way to handle the DNA marker data in polyploids is to treat each band as a marker allele of a single locus of a given genotype and to infer its allelic dose by studying the segregation ratio among progenies (Wu et al. 1992). In our case, the ASGR of *P. squamulatum* has been confirmed to be hemizygous (heterozygous); therefore, a band present only in the ASGR will be inherited by approximately half of the gametes and is considered single dose. When apomictic *P. squamulatum* is crossed with sexual tetraploid pearl millet, the segregation ratio for apomixis vs. sexuality expected in the progeny is also approximately 1:1, although segregation distortion resulting in a deficiency of apomicts has been reported (Ozias-Akins et al. 1998). Single-dose markers were the only markers used for linkage analysis in this study. Bands on the gel image were manually scored as 1 (band present) or 0 (band absent). Goodness-of-fit was assessed using χ^2 at a significance level of 5% to identify single-dose markers by their 1:1 segregation ratio that were present in *P. squamulatum* and absent in pearl millet and segregating among the progeny.

To analyze the scored markers, segregation distortion tests and linkage analyses were performed by using JoinMap 3.0 (Van Ooijen and Voorrips 2001) with the parameters set for BC1-derived progeny since the mapping of single-dose markers in polyploids is equivalent to backcross (BC) mapping in diploids. SSAP markers combined with one previous AFLP marker and one SCAR marker were used for linkage analysis. Map distances expressed in centi-Morgans (cM) were estimated based on the recombination fraction using the Kosambi function. Because suppression of recombination in the ASGR has been reported (Ozias-Akins et al. 1998),

marker grouping was initiated with a logarithm of odds (LOD) score of 10, and the parameter for showing weak linkage was changed from a default value of 0.45 to a more stringent value of 0.35. The LOD threshold was then reduced in a step-wise manner to a LOD of 5. In this study, genetic mapping was limited to only the largest linkage group that contains the ASGR-linked AFLP and SCAR markers, since both markers also have been cytogenetically mapped to the ASGR. Parameters used for marker ordering within a linkage group were Rec=0.30, LOD=10.0 and Jump=5.0.

Marker recovery and SCAR development

Fluorescence-labeled amplified PCR product (5-7 µl) was separated on a 6.5% polyacrylamide gel, then fragments were excised using Odyssey infrared imaging system (*LI-COR* Bioscience, Lincoln, Nebraska) and immersed into 25 µl TE buffer (pH 8.0). After three cycles of freezing and thawing, the gel slice was pelleted by centrifuging at 15,000 *g* for 25 min. An aliquot of 2 µl was used for a 20 µl selective amplification reaction. The PCR conditions were 3 min at 94°C; 40 cycles of 30s at 94°C, 30s at 56°C, 1 min at 72°C; and 7 min at 72°C for the final extension. The PCR product was first run on a *Li-COR* 4300 DNA Analyzer to confirm the integrity of recovered fragments. After confirmation, another round of PCR with corresponding non-labeled LTR-specific primers was performed with the same PCR profile described above, and PCR products were separated on 1.5% agarose gels (Invitrogen, USA) and purified with the QIAquick gel extraction kit (QIAGEN, Valencia, CA). Gel-purified fragments were ligated with the PCR®4-TOPO® vector (Invitrogen, USA), and transformed into one shot TOP10 *E. coli* DH5α following the manufacturer's instructions (Invitrogen, USA). Sequencing was performed with ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in Riverbend Research Lab, University of Georgia. Sequence analysis and primer design

were conducted with Vector NTI/Contig express 7.0 (Invitrogen, USA). Newly developed SCARs were screened among two different F1 populations (5 sexual and 5 apomictic plants from each).

Expression analysis of the ASGR retrotransposon by RT-PCR

SCARs were also applied to cDNA for RT-PCR if the primers were from the encoding region, if applicable, of recovered SSAP markers. About 1.2 µg of root, anther, leaf and two stages of ovaries (DAP 0 and DAP 1) total RNA were used for cDNA synthesis with the SuperScript II first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, California, USA). Controls without reverse transcriptase were included for all samples. A 2 µl aliquot of cDNA was used in a 20 µl RT-PCR reaction. A set of unlabelled primers (1156/1205) was used to analyze expression of the retrotransposon with the following PCR conditions: initial denaturation step at 94°C for 3 min, followed by 38 cycles of 94°C for 30 s, 55°C 30 s and 72°C for 1 min. All PCR products were separated on a 2% agarose gel.

Results

Identification and Sequencing of LTRs

Given that the gene encoding region of retrotransposons is relatively conserved among different retrotransposons (Pearce et al. 1999), BlastX with sequence contigs from ASGR-mapped BAC clones was first conducted. The BlastX similarity search showed that 5 contigs of P800, 6 contigs and 9 singletons of P602 have similarity to the *Opie-2* like LTR retrotransposon of rice (*Oryza sativa*) (NP912535, E-value $<e^{-08}$) (see supplemental Figs. 3.1 and 3.2). The BlastN search that was performed with contigs from these two BAC clones found that 5 contigs of P602 and 7 contigs of P800 showed similarity to the Ofovin retrotransposons in a pearl millet (*P. glaucum*) BAC clone (AF488414) (Fig.3.1 and data not shown). Pearl millet is a sexually

reproductive crop species related to *P. squamulatum*. BlastX analysis of Ofovin revealed that it is a *Ty-1*/*copia* type mobile element, which has high similarity with another *Opie-2* like retrotransposon from rice (AAN60494, E=0). Only two contigs of P800 showed similarity to both ends of Ofovin_311G2-1, one copy of a complete Ofovin retrotransposon in the pearl millet BAC (Fig. 3.1). Dot matrix analysis conducted with Ofovin_311G2-1 showed that the ends were direct repeats characteristic of LTR regions (Fig. 3.2). Its first 2500 bp was aligned to itself using blast2seq, and showed that the first 1818 bp and last 1818 bp shared 99.78% identity, more clearly delineating the 5' and 3' LTRs. The reverse complementary sequence (sense strand) of Ofovin_311G2-1 was used for further characterization using web-based software, LTR_FINDER (Fig. 3.3a). Both LTRs terminated with TG at the 5'end and CA at the 3'end. A 5-bp direct Target Site Repeat (TSR) flanked each LTR. A primer binding site was identified downstream of the 5'LTR and showed high similarity to 18 bp at the 3'end of tRNAmet (Fig. 3.3b), while the polypurine tract was immediately upstream of the 3'LTR (Fig. 3.3a).

The largest P800 contig, CTG35-2-33 was chosen for further study since the region of another contig showing similarity to the LTR of Ofovin was almost included in CTG 35-2-33 (Fig. 3.1 and data not shown). One set of primers (882/883) based on the conserved regions between this contig and four copies of Ofovin LTRs was subsequently designed, while another set of primers (898/901) was generated from the sequence specific region of CTG35-2-33 (Table 3.1). The expected sizes of amplified fragments from CTG35-2-33 were ~1600 bp for 882/883 and ~470 bp for 898/901.

In order to check if the sequence-specific region of CTG35-2-33 compared with the Ofovin LTRs was specific to the apomictic genotypes, primers 898/901 were first applied to 19 ASGR-linked BACs, two apomictic (both carrying the ASGR), and three sexually reproductive

plants. The amplifications were successful in apomictic Ps26 and the backcross line 58 containing two *P. squamulatum* chromosomes as well as the retrotransposon-containing BACs but not in three genotypes of sexual pearl millet, indicating that this region of CTG35-2-33 is probably specific to apomictic genotypes (Fig. 3.4a). However, PCR product was generated from sexual and apomictic F1 individuals demonstrating that the region was not specific to the ASGR (data not shown). Furthermore, Southern blotting with the amplicon as a probe did not reveal any candidate ASGR-specific band (band shared only between apomictic Ps26 and line 58) (Fig. 3.4b). We concluded that the primers 898/901 are specific to *P. squamulatum* but not the ASGR.

Primers 882/883 were used to obtain additional LTR sequences because 1) this set of primers was from the conserved region between Ofovin LTRs and CTG 35-2-33 which increased the probability of amplifying LTRs from multiple members of the *Opie-2*-like retrotransposon family; 2) the product produced by these primers spanned ~88% of the Ofovin LTR. Initially, 36 end sequences of LTR clones generated from the Ps26 genome were clustered into two distinct groups, one containing CTG35-2-33 and 16 other sequences and one containing the Ofovin LTR and 17 sequences (Fig.3.5). Eight clones (5 from CTG35-2-33 and 3 from Ofovin) were selected for further sequencing. Seven products amplified with primers 882/883 from 5 ASGR-mapped BACs also were sequenced in order to compare the same sequence region from clones generated by two approaches. Six LTR primers for SSAP were finally generated: two from the alignment of all sequences (Table 3.1, Fig. 3.6 and supplemental Fig. 3.3), two from the Ofovin LTR group (Table 3.1, Fig. 3.6 and supplemental Fig. 3.4), and two from the BAC group (Table 3.1, Fig. 3.6 and supplemental Fig. 3.5). LTR-primers were designed from the sequence close to either the 5' end or 3'end to maximize the proportion of sequence flanking the LTR. Restriction sites of

enzymes which were used in this study (*Eco*R I, *Pst* I and *Mse* I) were avoided during primer design.

Generation of Sequence Specific Amplified Polymorphism

*Eco*R I/*Mse* I-LTR/ *Mse* I SSAP

In this type of SSAP, *Eco*R I/*Mse* I enzymes were used for digestion, and LTR specific primers paired with *Mse* I adapter primers plus two selective nucleotides were used for selective amplification. Initially, different primer extensions were tested for developing SSAP. The *Mse* I/*Eco*R I adapter primer without any selective nucleotide and with one selective nucleotide were tested for pre-amplification. For selective amplification, *Mse* I +1, +2, or +3 selective nucleotides were tested. Based on the scorable polymorphisms and reproducibility of reactions, the *Mse* I/*Eco*R I +0 selective nucleotide for pre-amplification and *LTR*/*Mse* I +2 selective nucleotides for selective amplification were chosen for this study. In total, 96 primers (16 *Mse* I +2 selective nucleotide primers by 6 LTR primers) were screened with 5 sexual and 5 apomictic F1 plants plus two parental plants to determine the most informative combinations. Thirty-two primer combinations were chosen to screen the F1 population along with two parents (Fig. 3.7). One hundred thirty-five single-dose markers were generated (Table 3.2). Primer combinations producing these single-dose markers were formed by all 16 *Mse* I +2 primers paired with only two LTR-specific primers (1086 and 1155), both of which were from the CTG35-2-33/ASGR-linked BAC group (Table 3.2, Fig. 3.6 and supplemental Fig. 3.5). An average of 4.2 single-dose markers was produced per primer combination with a range from 0 to 12. Ninety-three of these markers (~68.9%) were shown to be closely linked with apomixis, and when the missing data were eliminated, seventy-eight markers (~57.8%) were shown to fully co-segregate with apomixis.

Pst I/*Mse* I-LTR/ *Mse* I SSAP

Pst I/*Mse* I enzymes were used for digestion in *Pst* I/*Mse* I – LTR/*Mse* I SSAP. Pre-amplifications were obtained with *Pst* I/*Mse* I adapter primers without any selective nucleotides, while *Mse* I adapter primers plus two selective nucleotides with LTR specific primers were used for selective amplifications. One hundred forty-two single-dose markers were generated from twenty-nine primer combinations (Table 3.2). Eighty-five markers (~59%) were linked with apomixis, and seventy-three (~51%) were completely linked with the trait when the missing data of F1 individuals for some markers was not considered. Approximately 4.9 bands per primer combination were generated with a range from 0 to 11. Ninety-six markers (67%) from this type of SSAP were found to have similar sizes and segregation patterns as *Eco*R I/*Mse* I-LTR/ *Mse* I SSAP markers, which was not unexpected given that representation of fragments in the pre-amplifications might be similar but not identical. Seventy-seven out of 96 markers were linked with apomixis. The number of markers sharing similar segregation patterns could be underestimated since some markers were scored for one type of SSAP, but not for another depending on image quality.

Pst I/*Mse* I - LTR/*Pst* I SSAP

This type of SSAP is the same as *Pst* I/*Mse* I-LTR/*Mse* I SSAP except that *Pst* I adapter primers plus one selective nucleotide were used with LTR specific primers for selective amplifications. Twelve F1 plants (4 sexual, 6 apomictic and 2 parental plants) were used for screening 24 primer combinations (Table 3.3). Eight informative primer combinations were obtained, and these informative primer combinations were not confined to only two LTR specific primers as was observed for *Eco*R I/*Mse* I-LTR/*Mse* I SSAP, although the average number of polymorphic bands per primer combination was less. Thirteen single-dose markers were

generated from six primer combinations (Table 3.3). Six markers were shown to be closely linked with apomixis, two of which completely co-segregated with the trait.

Genetic mapping of the ASGR in *P. squamulatum* using retrotransposon-based markers

A total of 290 SSAP markers combined with a previous SCAR marker Ugt197 and an AFLP marker PQ355 was used for genetic linkage analysis. Because suppression of recombination in the ASGR has been reported (Ozias-Akins et al. 1998), stringent parameters for linkage first were tested (see Materials and Methods). Four individuals which showed missing genotype data for over 20 (5%) markers and two individuals showing a weird band pattern in all images were excluded from the dataset leaving data from 83 F1 individuals to construct the map. Twenty-seven linkage groups were formed at a LOD score of 10, and the largest group contained 186 (63.7%) of the markers while there were only six markers in the second largest group. Thirty-eight markers (13%) were unlinked. One hundred forty-one identical markers (identical band scoring) were automatically excluded from the dataset. Ninety-five out of 141 markers were identical to Ugt197 in their segregation pattern. One hundred forty excluded markers were from the largest group, which left 46 markers at LOD 10.

Using the same dataset in which the identical markers (identical band scoring) were excluded, the LOD threshold was dropped from 10 to 5 in a stepwise manner, resulting in one additional marker (P86-1105-334) on the largest linkage group. These 47 markers remained linked up to a LOD value of 9.0 where P86-1105-334 was split from this group.

The group containing 46 markers at LOD 10 was used for construction of a genetic linkage map using a stringent set of parameters (Rec=0.30, LOD=10.0 and Jump=5.0) (Fig. 3.8). Forty-six markers were distributed onto 10 different loci and covered 19 cM, while 45 markers spanned only 9 cM. Ugt197, one SCAR marker fully co-segregating with apomixis, was mapped

to a locus containing 21 other co-segregating markers. The remaining 23 markers including PQ355 were distributed onto 8 different loci flanking the Ugt197 locus (4 loci each side) (Fig. 3. 8). PQ355, an AFLP marker, has been shown to recombine in 2 out of 193 F1 individuals, one sexual F1 and one apomictic F1 (Goel et al. 2006) and has been cytogenetically mapped to a position more proximal to the centromere of the ASGR-carrier chromosome than Ugt197. A low frequency of genotyping error is unavoidable in AFLP-based marker data (Remington et al. 1999; Bonin et al. 2004; Pompanon et al. 2005), and since recombination around the ASGR previously has been observed only on one side of Ugt197, we suspected that some of the putative recombination events could be erroneous. In order to confirm recombination, SCAR development was pursued for several markers.

Marker recovery and SCAR development

Combining information from the genetic map constructed in this study and the physical map described by Goel et al. (2006), SSAP markers representing six markers were selected for potential SCAR development, five of which showed recombination in some F1 plants (Fig. 3.8), and a sixth (Pst56-1205-400) generated from *Pst* I/*Mse* I - LTR/ *Pst* I SSAP that co-segregated with apomixis (data not shown). Sequence analysis showed that five of the markers displayed similarity by BlastN to the Ofovin LTR from pearl millet BAC clone (AF488414) as might be predicted. Only the marker that co-segregated with apomixis showed more significant similarity to a transposable element protein in rice (*Oryza sativa*) (AAQ56333, E = 8e-11, Table 3.4), when the BlastX was performed. Only one of the six markers, E86-1080-295, could not be converted to a SCAR because of the lack of polymorphism. SCAR markers were either dominant (present in apomictic F1, absent in sexual F1) or polymorphic in amplification pattern within the F1 population (Fig. 3.9b, Fig. 3.10a, supplemental Fig. 3.6a,b,c). One SSAP marker (E86-1104-522)

initially was scored as recombining in three different F1 plants (A46, S27 and S50), while recombination of its corresponding SCAR was detected in only two plants (A46 and S27, Fig. 9a,b). The other three SCAR markers did not show any recombination in F1 plants contrary to what was observed for their corresponding SSAP markers (supplemental Fig. 3.6a,b,c). Therefore, recombination was confirmed in individuals A46 and S27 with a second marker in addition to PQ355, but not for markers distal to Ugt197. The potential for recombination distal to Ugt197 would need to be further tested by converting other SSAP markers to SCARs and by testing on a larger population. Because 73.6% of the sequence of Pst56-1205-400 showed similarity to a transposable element protein (Tables 3.1 and 3.4), its expression was tested by RT-PCR using the SCAR primers. Expression was observed in root, anther, leaf, and non-fertilized and fertilized ovaries (Fig. 3.10b).

Discussion

Identification of LTR sequences from the ASGR

Most retrotransposon-based markers require sequence information from long terminal repeats (Kumar and Hirochika 2001; Pearce et al. 1999; Schulman 2004). The method developed by Pearce et al. (1999) was extensively used for isolating LTR sequences from *Ty1/copia* type retrotransposons in a variety of plant species (Acquadro et al. 2006; Berenyi et al. 2002; Bousios et al. 2007; Lou and Chen 2007; Pearce et al. 2000; Syed et al. 2005). The principle of this method is to take advantage of the conserved RNase H motif which is upstream of the 3' LTR. After digestion with a frequent-cutter enzyme, adapters are ligated to the digested genomic fragments; amplifications are conducted with a biotinylated RNaseH motif primer and adapter primers. However, this method is limited to the retrotransposons whose LTRs are within 500 bp of the RNase H motif, and therefore is likely to exclude *Opie*-like elements, in which the

intervening sequence between the RNase H motif to the 3' LTR is over 1 kb (Pearce et al. 1999). Another pitfall is that the sequences recovered with this method are not specific to a retrotransposon family, which would complicate the development of SSAP using a particular LTR, for example, the ASGR abundant retrotransposon (Pearce et al. 1999). We did not sequence a full-length LTR-retrotransposon because preliminary analysis of BAC clones suggested that the elements could be greater than 12 kb in length; therefore, we do not have data on the distance between RNase H and the 3' LTR and chose an alternative means for generating LTR sequences.

We took advantage of one complete copy of a related retrotransposon, Ofovin, from a pearl millet BAC clone (AF488414) for identification of ASGR-abundant retrotransposon LTR sequences. Ofovin is a *Ty-1*/*copia* type mobile element, which also has high similarity with an *Opie-2* like retrotransposon from rice (AAN60494, E=0). *Opie*-2-like retrotransposons were found to be abundant in the ASGR-linked BAC clones (Akiyama et al. 2004). Some contigs of P800 and P602 showing high similarity to another *Opie-2*-like retrotransposon from rice (NP912535, E-value $<e^{-08}$) also shared some similarity to Ofovin (Fig. 3.1 and supplemental Fig. 3). Because the intervening space between the RNase H motif and 3'UTR of Ofovin is over 1.5 kb, the method described by Pearce et al. (1999) is not suitable for this study. Variations within a subgroup of a retrotransposon family are not as significant as inter-subgroups in the same family although Ty-1/*copia* retrotransposons are highly heterogeneous in the plant kingdom (Flavell et al. 1992; Flavell et al. 1997; Kumar and Bennetzen 1999). LTR sequences were successfully generated from the *P. squamulatum* genome and ASGR-BAC clones using the primers which were from the conserved region between CTG35-2-33 and four copies of the Ofovin LTR, indicating that within the retrotransposon subgroup, conserved regions of the LTR

could also be used for isolation of LTRs from different species. Based on the alignment, sequences isolated by PCR from genomic DNA and ASGR-linked BAC clones clustered into different groups (Fig. 3.5 and Fig. 3.6). The lack of a genomic PCR clone in the BAC cluster could be due to the genome size of and distribution of elements in *P. squamulatum* vs. the number of clones that were sequenced, which were too few to adequately sample across the genome. LTR sequences isolated from *P. squamulatum* and ASGR-linked BACs showed moderate similarity to pearl millet Ofovin; however, whether the sequences within the Ofovin group are part of the same retrotransposon subfamily as Ofovin and whether they are related in origin are still undetermined, although it is possible for interspecific hybridization to result in horizontal transmission between genomes (Roulin et al. 2008).

Generation of Sequence Specific Amplified Polymorphisms

In this study, three types of SSAP were generated from the digestion of two different enzyme combinations, *EcoR* I/*Mse*I and *Pst* I/*Mse* I. The ASGR is highly heterochromatic and abundant in repetitive DNA which usually is highly methylated (Avramova 2002), probably resulting in the inhibition of digestions with some DNA methylation sensitive enzymes such as *Pst* I. Digestion with *Pst* I was expected to be more frequent in euchromatic regions than heterochromatic regions, with the possibility to target genic regions in the ASGR. Interestingly, in *Pst* I/*Mse* I-LTR/ *Mse* I SSAP , over 67% of the markers generated likely were duplicating markers amplified in *EcoR* I/*Mse* I-LTR/ *Mse* I SSAP, since they shared very similar fragment sizes and segregation patterns. This result suggested that digestions with either *Pst* I or *EcoR* I combined with *Mse* I released similar fragments containing LTR-specific primers and also indicated that segregation of these markers probably was due to sequence variation at the flanking or internal restriction sites, but not DNA methylation differences. The composition of

the selective bases on the *Mse* I primer did not have as strong impact on the number of bands detected and the level of polymorphism as did the sequence of LTR primers (Table 3.2). Markers generated in these two types of SSAP were confined to two LTR-specific primers (1155 and 1086) which were from the BAC sequence alignment (Table 1 and supplemental Fig. 3.5). Given that >60% of the SSAP markers generated by these primers were linked with apomixis, this approach had unprecedented efficiency for targeting a genomic region, the ASGR. However, in the third type of SSAP, *Pst* I/*Mse* I - LTR/*Pst* I SSAP, LTR primers from some groups other than the BAC group also were informative, although the average number of polymorphic bands was less than in the other two types of SSAP. Whether this was caused by DNA methylation is still undetermined.

Genetic mapping of the ASGR in *P. squamulatum* using retrotransposon-based markers

Although 290 markers were generated from three types of SSAP, one hundred forty markers were automatically removed by the Joinmap software since these markers were shown to have the same segregation pattern as other markers in the data set. The marker distribution on the genetic map indicated that these SSAP markers were strongly clustered although the clustering based on the genetic map could be misleading since the physical size of the ASGR is known to be >50 Mb. It also is possible that these markers do not span the ASGR, which can only be estimated once BACs containing these markers have been isolated. Clustering and nesting of retrotransposons have been reported in other genetic and physical mapping studies (Bouck et al. 2005; Boyko et al. 2002; Manninen et al. 2000; Yu and Wise 2000). Retrotransposon clusters, particularly in intergenic regions, often coincide with suppression of recombination, which reflects a notable feature of the grass genome in which gene islands are embedded among repetitive elements (Dooner and He 2008; Panstruga et al. 1998).

Suppression of recombination has been observed at the apomixis "locus" of many species. It was first found in *P. squamulatum*, and subsequently reported in diplosporous *E. annuus* (Noyes and Rieseberg 2000), *Tripsacum dactyloides* (Grimanelli et al. 1998), aposporous *Paspalum simplex* (Labombarda et al. 2002), and *Cenchrus ciliaris* (Akiyama et al. 2005; Goel et al. 2006; Jessup et al. 2002). All of these studies demonstrated that a cluster of molecular markers is strictly linked to the apomeiosis trait. Suppressed recombination in the ASGR, whose position is proximal to the short arm telomere of one chromosome in *P. squamulatum,* has been described and extensively discussed (Akiyama et al. 2004; Goel et al. 2006; Goel et al. 2003; Ozias-Akins et al. 2003; Ozias-Akins et al. 1998). To date, only two markers, Ugt204 and PQ355, have been shown to recombine with the ASGR. Ugt204 maps at a considerable genetic distance (~24 cM) from the ASGR (Ozias-Akins et al. 1998), while PQ355 is positioned 2.02 cM away from the ASGR and was physically mapped to a position proximal to the centromere (Goel et al. 2006). The BAC P1300 containing the recombinant marker PQ355 hybridized not only with the ASGR-carrier chromosome but also with its apparent homolog, indicating that its hybridization pattern is not hemizygous as observed for the majority of non-recombinant BACs (Akiyama et al. 2004; Goel et al. 2006; Goel et al. 2003). One of the new SSAP markers, when converted to a SCAR was confirmed to recombine and co-segregate with PQ355. Other markers, however, failed to show recombination when converted to SCARs. One of these, Pst55-1205-440, was initially scored as recombining in 11 apomictic F1s but not any sexual F1 (data not shown), but its corresponding SCAR marker did not show any recombination in all tested F1 plants (Supplemental Fig. 3.6 c). This result is most likely due to DNA methylation differences among the F1s since Pst55-1205-440 originated from the *Pst* I/*Mse* I - LTR/*Pst* I SSAP in which the DNA methylation sensitive enzyme, *Pst* I, was used for digestion. It is known that wide

hybridization can cause DNA methylation changes in the progenies of some crosses (Ainouche et al. 2003; Liu et al. 2004). We have observed expression differences for *ASGR-BBML* in F1 hybrids compared with parental *P. squamulatum* where expression was detected in the leaves of some F1s, but was not detectable in the leaves of the apomictic parent (see Chapter 4). It is not yet known whether these expression differences are related to changes in methylation.

Because the size of the ASGR has been estimated to be over 50 Mb (Akiyama et al. 2004), it would be costly to sequence but feasible if it could be spanned with BAC contigs. Low recombination excludes high resolution genetic mapping, however, and sequence analysis alone would not be sufficient to distinguish only a few candidate genes. Nevertheless, the recovery of a large number of SSAP markers has greatly increased the potential to physically map the ASGR provided that the markers are randomly distributed.

One SSAP marker (Pst 56-1205-400) that co-segregated with apomixis and was successfully converted to a SCAR showed sequence similarity to a *Ty-3*/*gypsy* type retrotransposon protein from rice (AAQ56333) and expression was observed in leaf, anther, root and ovaries of Ps26, indicating that the retrotransposon is transcriptionally active and specific only to the ASGR. No evidence for transposition has been produced thus far; however, the transposition power of this retrotransposon could be detected by checking its presence in progeny of BC8, an eighth backcross line between Ps26 and IA4X that has been confirmed to have only a single chromosome of *P. squamulatum* (unpublished data). If transposition has occurred, the marker may no longer be apomict specific.

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Table 3.1 LTR-specific and linker/adapter primers used in this study

Primer ID	Primer sequence	Source of sequence/Purpose
882	GCAGGTGAAGAACAAGCG	Conserved region between CTG35-2-33 and 4 copies of Ofovin LTRs
883	AGTGAATGGGAGCCGATAA	Conserved region between CTG35-2-33 and 4 copies of Ofovin LTRs
898	GAGACAACGTGGACATGGAG	Specific region of CTG35-2-33 compared with 4 copies of Ofovin LTRs
901	TTGGCGAATTTTAATTCTCACACTC	Specific region of CTG35-2-33 compared with 4 copies of Ofovin LTRs
1087	TTCGCGCTTGTTCTTCACCTGC	IRDye 700 labeled LTR-primer from the conserved region of all sequences
1086	GTTGTTAGCCGCCACCTTGC	IRDye 700 labeled LTR-primer from BAC group
1153	ACTTCATCTTGCTGTTCTTGCCACCAT	IRDye 700 labeled LTR-primer from Ofovin group
1154	GTAGCCTCCGCCAATCACCACTCAT	IRDye 700 labeled LTR-primer from Ofovin group
1155	AGCCTACTTGCCTCTCCACT	IRDye 700 labeled LTR-primer from BAC group
1156	ATTTTTATCGGCTCCCATTCACT	IRDye 700 labeled LTR- primer from the conserved region of all sequences
1205	GACTGCGTACATGCAGA	*Pst* I adapter primer plus A
1206	GACTGCGTACATGCAGT	*Pst* I adapter primer plus T
1207	GACTGCGTACATGCAGC	*Pst* I adapter primer plus C
1208	GACTGCGTACATGCAGG	*Pst* I adapter primer plus G
1072	CTCGTAGACTGCGTACC	*Eco*R I linker 1[a]
1073	AATTGGTACGCAGTCTAC	*Eco*R I linker 2
1076	GACTGCGTACCAATTC	*Eco*R I adapter primer
1133	CTCGTAGACTGCGTACATGCA	*Pst* I linker 1
1134	TGTACGCAGTCTAC	*Pst* I linker 2
1152	GACTGCGTACATGCAGAC	*Pst* I adapter primer
1074	GACGATGAGTCCTGAG	*Mse* I linker 1
1075	TACTCAGGACTCAT	*Mse* I linker 2
1077	GATGAGTCCTGAGTAAC	*Mse* I adapter primer
1127	ACGAGGATTTCACCAACAGC	ADF forward primer
1128	AACGCATAGACGACGCCT	ADF reverse primer

[a] Equal volumes of *Eco*R I linker 1 and 2 were mixed and denatured at 94°C for 5 min. Subsequent annealing at room temperature allowed the adapter to form. The same procedure was used to form *Pst* I and *Mse* I adapters.

Table 3.2 Number of single-dose markers generated from different LTR-specific/*Mse* I primer combinations in *EcoR* I/*Mse* I-LTR/*Mse* I SSAP [a] and *Pst* I/*Mse* I-LTR/*Mse* I SSAP[b]

Primers	Digestion	GT	GC	GA	GG	CA	CT	CG	CC	TA	TC	TG	TT	AC	AG	AT	AA
E1086[c]	*Eco*R I/*Mse*I	12	12	4	6	6	3	7	5	9	6	5	0	7	2	2	3
E1155[d]	*Eco*R I/*Mse*I	7	5	1	6	2	1	5	4	4	0	0	0	6	1	4	0
P1086[e]	*Pst* I/*Mse*I	11	5	11	10	10	3	7	5	12	7	6	0	6	3	2	2
P1155[f]	*Pst* I/*Mse*I	6	5	2	4	3	2	3	4	4	2	3	0	4	na[g]	na	na

[acd] *EcoR* I/*Mse* I enzymes were used for digestion, and LTR specific primers and *Mse* I adapter primers plus two selective nucleotides were used for selective amplification

[bef] *Pst* I/*Mse* I enzymes were used for digestion, and LTR specific primers and *Mse* I adapter primers plus two selective nucleotides were used for selective amplification

[g] not assayed

Table 3.3 Number of single-dose markers generated from different LTR specific/*Pst* I primer combinations in *Pst* I/*Mse* I - LTR/ *Pst* I SSAP[a]

Primers	Digestion	1086	1087	1153	1154	1155	1156
Pst1205[b]	*Pst* I/*Mse*I	-[c]	-	-	-	3	3
Pst1206	*Pst* I/*Mse*I	na[d]	2	na	-	5	2

[ab] *Pst* I/*Mse* I enzymes were used for digestion, and LTR specific primers and *Pst* I adapter primers plus one selective nucleotide were used for selective amplification.
[c] not an informative primer combination
[d] not assayed

Table 3.4 BlastX and BlastN similarity of sequenced markers.

	BlastX	BlastN
E86-80-295	N[a]	80% of sequence to Ofovin LTR of pearl millet (AF488414) E=1e-48 20% of sequence to *Opie-2* like retrotransposon *gag-pol* of *P. squamulatum* (AY375366) E = 7e-08
E86-04-522	unclassified *gag-pol* polyprotein of rice (*Oryza sativa,*ABF96035) E= 2e-04	*Zea mays* 25S rRNA gene and transposon-like sequence (AJ309824) E= 0.003
E86-36-410	Ty1-*copia gag-pol* protein of rice (*Oryza sativa*, ABG00000) E=0.006	65% of sequence to Ofovin LTR E=2e-60 18% of sequence to *Opie-2* like retrotransposon *gag-pol* of *P. squamulatum* (AY375366) E=2e-23 22% of sequence to Sorghum bicolor clone BAC (AY661656) E =8e-09
E86-36-385	N	75% of sequence to Ofovin LTR E=1e-63 18% of sequence to *Opie-2* like retrotransposon *gag-pol* of *P. squamulatum* (AY375366) E=2e-15
Pst55-1205-440	N	46% of sequence to Ofovin LTR E=2e-36 LTR 32% to Sorghum bicolor clone SB20O07 b1-1, b1-2, putative genetic modifier (AY542311) Expect = 8e-10
Pst56-1205-400	*gypsy*-type retrotransposon protein of rice (Oryza sativa, AAQ56333) E = 8e-11 Transposable element protein of rice (Oryza sativa, ABA96640) E = 8e-11	14% of sequence to Ofovin LTR Expect = 3e-08

[a] No significant hit

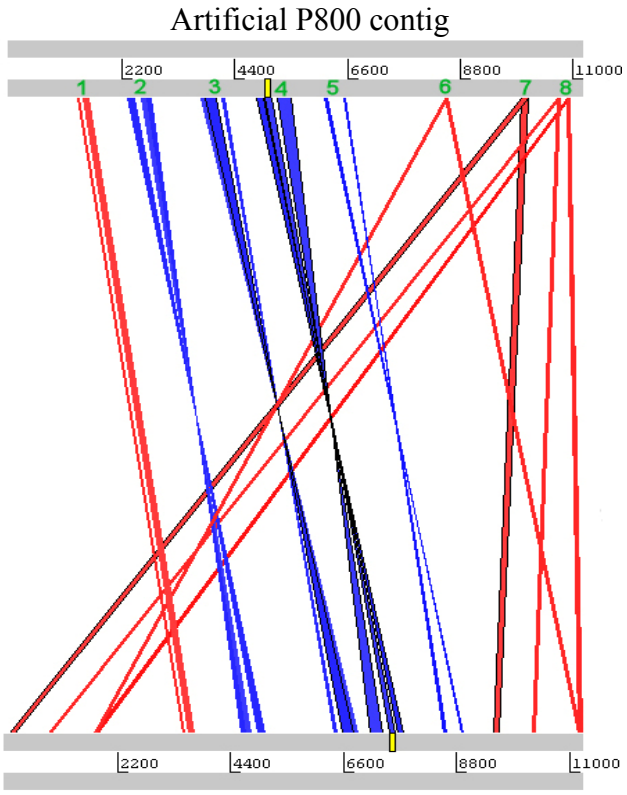


Fig. 3.1 Comparison of artificial P800 contigs with Ofovin_311G2-1. Numbers (1-8) across top of the image represent different contigs except the numbers seven and eight represent one contig, CTG35-2-33.

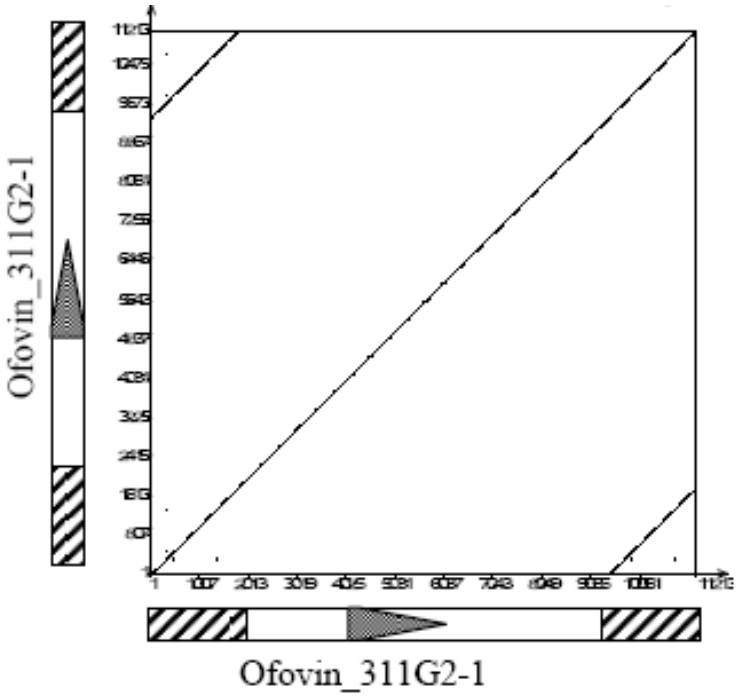
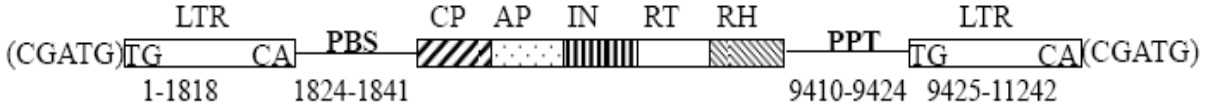


Fig. 3.2 Dot matrix analysis of Ofovin_311G2-1. LTRs are indicated with hatched boxes. Arrows show the orientation of full-length Ofovin_311G2-1.

a



b

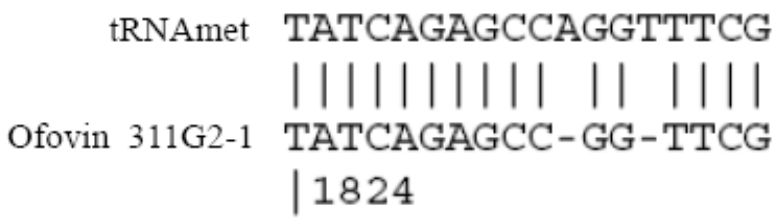
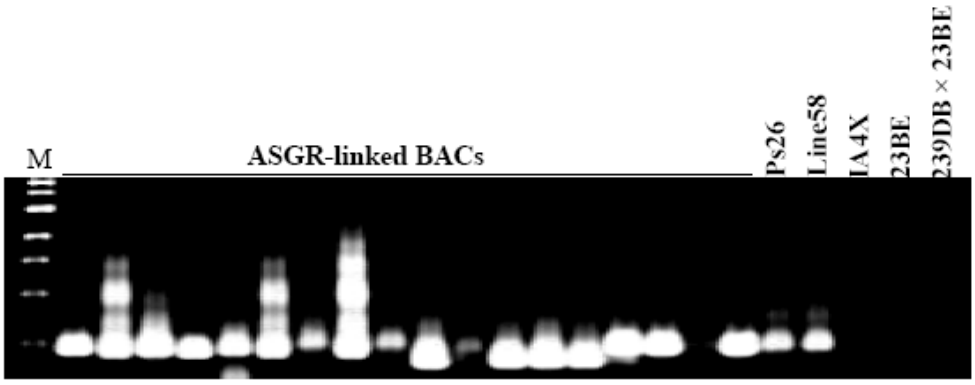


Fig. 3.3 a) Structure of Ofovin. The diagram was constructed with the complementary strand of Ofovin_311G2-1(AF488414) plus 50 bp upstream and downstream. Numbers indicate the sequence range of different parts of Ofovin. The LTR terminates with a short inverted repeat TG/CA. The short sequences in parenthesis are genomic Target Site Repeats. PBS: primer binding site; CP: capsid-like protein; AP: aspartic proteinase; IN: integrase; RT: reverse transcriptase; RH: RNase H; PPT: polypurine tract. b) Homology of PBS and tRNAmet.

a



b

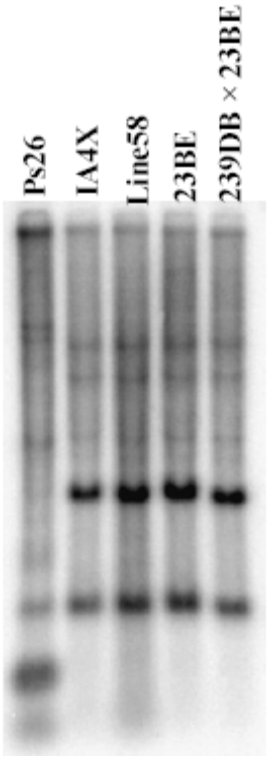


Fig. 3.4 Detection of specificity of the non-homologous region between CTG35-2-33 and Ofovin using PCR (a) or Southern blotting (b). Ps26 and Line58 are apomictic, both having the ASGR-carrier chromosome; IA4X and 239DB x 23BE are sexual tetraploid pearl millets; 23BE is a sexual diploid pearl millet.

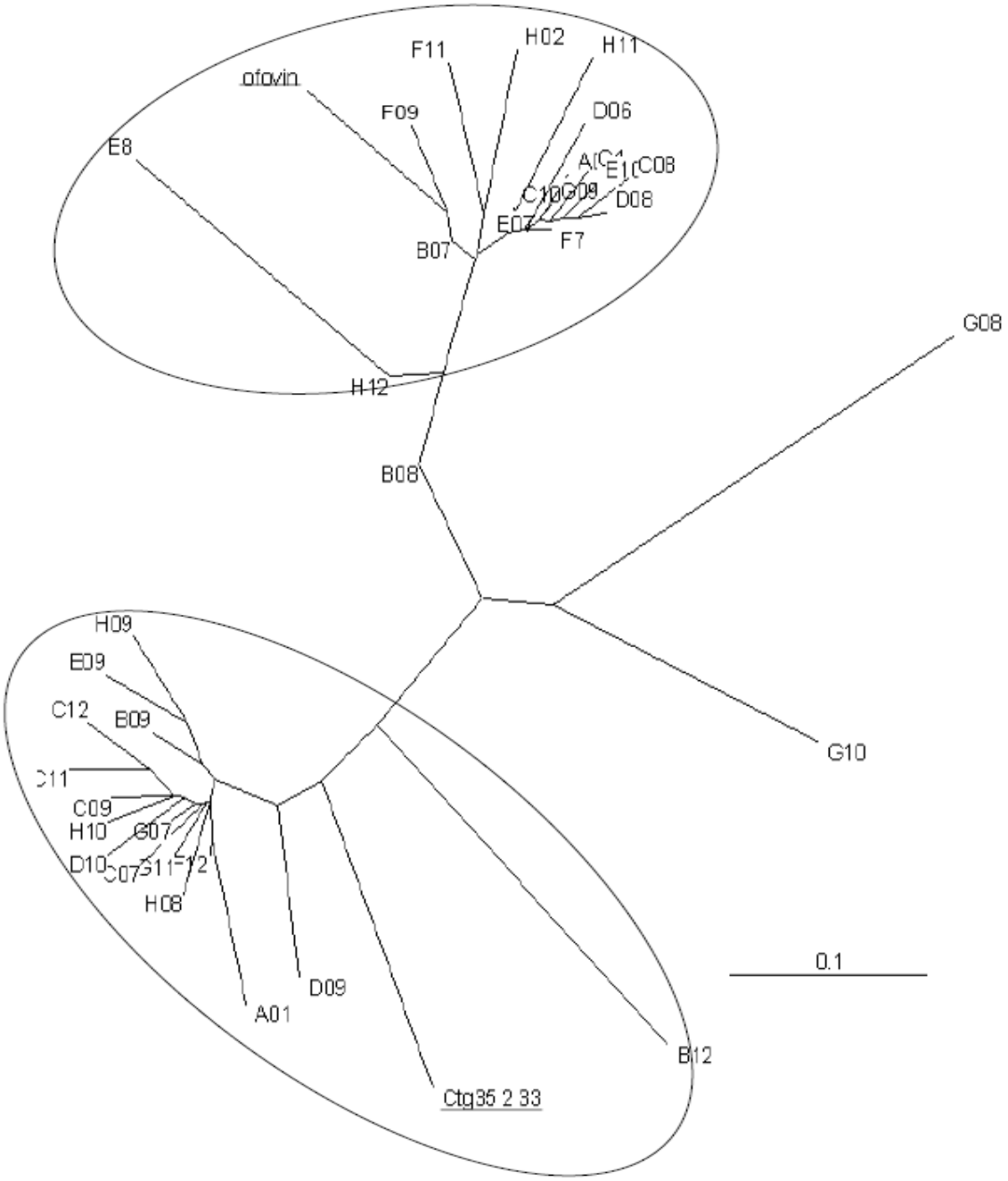


Fig. 3.5 Phylogenetic analysis of LTR sequences generated from the *P. squamulatum* genome. Ofovin: LTR of Ofovin_311G2-1 (AF488414); CTG35-2-33: a contig of P800.

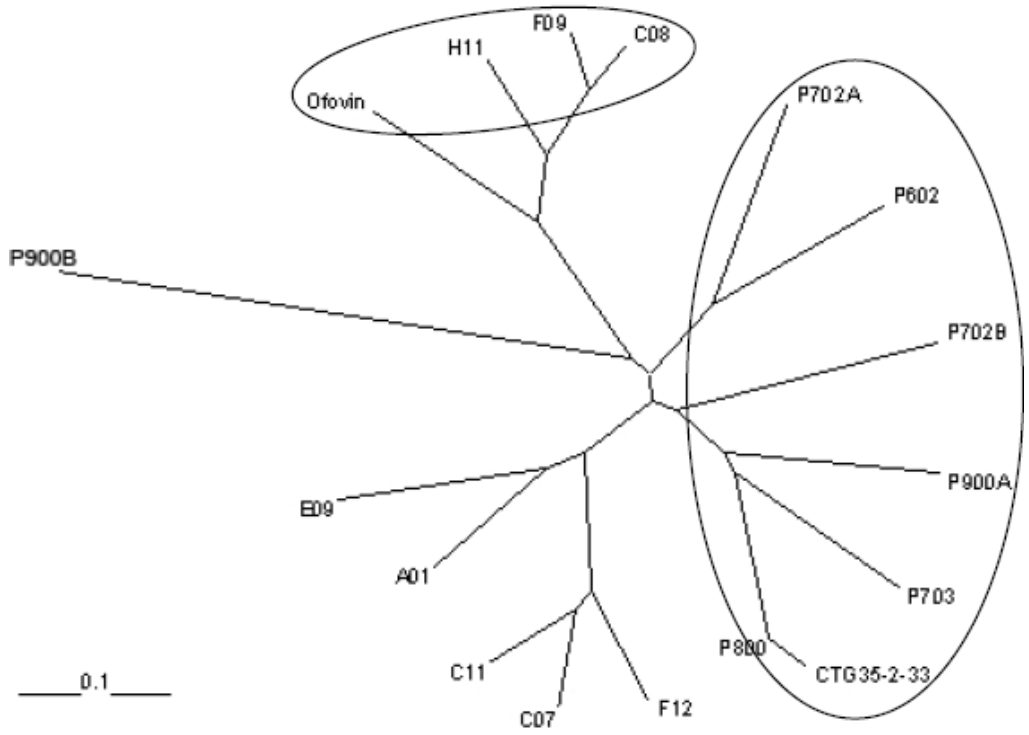


Fig. 3.6 Phylogenetic analysis of LTR sequences generated from the *P. squamulatum* genome and ASGR-linked BACs. LTR primers were designed based on the alignment of the two circled groups and all sequences here. Ofovin: LTR of Ofovin_311G2-1 (AF488414); CTG35-2-33: a contig of P800. P602-P900: sequences from ASGR-linked BACs generated with primers 882/883. All other sequences were from randomly picked clones generated from Ps26 genomic DNA amplified with primers 882/883.

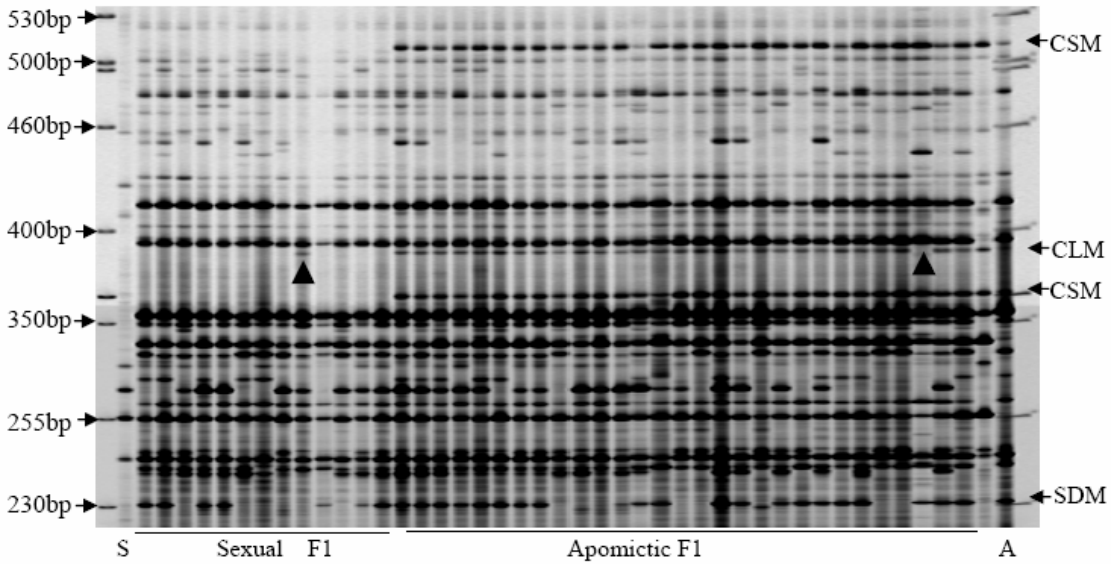


Fig.3.7 Partial SSAP profile produced by primer combination E86-1138 on a 43-plant subset of the mapping population derived from *P. glaucum* × *P. squamulatum.* CLM, ASGR closely linked marker; CSM, ASGR co-segregating marker; SDM, single-dose marker unlinked with apomixis; S, IA4X, a sexual parent; A, Ps26, apomictic parent. Recombination of the CLM marker happened in a sexual F1 and an apomictic F1 (arrow heads).

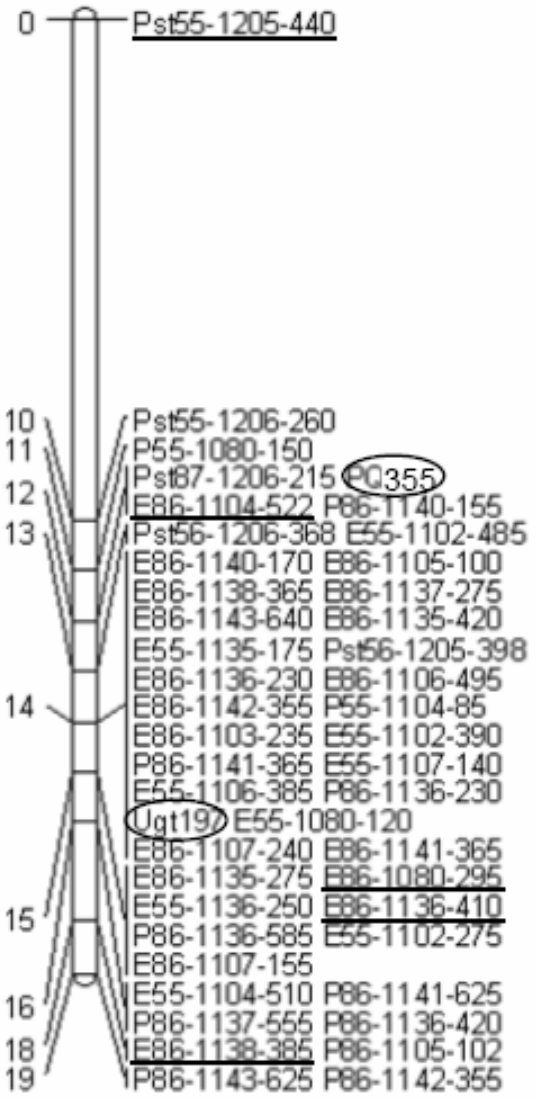


Fig. 3.8 Genetic linkage map of ASGR retrotransposon-based markers. The letters in markers (E, P and Pst) indicate that the marker was generated from *EcoR* I/*Mse* I-LTR/ *Mse* I SSAP, *Pst* I/*Mse* I-LTR/ *Mse* I SSAP and *Pst* I/*Mse* I - LTR/ *Pst* I SSAP, respectively. The first two digits correspond to the last two digits of LTR specific primers; the four digits in the middle indicate the ID of *Mse* I+2 primers or *Pst* I+1 primers; the last two or three digits indicate the estimated sizes of bands. Circled markers are the two previously published and cytogenetically mapped to the ASGR. Markers underlined were chosen for developing SCARs.

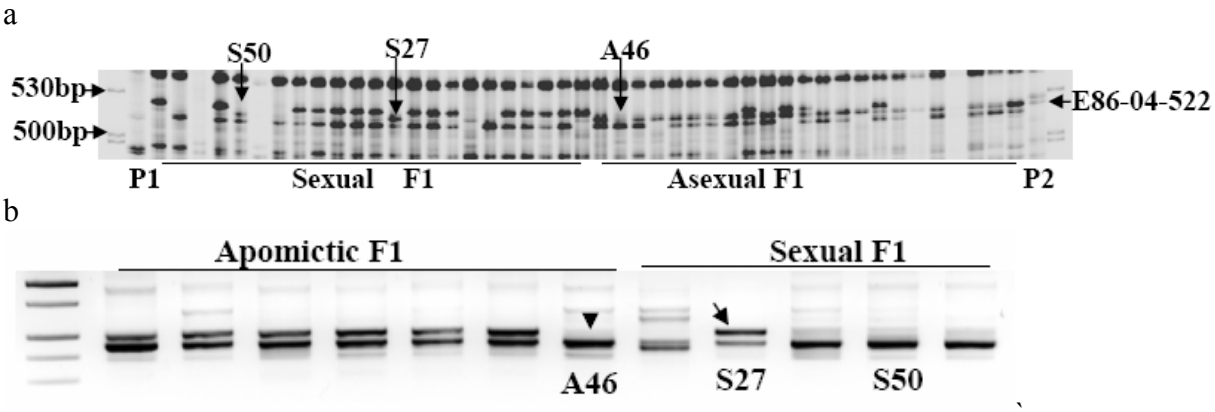


Fig. 3.9 a) SSAP marker (E86-1104-522) showing recombination in three F1 plants (S50, S27 and A46) was recovered and converted into a SCAR marker; b) SCAR marker (E86-1104-522) was used for screening 12 F1 plants including the three recombinants. Recombination was confirmed in only 2 of 3 F1 plants with this SCAR (indicated by arrow head and arrow). P1, IA4X; P2, Ps26.

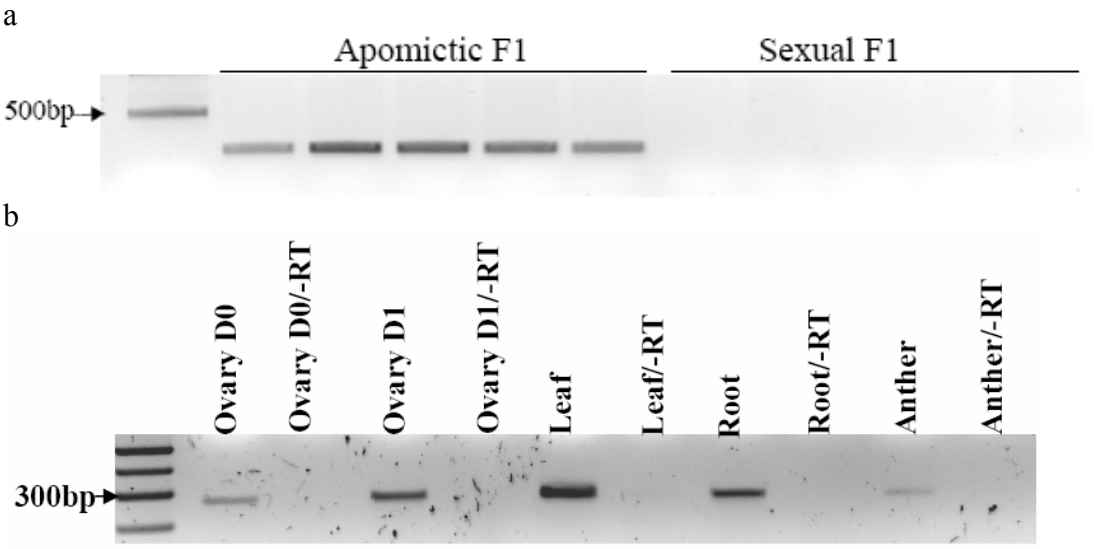


Fig. 3.10 a) SCAR marker developed for Pst56-1205-400, a non-recombining SSAP marker, and screened on 10 F1 individuals. b) Detection of expression of an ASGR-specific LTR-retrotransposon using SCAR converted from Pst56-1205-400. All tissues were from Ps26. Ovary D0, Ovaries at the stage of D0, i.e. anthers beginning to exsert but prior to pollen shed; Ovary D0/-RT, no RT control to check for DNA contamination; Ovary D1, ovary at 1 day after pollination;

Supplemental Figures

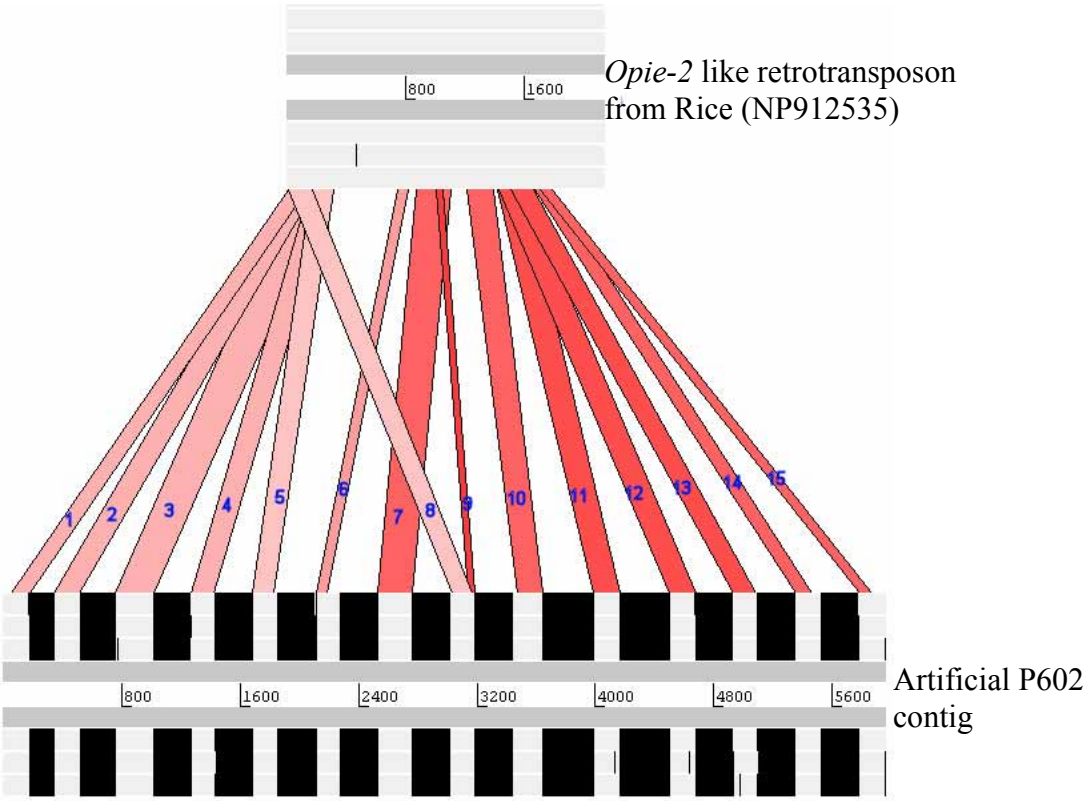
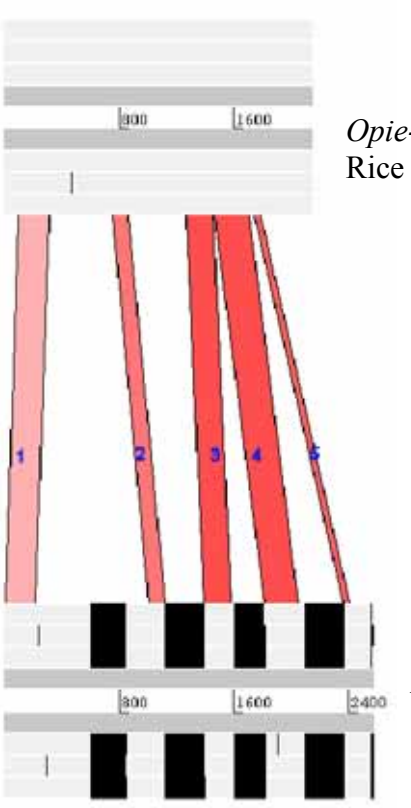


Fig. 3.1 Comparison of artificial P602 contigs with an *Opie-2* like retrotransposon from rice (NP912535) at the amino acid level. Numbers in the image are representing different contigs or singletons.



Opie-2 like retrotransposon from Rice (NP912535)

Artificial P800 contig

Fig. 3.2 Comparison of artificial P800 contigs with an *Opie-2* like retrotransposon from rice (NP912535) at the amino acid level. Numbers in the image are representing different contigs

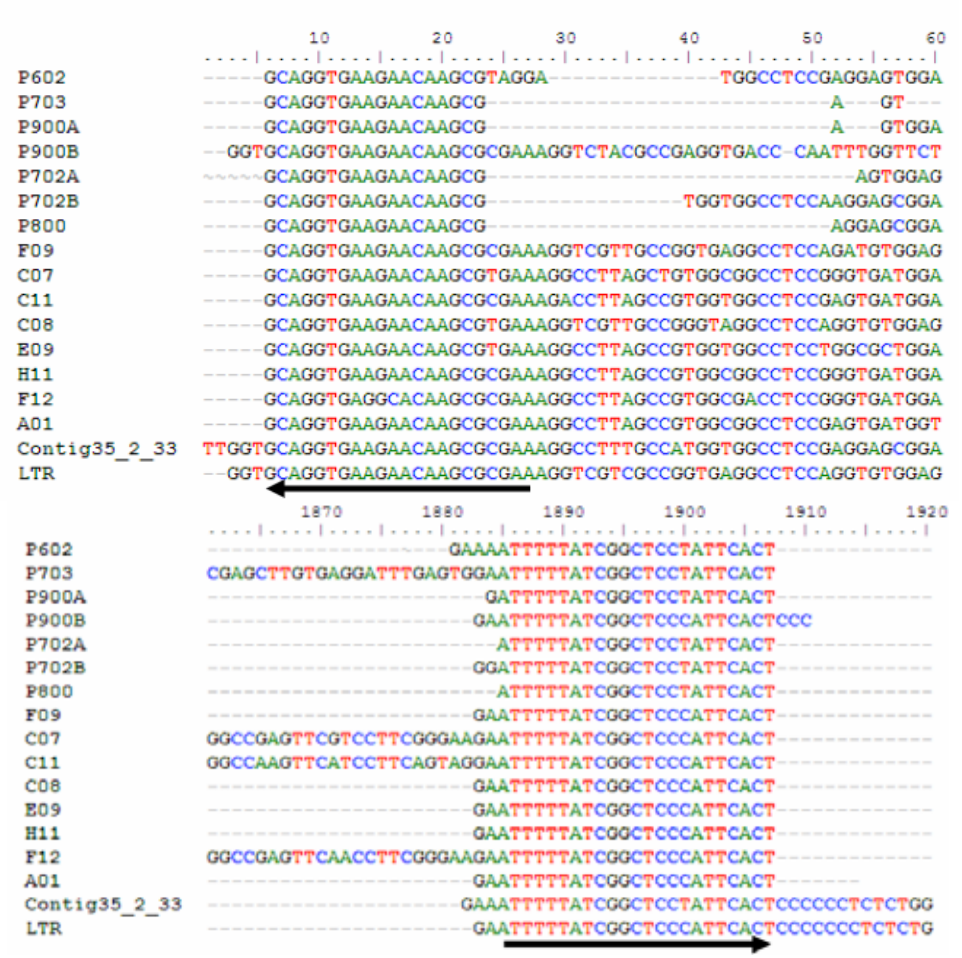


Fig.3.3 LTR-specific primers 1087 (upper panel, indicated by underline) and 1156 (lower panel, indicated by underline) were from the alignment of all sequences. Arrows show the orientation of primers.

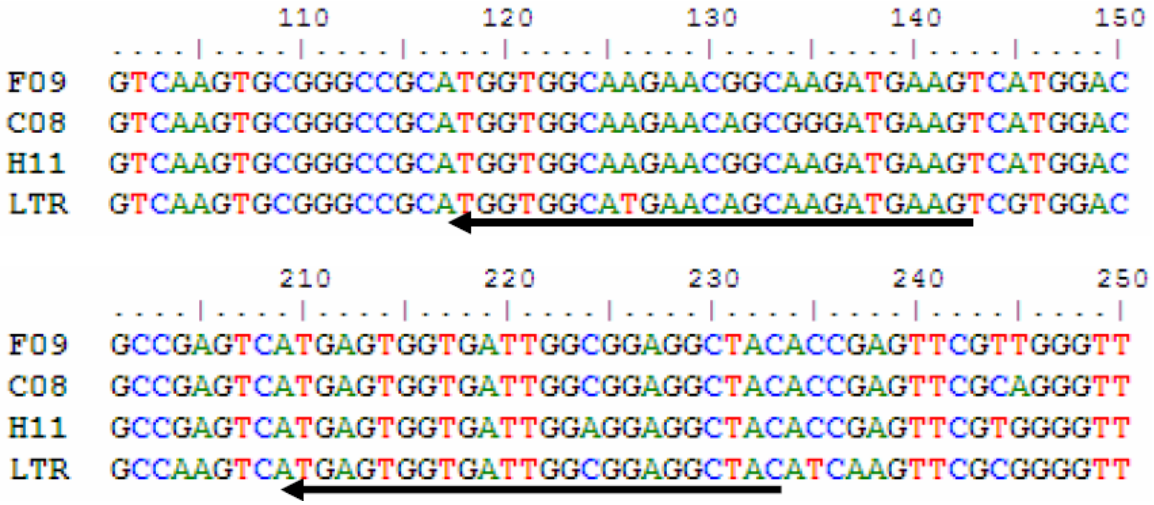


Fig. 3.4 LTR-specific primers 1153 (upper panel, indicated by underline) and 1154 (lower panel, indicated by underline) were from alignment of Ofovin group sequences. Arrows show the orientation of primers.

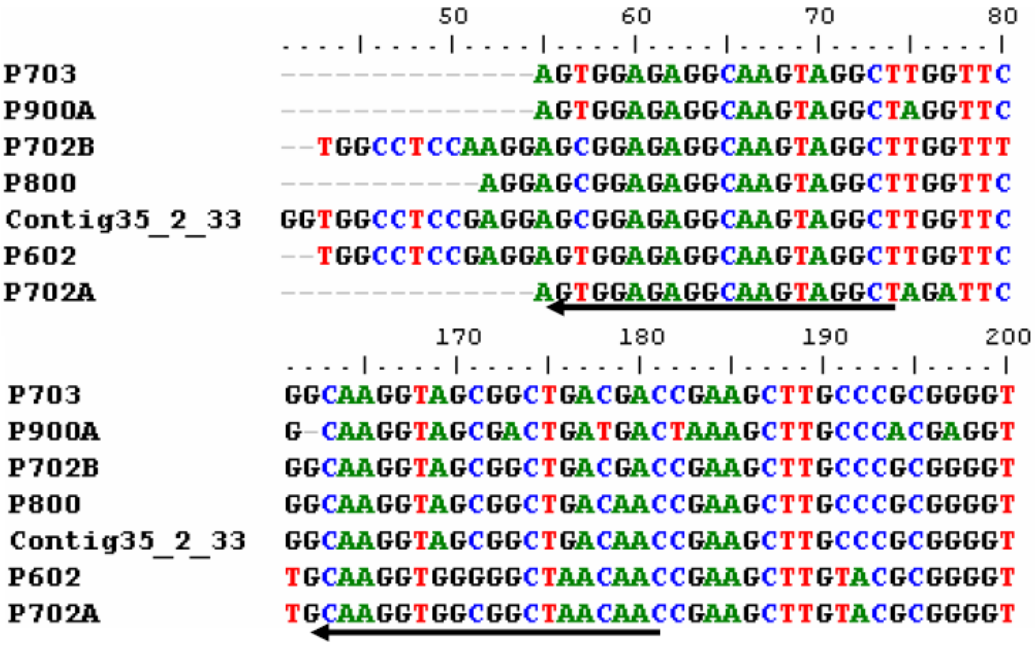


Fig. 3.5 LTR-specific primers 1155 (upper panel, indicated by underline) and 1086 (lower panel, indicated by underline) were from alignment of ASGR-linked BAC group sequences. Arrows show the orientation of primers.

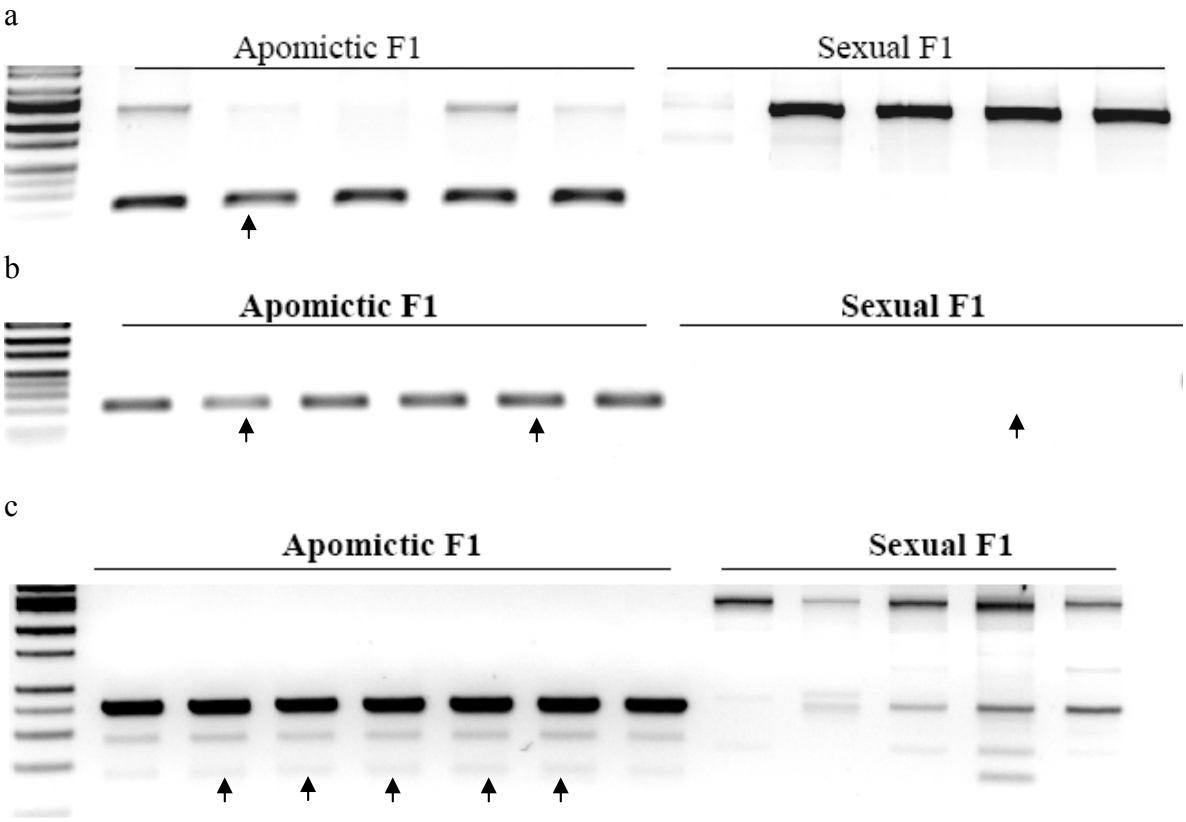


Fig 3.6 SCAR marker E86-1136-410 (a), E86-1138-385 (b), and Pst55-1205-440 (c) were used for screening among individuals from the F1 population. Arrows show the F1s in which recombination was indicated by SSAP markers but not corresponding SCARs.

CHAPTER 4

CHARACTERIZATION OF THE ASGR BABY-BOOM-LIKE GENE IN PROGENIES OF A TETRAPLOID PEARL MILLET (*PENNISETUM GLAUCUM*) × *P. SQUAMULATUM*

Introduction

In flowering plants, seeds normally are derived through sexual reproduction from the event of double fertilization in which the reduced egg cell receives one sperm to form a diploid embryo while the binucleate central cell fuses with another sperm to form a triploid endosperm (Chaudhury et al. 2001). However, sexual reproduction is not the universal mode in the plant kingdom but can be supplanted with asexual reproduction through seeds, a natural phenomenon called apomixis (Asker and Jerling 1992; Nogler 1984a). Apomixis has basically two forms: sporophytic and gametophytic. In sporophytic apomixis, no unreduced embryo sac forms, but asexual and sexual reproduction co-exist because the development of an asexually derived adventitious embryo relies on endosperm formation upon fertilization of the central cell from a reduced embryo sac. Gametophytic apomixis is subdivided into two categories, apospory and diplospory. Both forms of gametophytic apomixis result from the production of unreduced embryo sacs and parthenogenetic development of an egg into an embryo resulting in clonal progeny of the maternal genotype (Bhat et al. 2005; Bicknell and Koltunow 2004; Ozias-Akins 2006). Apomixis has been attracting and challenging scientists for decades due to its potential impact on crop improvement and study of plant development and evolution (Meeûs et al. 2007; Spillane et al. 2004; Spillane et al. 2001; van Dijk and van Damme 2000). Because introgression of apomixis to crop species from their wild apomictic relatives has proved complex (Bicknell and Koltunow 2004), understanding the genetic mechanisms of apomixis involved in natural apomixis will greatly facilitate the utilization of this trait in crop breeding (Ozias-Akins and van Dijk 2007).

There are three essential elements for apomixis: apomeiosis (circumvention of meiosis), parthenogenesis (autonomous development of the embryo) and pseudogamous or autonomous

endosperm development (Grossniklaus et al. 2001; Ozias-Akins 2006). Two or more of these elements have been shown to be inherited independently such as in *Taraxacum officinale* (van Dijk 2003; van Dijk et al. 1999) *Erigeron annuus* (Noyes and Rieseberg 2000), *Hieracium caespitosum* (Catanach et al. 2006), and *Poa pratensis* (Albertini et al. 2001) or as a single dominant trait such as in *Pennisetum ciliare* (Jessup et al. 2002; Sherwood et al. 1994), *Pennisetum squamulatum* (Ozias-Akins et al. 1998), *Paspalum notatum* (Martinez et al. 2001), *Tripsacum dactyloides* (Grimanelli et al. 2001), *Ranunculus auricomus* (Nogler 1984b), and *Panicum maximum* (Savidan 2000). Isolation of candidate genes has been attempted through comparative study of differential gene expression between ovules from sexual and apomictic genotypes (Albertini et al. 2004; Chen et al. 1999; Pessino et al. 2001; Rodrigues et al. 2003; Singh et al. 2007). Some candidate genes were also revealed from sequencing of BAC clones (Conner et al. 2008). Although some candidate genes were isolated from natural apomicts, only a few have been shown to specifically express in apomeiotic embryo sacs (Alves et al. 2007; Chen et al. 2005; Singh et al. 2007). None have been thoroughly characterized for function in the apomictic plants. At the same time, forward and reverse genetics have been applied to sexually reproductive plants to discover gene(s) involved in one or more elements of apomixis. Many mutants have been identified to have features similar to those of apomicts (Spillane et al. 2001). For example, in the Arabidopsis *dyad/swi1* mutant, the megaspore mother cell entered meiosis, but chromosomes failed to undergo synapsis**,** resulting in the production of two diploid cells in place of four haploid megaspores (Agashe et al. 2002; Ravi et al. 2008; Siddiqi et al. 2000). Several genes such as *LEAFY COTYLEDON* (*LEC1*) (Lotan et al. 1998), *LEAFY COTYLEDON2 (LEC2*) (Stone et al. 2001), *BABY BOOM* (*BBM*) (Boutilier et al. 2002), *WUSCHEL*(*WUS*) (Zuo et al. 2002), and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE*

(*AtSERK1*) (Hecht et al. 2001) were identified to be involved in embryo initiation; over-expression of these genes has been shown to induce embryogenic cell fates in Arabidopsis. Also in Arabidopsis, the FIS gene family was identified to play an important role in endosperm development. In three mutants, *fertilization-independent endosperm* (*fie*), *medea* (*mea*), and *fertilization independent seed2* (*fis2*), endosperm can autonomously develop in the absence of fertilization of the central cell whereas embryo development is initiated but arrested without fertilization of the egg cell (Chaudhury et al. 1997; Grossniklaus et al. 1998; Ohad et al. 1996). Although mutations in sexually reproductive plant species can enhance the progress in understanding apomictic reproduction, no mutant identified thus far displays the full spectrum of apomixis elements.

We are working on a grass species, *P. squamulatum,* which can reproduce through apospory. Apospory is inherited as a dominant Mendelian trait in *P. squamulatum*. A greater than expected number of molecular markers co-segregate with apomixis, defining an apospory specific genomic region (ASGR) (Goel et al. 2006; Goel et al. 2003; Ozias-Akins et al. 1993; Ozias-Akins et al. 1998). Genetic and molecular cytogenetic characterization of the ASGR showed that it is hemizygous, physically large (over 50 Mb), and highly heterochromatic (Akiyama et al. 2004; Goel et al. 2006; Goel et al. 2003). Although the ASGR has an abundance of repetitive elements such as retroelements, genes also have been revealed in the ASGR. One of these genes has been named the *ASGR-BABY-BOOM-Like* gene (*ASGR-BBML*) (Conner et al. 2008). The first *BABY-BOOM* (BBM) gene was isolated from microspore cultures of *Brassica napus*, and was shown to promote conversion of cells from vegetative to embryonic growth (Boutilier et al. 2002). Two copies of *ASGR-BBML* were identified from the polyhaploid BAC clones. Both of them were predicted to encode a 542 amino acid protein containing two AP2

domains (Conner et al. 2008). Studies on *Baby-Boom-Like* genes isolated from other species suggested that they may play a role in somatic and zygotic embryo development (Boutilier et al. 2002; Morcillo et al. 2007; Srinivasan et al. 2007). The function of *ASGR-BBML* in *P. squamulatum* is unknown, but by homology, it is a candidate for control of parthenogenesis. In this study, we used RNA interference to analyze the role of *ASGR-BBML* in the development of ovules/embryos in mature embryo sacs of F1 plants of a tetraploid pearl millet (*P. glaucum*) × *P. squamulatum*.

Materials and Methods

Plant materials

An induced sexual tetraploid pearl millet (*P. glaucum*) (IA4X, 2n=28) and an obligate apomictic *P. squamulatum* (Ps26, PI 319196, 2*n*=56) were used in this study. A red tetraploid pearl millet (Tift23B) genotype showing red stems and leaves (a dominant phenotypic marker) was used as a pollen donor in test crosses. An apomictic individual (Line58) from the fourth back cross between BC3 (Dujardin and Hanna 1989) and tetraploid pearl millet was also included in this study for *ASGR-BBML* gene isolation. The *Arabidopsis thaliana* ecotype Columbia was used for over-expression.

Nucleic acid extraction and purification

Genomic DNA was extracted from leaf tissue using a previously described method (Ozias-Akins et al. 1993). Plasmid DNA was extracted and purified with the QIAGEN plasmid mini kit following the manufacturer's instructions (QIAGEN, Valencia, CA). Leaf and ovary RNA were isolated with the QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA). Leaf tissues were collected from the terminal leaf of a tiller with 5-8 expanded leaves. Ovaries were collected from plants at anthesis (as DAP 0, i.e. anthers beginning to exsert but prior to pollen

shed), at DAP 1 (1 day after pollination) and at DAP 2 (2 days after pollination). Thirty ovaries of each stage were collected and placed in 80 µl of RLT buffer from the QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA) then stored at -80 °C until use.

DNA quantification was conducted with a Fluorocount Microplate Fluorometer using Hoechst 33258 (Packard, Meriden, CT, USA) following the instruction of Molecular Cloning Protocols (Sambrook and Russell 2001), while RNA was quantified with RiboGreen® RNA Quantitation Kit following the manufacturer's protocol (Molecular Probes, Eugene, OR, USA).

RNAi vector construction

A binary expression vector pMCG161 (Fig.4.1) (http://www.chromdb.org) was used for RNAi vector construction. This vector contains a chloramphenicol resistance gene for bacterial selection, a phosphinothricin acetyl transferase (Basta resistance or BAR) gene driven by a maize (*Zea mays* L.) ubiquitin promoter conferring herbicide resistance, and a CaMV (Cauliflower mosaic virus)-35S promoter driving expression of the RNAi-inducing dsRNA. The RNAi fragment includes an intron from the rice waxy a gene to stabilize the inverted repeat of the target gene fragments and a terminator from the octopine synthase (OCS) gene which is downstream of the antisense insert. The target gene fragments were amplified from BAC P207 with primers BBM-RNAi-F (GTACTAGT***GGCGCGCC***CCCTCAATGCTGTCACGAACTT, underlined and italic bold are *Spe* I and *Asc* I restriction sites, respectively) and BBM-RNAi-R (GTGCGATCGC***CCTAGG***CAACACCTGTCATGTCCTGAA, underlined and italic bold are *Asis* I and *Avr* II restriction sites, respectively). Designing of this set of primers was based on the alignment between genomic sequence of *ASGR-BBML* in P207 BAC clone and the predicted mRNA sequence produced with *ASGR-BBML* genomic sequence using FGENESH (http://www.softberry.com/berry.phtml). The amplicon from P207 was 425 bp in length and

covered ~52% of the last predicted exon of *ASGR-BBML*. Amplification was performed in a reaction volume of 50 µl, including ~80 ng of P207 DNA, 0.25 µM each of the BBM-RNAi-F and BBM-RNAi-R primers, 0.2 mM of each dNTP, 1.25U PrimeSTAR HS DNA polymerase (Takara, Madison, WI, USA), in 1× PCR buffer (10 mM Tris-HCL, 50 mM KCl, 1.0 mM $MgCl_2$). PCR was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following condition: an initial denature at 94°C for 3 min followed by 32 cycles of 98°C, 10s; 68°C, 30s. A final extension was carried out at 68°C for 7 min. PCR purification was conducted with QIAquick PCR purification kit following the manufacturer's instructions (QIAGEN, Valencia, CA, USA). Both pMCG161 vector and purified PCR product were digested with *Asc* I and *Avr* II simultaneously at 37°C for 16 hr in a reaction volume of 50 µl, including ~1.5 µg vector DNA or 1.2 µg PCR product, 15U of *Asc* I and *Avr* II each in 1× NEBuffer 4 (50 mM potassium acetate, 20 mM Tris- acetate, 10 mM magnesium acetate, 1 mM DTT). Ligation of the *ASGR-BBML* fragment in sense direction to pMCG161 was subsequently performed at 4°C overnight in a 10 µl reaction containing 1× ligation buffer (30 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM DTT and 1 mM ATP), 50 ng digested pMCG161 vector, 5 ng digested PCR product and 3 U T4 DNA ligase (Promega, Madison, WI, USA). The plasmid carrying the sense *ASGR-BBML* insert was transformed into DH5α *E. coli* competent cells via the heat-shock method. Ligation and transformation were confirmed by a PCR with primers of BBM-RNAi-F and BBM-RNAi-R. The plasmid DNA was digested with *Asc* I to make sure that only a single copy of the insert was ligated to the vector. Plasmid DNA carrying the sense *ASGR-BBML* insert and PCR product from P207 with BBM-RNAi-F and BBM-RNAi-R were also digested with both *Spe* I and *Asis* I using similar reaction conditions as described above except that NEBuffer 4 was replaced with NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM

$MgCl_2$, 1 mM DTT) supplemented with 100 µg/ml of BSA. Ligation of the *ASGR-BBML* fragment in antisense orientation to the plasmid DNA carrying sense *ASGR-BBML* insert and its subsequent transformation were conducted as described above. *Asc* I was used for digestion of plasmid DNA carrying both sense and anti-sense *ASGR-BBML* inserts to confirm the ligation and transformation. The digestion should release an ~2.1 kb fragment (sense and anti-sense *ASGR-BBML* plus the 1.19 kb rice waxy a intron) from the plasmid.

Embryogenic callus induction and plant transformation

Seeds of IA4X were placed in a Petri-dish containing damp filter paper for germination at room temperature. Germinated seeds were grown in a 25 cm diameter × 29.5 cm deep pot in the greenhouse. Inflorescences of IA4X were collected at the "milky" stage, i.e., the endosperm of seeds was still liquid, not solidified, and surface sterilization of immature seeds was carried out in 70% ethyl alcohol for 30 s, followed by 20% Clorox (stock contains 6.0% sodium hypochlorite) supplemented with a few drops of Tween-20 for 20 min. Sterilization was followed by 4 rinses with sterile deionized water for 20 min each. Immature embryos were dissected from the seeds under a stereo microscope and placed onto induction medium (MS medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 7.5 g/L agar, 2 mg/L 2,4-dichlorophenoxyacetic acid, and 2 ml/L plant preservative mixture (PPM; Plant Cell Technologies, Washington, D.C. USA), pH 5.8, with the scutellum side up. All immature embryos were cultured in the dark at 25±1°C for induction of embryogenic callus. After a 3-week induction, embryogenic calluses were transferred onto fresh induction medium for 7-10 days before bombardment. Calluses were arranged within a 2.5-cm-diameter circle and treated on osmotic medium (induction medium supplemented with 0.35 M sucrose) for 4-6 hr immediately before bombardment.

DNA delivery was performed with the method described by Goldman et al. (2003) using a PDS-1000 He biolistic device (Bio-Rad, Hercules, CA, USA). In brief, 0.6 μm gold particles (100, 120 or 500 μg/shot) (Bio-Rad) were coated with plasmid DNA (~500 ng/shot) using a previously reported protocol (Goldman et al. 2003). The DNA-coated gold particles were finally resuspended in 100% ethanol with an approximate volume which is equal to 8 times the number of planned shots plus 20 μl. An 8 μl aliquot of DNA-coated gold particles was placed onto the center of macrocarriers, which were placed in their holders after the evaporation of ethanol. Each plate was bombarded twice at a helium pressure of 1350 psi by rotating the plate 90°. A distance of 6-cm between rupture disk and stopping screen and between stopping screen and target plates was maintained. Calluses were cultured on the osmotic medium for another 16 hr before they were transferred onto pre-selection medium (induction medium plus 0.01 mg/L 6-benzylaminopurine) for 10-14 days.

Selection and regeneration of transformed plants

After culturing on pre-selection medium, calluses subsequently were cultured on selection media (pre-selection medium plus 15 mg/L glufosinate ammonium, diluted from Liberty 200 SL containing ~200 g/L glufosinate ammonium, Bayer Cropscience, Kansas City, MO, USA) for 8-9 weeks in dim light (~60 μmol m^{-2} s^{-1}) to select herbicide-resistant tissues. For regeneration, rapidly growing calluses were transferred onto regeneration medium (MS medium supplemented with 0.1 mg/L thidiazuron and 0.1 mg/L 6-benzylaminopurine) and cultured in the light (100 μmol m^{-2} s^{-1}) at 26±1°C. Regenerated shoots were transferred onto a second selection medium (basal MS medium supplemented with 8 mg/L glufosinate ammonium and 50 mg/L chlorophenol red). Chlorophenol red is a pH indicator dye whose solution color is red at a pH of 6.0 or higher or yellow at pH<6.0. Plantlets resistant to herbicides can cause medium

acidification and yellowing of chlorophenol-containing culture media (Kramer et al. 1993). Vigorous plantlets were transplanted to 9 cm diameter ×11.5 cm deep pots and covered with clear plastic cups for 10-14 days of acclimation in a greenhouse. Diluted Liberty (500 mg/L) was sprayed on the putative transgenic plants 3-4 weeks after transplanting to the greenhouse. Molecular confirmation of putative transgenic plants was also performed by PCR with primers BBM-RNAi-F and BBM-RNAi-R using the following conditions: an initial denature at 94°C for 5 min followed by 35 cycles of 94°C, 30s; 55°C, 30s, 72°C, 30s; a final extension was performed at 72°C for 7 min.

Introgression of apomixis into transgenic plants

Transgenic IA4X plants were used as maternal parents for crossing with apomictic Ps26. Heads were bagged at least 7-10 d before anthesis. Pollen was collected in glassine bags from Ps26 before noon and used to pollinate transgenic IA4X. Seeds were collected from heads 5-6 weeks after pollination. All seeds were dried at 34°C for 36 hr and stored at 4°C for two weeks or longer before attempting germination. For germination, 190 seeds from 8 independent lines were treated for 2 hr in a solution containing 1% (v/v) 2-chloroethanol and 2% (v/v) Clorox (6.0% sodium hypochlorite), followed by a 45-minute rinse with tap water. Seeds were blotted dry with paper towels and sown in the greenhouse. Genotypes of 190 plants were tested with several different sets of primers: Ps26 specific primers (1032/1035, Table 1) to check if the plants were originated from the cross between transgenic IA4X and Ps26; BAR gene primers (992/993, Table 1) to check if the plants were carrying the BAR gene; RNAi sense insert (1222/1223) and antisense insert primers(1224/1225) (Table 1) to check if plants were carrying the *ASGR-BBML* fragment in both sense and antisense orientations; ASGR specific primers (Q8M and Ugt197, Table 1) to check if the plants were carrying the ASGR. Seventy-five plants

which were initially chosen for further analysis were reconfirmed with PCR as described above. The reproductive mode of 49 plants also was characterized by clearing of ovules in methyl salicylate (Young et al. 1979).

Detection of *ASGR-BBML* gene expression with semi-quantitative RT-PCR

In the semi-quantitative RT-PCR, actin depolymerizing factor 3 (ADF3) was used as an internal control. To avoid the amplification plateau for analysis, cycle number (20, 23, 26, 29, 32, 35, 38 and 40) for the exponential range of amplification of ADF3, *ASGR-BBML* and OCS (octopine synthase) was tested with a standard reaction condition: 0.25 μM of each primer, 0.2 mM of each dNTP, 0.025U/μl Jumpstart *Taq* polymerase (Sigma-Aldrich, MO, USA) and 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$). To determine the cycle number for OCS and ADF3, 3.6 μg of total leaf RNA from a transgenic plant with the genotype of -/RNAi was used for reverse transcription in a 20 μl reaction with SuperScript II first-strand synthesis system (Invitrogen, Carlsbad, California, USA). Total ovary RNA (5 μg) from Ps26 (at DAP 0) was used to determine the cycle number for *ASGR-BBML*. A 2 μl aliquot of cDNA was used in a 20 μl PCR reaction. However, when expression of OCS in leaves of transgenic F1s was detected, 1 μg of total RNA was used for reverse transcription; when detection of expression of OCS, ADF3 and *ASGR-BBML* in ovaries of transgenic F1s was performed, 3.3 μg ovary total RNA was used for cDNA synthesis. All amplifications were performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). Amplified products were separated on a 2% agarose gel (Invitrogen, Carlsbad, CA). Gels were stained for 30 min in TBE buffer containing 0.5 μg/ml ethidium bromide and cleared in distilled water for 10 min. For imaging, gels were exposed to the UV light for 2.5s with Molecular Imager Gel Doc XR System (Bio-Rad Laboratories, Hercules, CA, USA).

Amplified products were also separated on a 1.5% agarose gel for *ASGR-BBML* and ADF3 or 2.5% agarose gel for OCS. Fragments were subsequently transferred to Genescreen Plus nylon membranes (NEN Life Sciences, Boston, MA) using the alkaline transfer method (Sambrook and Russell 2001). Probes were labeled with α-^{32}P dCTP using a PCR-based method with *ASGR-BBML* (779/780, Table 1), ADF3 (1127/1128, Table 1), or OCS (1126/1127, Table 1) specific primers in a 20 µl reaction, including 100 ng of *ASGR-BBML*, ADF3 or OCS-containing plasmid DNA, 1.25 U of Jumpstart *Taq* polymerase (Sigma-Aldrich, St. Louis, MO, USA), 0.25 µM of each primer, 0.5 mM of each dNTP (excluding dCTP), 3 µl of α-^{32}P-labeled dCTP (3000 Ci/mmol) and 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2). Probes were purified with Sephadex G-50 and denatured at 95°C immediately before adding to hybridization buffer. Membranes were pre-hybridized in a hybridization buffer (6×SSC, 1% SDS, 100 µg/ml heat-denatured salmon sperm DNA) at 65°C overnight. The denatured α-^{32}P-labeled probe was then added into the hybridization buffer and incubated overnight at 65°C. Imaging of hybridization was conducted with a Storm phosphorimager (Amersham Biosciences, Pittsburgh, PA, USA) after two washes (2×SSC, 0.1% SDS; and 1× SSC, 0.1% SDS) at 65°C for 15 min each. Quantification of each band was conducted with ImageQuant v5.0 following the manufacturer's instructions (Amersham Biosciences, Bukinghamshire, England).

Northern Blotting

Total ovary RNAs (5 µg) from six transgenic plants at DAP 0 and Ps26 at DAP 0, DAP 1 and DAP 2 were separated on a denaturing gel (1.2% agarose, 0.2 M formaldehyde) and transferred to Genescreen Plus nylon membranes (NEN Life Sciences, Boston, MA) according to the protocol described by Sambrook and Russell (2001). Labeling, purification and denaturing of probes was as described above. Membranes were pre-hybridized and hybridized at 42°C

overnight using the same hybridization buffer (5× SSC; 50% formamide; 5× Denhardt's solution; 1% SDS, 100 μg/ml heat-denatured salmon sperm DNA). The hybridization signal was detected with a Storm phosphorimager (Amersham Biosciences, Pittsburgh, PA) after two washes (2×SSC, 0.1% SDS; and 1× SSC, 0.1% SDS) at 42°C for 15 min each.

Histological Analysis

Half of the spikelets from heads of four transgenic plants were manually de-styled at anthesis (DAP 0), i.e., anthers beginning to exsert but prior to pollen shed) prior to pollination with red TIFT23B. Spikelets (de-styled and non-de-styled) were collected at 2 days after pollination and fixed in FAA (47.5% (v/v) ethanol, 5% glacial acetic acid, 3.7% (v/v) formaldehyde). Spikelets in FAA were subjected to a 30-min vacuum treatment at 15 mm Hg prior to a 24-hr fixation at room temperature. Fifty to sixty ovaries from each plant were dissected for each treatment (de-styled or non-destyled) and stored in 70% ethanol containing saturated erythrosin B until dehydration. Dehydration was initiated with TBA 1 (40% ethanol, 10% tertiary butyl alcohol (TBA), 50% distilled H_2O) for 2 hr, then transferred through TBA2 (50% ethanol, 20% TBA, 30% distilled H_2O) for 8 hr, TBA3 (50% ethanol, 35% TBA, 15% distilled H_2O) for 1 hr, TBA 4 (45% ethanol, 55% TBA) for 1 hr, TBA 5 (25% ethanol, 75% TBA) for 1 hr, TBA 6 (100% TBA) for 1 hr. Ovaries were transferred to fresh 100% TBA for another 8 hr prior to being transferred to TBA: paraffin oil (Fisher, Pittsburgh, PA, USA) (1:1) at 58°C overnight. Ovaries were taken through three changes of pure Paraplast X-tra (Fisher, Pittsburgh, PA) for 48 hr each before embedding. Sectioning was carried out at 9 μm for all samples. Samples were stained with safranin O/fast green using a modified protocol from the one described by Jensen (Jensen 1962). In brief, embedded and sectioned samples were de-waxed in two changes of 100% histoclear for 5 min each. Samples were transferred to 100%

ethanol, then coated with 0.05% nitrocellulose (diluted from collodion, Fisher, Pittsburgh, PA) in ether-alcohol (50% diethyl ether, 50% ethanol) for 30 s. Rehydration of samples was accomplished by transfer through 70% ethanol, 30% ethanol and distilled water for 5 min each. Staining first was performed in Safranin O solution (4 g Safranin O, 100 ml distilled water, 100 ml 95% ethanol, 4 g sodium acetate) for 5 min. Samples then were subjected to dehydration in the following series of solutions for 5 min each: two changes of distilled water, 50% ethanol, 95% ethanol, 100% ethanol. Samples subsequently were stained in fast green solution (1 g fast green, 100 ml 100% ethanol, 100 ml cellosolve and 100 ml clove oil) for 4 sec and immediately placed into pure clove oil for 10 sec, then quickly transferred into clearing mix (50% clove oil, 25% ethanol, 25% histoclear). Slides were finally cleared in histoclear for 5 min and mounted with permount.

Observation of pollen tube growth in pistils of transgenic plants

Inflorescences were collected from two transgenic plants (500S4#2T-8 and 500S2#2T-9) 4 hr after pollination with red TIFT23B and fixed in FAA overnight (as described above). Thirty-three and twenty-eight ovaries from 500S4#2T-8 and 500S2#2T-9, respectively, were dissected under a microscope and placed in 70% ethanol. Rehydration of ovaries was subsequently conducted by transferring through 50%, 30% ethanol and distilled water for 10-15 min each. Ovaries were cleared with 5 M NaOH for 8 hr at room temperature and washed carefully with distilled water for 10-15 min. Ovaries then were stained with 0.1% (w/v) aniline blue in 108 mM K_3PO_4 (pH ~11) for 2 hr in the dark. Observation was performed with the Zeiss Axioskop 2 plus microscope (Carl Zeiss, Jena, Germany) under UV light condition.

5’ and 3’ RACE

Gene specific nested primers were designed for both the 5’ and 3’ ends of *ASGR-BBML* using a recovered cDNA fragment of *ASGR-BBML* (unpublished data). Using the GeneRacer® RLM-RACE kit (Invitrogen, Carlsbad, CA), ~1.5 µg of total leaf RNA from line 58 was treated with calf intestinal phosphatase to remove the 5’ phosphate. This can also eliminate truncated mRNA and non-mRNA from subsequent ligations. The treatment was followed by a phenol: chloroform extraction and purification. The sample was then subjected to a tobacco acid pyrophosphatase treatment to remove the cap structure from intact, full-length mRNA, also resulting in a free phosphate at the 5’end of full-length mRNA which is required for ligation to the RNA adapter. Purification was performed again with phenol:chloroform. A 44 nt RNA adapter, GeneRacer™ RNA Oligo, was ligated to the 5’ end of the decapped mRNA using T4 ligase (Invitrogen, Carlsbad, CA). Reverse transcription was then performed using SuperScript ™ III RT module supplied with the kit and *ASGR-BBML* gene specific primers (1024 and 1025, Table 1). Gene specific primers (984, 1026 and 1027, Table 1) and GeneRacer™ 5’ primer were used for outer 5’RACE amplification in a volume of 15 µl including 0.5 µl RT template, 0.6 µM GeneRacer™ 5’primer, 0.2 µM gene specific primer, 0.2 mM of each dNTP, 0.375 U Jumpstart *Taq* polymerase (Sigma-Aldrich, St. Louis, MO, USA) and 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$). The PCR included an initial denature at 94°C for 2 min; 5 cycles of 94°C for 30 s, 72°C for 5 min; 5 cycles of 94°C for 30 s, 70°C for 5 min; 25 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 5 min; followed by a final extension at 72°C for 10 min. The inner 5’RACE amplifications with nested gene specific primers (1027 and 1028, Table 1) and GeneRacer™ 5’nested primer were subsequently performed with the outer 5’RACE PCR product as template. The 20 µl PCR reaction for nested amplification contained 1 µl outer

5'RACE PCR product, 0.25 µM gene-specific nested primer, 0.25 µM GeneRacer™ 5' nested primer, 0.2 mM of each dNTP, 0.5 U Jumpstart *Taq* polymerase (Sigma-Aldrich, St. Louis, MO, USA) and 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$). The PCR profile for nested amplification was an initial denature at 94°C for 2 min; 25 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 2 min; followed by a final extension at 72°C for 10 min. Nested 5'RACE PCR products were separated with 1.5% agarose gels (Invitrogen, USA) and purified with QIAquick gel extraction kit (QIAGEN, Valencia, CA). Gel purified fragments were ligated with the PCR4-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into One Shot TOP10 *E. coli* DH5α following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Sequencing was carried out with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence assembly and primer design were performed with Vector NTI (Invitrogen).

The GeneRacer® RLM-RACE kit (Invitrogen, Carlsbad, CA) was also used for 3'RACE. Total leaf RNA (~1 µg) of line 58 was used for reverse transcription in a 20 µl volume: 1 µl of dNTP mix, 1 µl of GeneRacer™ oligo dT primer, 4 µl 5× RT buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOut™ (40 U/µl), 1 µl of SuperScript™ III reverse transcriptase. The reaction was incubated at 50°C for 1 hr followed by 70°C for 15 min to inactivate the reverse transcriptase. RNA was removed by adding 1 µl RNase H and incubating at 37°C for 20 min. The RT product was then used for an outer 3'RACE PCR reaction in a 20 µl volume using the same conditions as described for the outer amplification of 5'RACE. The product from the outer 3'RACE PCR was used as a template for a second round of PCR using a nested gene specific primer and the GeneRacer™ 3' nested primer under the same conditions as for nested 5'RACE PCR. Cloning and sequencing were also the same as described for 5'RACE.

Cloning of a full-length *ASGR-BBML* gene

An end-to-end amplification of the *ASGR-BBML* gene was performed with primers (1088 and 1089, Table 1) in a 20 µl PCR reaction, including 1.6 µl RT, 0.4 µM of each primer, 0.5 mM of each dNTP, 1 U Jumpstart REDAccu*Taq* LA DNA Polymerase (Sigma-Aldrich, St. Louis, MO) and 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$). The PCR included an initial denature at 96°C for 30 s, 35 cycles of 94°C for 15 s, 56°C for 30 s, 68°C for 2 min, followed by a final extension at 68°C for 10 min. Because of point mutations caused by Jumpstart REDAccu*Taq* LA DNA Polymerase, a different PCR was also performed with PrimeSTAR HS DNA polymerase (Takara, Madison, WI) in a 25 µl reaction, including 1.5 µl RT, 0.2 µM each of 1088 and 1089 primers, 0.2 mM dNTPs, 0.625 U PrimeSTAR HS DNA polymerase, in 1x PCR buffer (10 mM Tris-HCL, 50 mM KCl, 1.0 mM $MgCl_2$) (Takara, Madison, WI). The PCR was carried out under the following conditions: an initial denature at 94°C for 30 s, 32 cycles of 98°C for 10 s, 55°C for 15 s, 72°C for 2 min. Ligation, transformation, and sequencing were the same as described above. Point mutations caused by amplification with PrimeSTAR HS DNA polymerases also were found. One clone (Jumpstart clone 9) from the amplification with Jumpstart REDAccu*Taq* LA DNA polymerase showed no point mutation in the first 874 bp of *ASGR-BBML*. Another clone (PrimeSTAR clone 21) from the amplification with PrimeSTAR HS DNA polymerase, showed point mutations in the last 887 bp of *ASGR-BBML* but these were silent. There is a unique *BstX* I restriction site at position 767 bp of *ASGR-BBML* and in the TOPO vector. Digestion of two clones with *BstX* I produced a smaller fragment released from Jumpstart clone 9 containing the first 767 bp of *ASGR-BBML* and a larger fragment from PrimeSTAR clone 21 containing the last 887 bp of *ASGR-BBML* (Fig.4.16). Digestion of the two clones was separately carried out in a 10 µl reaction including

1× NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT), 450-600 ng plasmid DNA, 5 U *BstX* I (New England Biolabs, Beverly, MA) and incubated at 55°C for 4 hr. Digested fragments were gel-purified with the QIAquick gel extraction kit (QIAGEN, Valencia, CA). Ligation was performed in a 5 µl reaction containing ~60 ng of the 767 bp fragment and ~25 ng of the 887 bp fragment, 0.3 U T4 ligase (Promega, Madison, WI), 1× ligation buffer (30 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM DTT and 1 mM ATP) and incubated at 4°C overnight. Ligated plasmid was transformed into One Shot TOP10 *E. coli* DH5α (Invitrogen, USA). Plasmid DNA was extracted and digested with *Xho*I and *Xba*I (New England Biolabs) to distinguish if the plasmid contained the first 767 bp of *ASGR-BBML* from Jumpstart clone 9 because there were *Xba*I sites at each end of *ASGR-BBML* and a *Xho*I site in the first 767 bp of Jumpstart clone 9, but there was no *Xho*I site in the first 767 bp of *ASGR-BBML* in PrimeSTAR clone 21. Digestion with *Xho*I and *Xba*I would release 3 different fragments (3956 bp, 1514 bp and 140 bp). Sequencing was also carried out with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) for further confirmation.

Over-expression vector construction

A binary expression vector pBINPLUS35S was used for over-expression vector construction. This vector contains a kanamycin resistance gene for both bacterial and plant selection, a CaMV (Cauliflower mosaic virus) 35S promoter driving expression of the target gene, and a nopaline synthase (NOS) terminator. Both pBINPLUS35S vector and plasmid DNA containing the modified full-length *ASGR-BBML* gene (TOPO vector backbone, see above) were digested with *Xba* I in a 10 µl reaction including 1× NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT), 100 µg/ml BSA, ~250 ng pBINPLUS35S plasmid DNA or 600 ng modified full-length *ASGR-BBML* containing plasmid DNA, 5 U *Xba* I (New England Biolabs).

Dephosphorylation of 5' recessed ends of pBINPLUS35S was performed by adding 1μl APex™ heat-labile alkaline phosphatase (EPICENTRE Biotechnologies, Madison, WI, USA) and incubating for 10 min at 37°C. The modified full-length *ASGR-BBML* fragment and dephosphorylated pBINPLUS35S were gel-purified with the QIAquick gel extraction kit (QIAGEN, Valencia, CA) and ligated to one another in a 10 μl reaction including ~105 ng pBINPLUS35S, ~60 ng modified full-length *ASGR-BBML*, 0.6 U T4 ligase (Promega, Madison, WI) and 1× ligation buffer (30 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM DTT and 1 mM ATP) and incubated at 4°C overnight. The orientation of inserts was confirmed by digestion with five different restriction enzymes: *Xho* I - if sense ligation, a 1.5 kb fragment and a vector backbone would be released; if antisense ligation, a 161 bp fragment and a vector backbone would be released; *Sac* I - if sense, a 1.4 kb fragment and a vector backbone; if antisense, a 238 bp fragment and a vector backbone; *BamH* I - if sense, a 93 bp fragment and a vector backbone; if antisense, a 1.5 kb fragment and a vector backbone; *Sal* I - if sense, a 212 bp fragment and a vector backbone; if antisense, a 1.3 kb fragment and a vector backbone (Fig. 4.19). Ligated plasmid DNA was transformed to ElectroMAX DH10B Cells (Invitrogen) via electroporation following the manufacturer's protocol. Plasmid DNA was then introduced into *Agrobacterium tumefaciens* GV1301 via electroporation with a BTX electro cell manipulator (Harvard Apparatus, Holliston, MA, USA) using the ECM 600 electroporation protocol supplied with the machine for *Agrobacterium tumefaciens*.

Arabidopsis transformation

Seeds of *Arabidopsis thaliana* ecotype Columbia were grown in 12 × 5 × 5 cm plastic pots (1-2 plants per 25 cm^2) under long-day conditions (16 h light/8 h dark) at 24±1°C. Plants were watered when the top of soil looked dry but was still moist beneath the surface. At each

watering, the tray containing pots was flooded with Schultz plant food solution (7 drops/L water; Schultz, USA). After allowing to stand for several hours, the extra solution was removed from the tray. Watering was stopped ~3-4 d before infiltration, although plants were thoroughly watered one day prior to transformation. When plants had bolted but no flowers were yet open, transformation was conducted with a method modified from Clough and Bent (Clough and Bent 1998). In brief, *Agrobacterium tumefaciens* GV1301 harboring the over-expression vector was cultured for 36-48 hr at 28°C in a 50 ml tube in 10 ml LB broth supplemented with 50 mg/ml rifampicin, 25 mg/ml gentamycin, and 25 mg/ml kanamycin. One day prior to transformation, 7.5 ml GV1301 culture was used to inoculate 750 ml LB liquid medium (same composition as above) and grown for 24 hr. Bacterial cells were pelleted at 7,000 rpm for 15 min and resuspended in 1.5 L of 5% (w/v) sucrose, 0.03% (v/v) Silwett L-77 (OSI Specialities, Danbury, CT, USA). The Agrobacterium suspension was placed into a large plastic tray, and the Arabidopsis pots were inverted to immerse the tops of plants for ~1 min with gentle rocking of the tray. After infiltration, pots were placed sideways on paper towels in a new plastic tray and covered with Saran wrap for 24 hours under light. At maturity, seeds were collected and desiccated over Drierite for ~1 week before sterilization. About 2500 seeds were placed into a sterile 15 ml conical tube containing 7 ml of 50% Clorox, 0.02% Triton X-100. Tubes were rotated by hand for 6 min followed by 3 rinses with sterile distilled water. After the last rinse, three milliliters of sterile distilled water was added into the tube and mixed well. Seeds along with water were then quickly poured onto the culture plate (0.5 × MS, 0.8% (w/v) agar, 50 μg /ml kanamycin, pH 5.7). Plates were swirled to distribute seeds and excessive water was pipetted off. Plates were sealed with Parafilm and placed at 4°C for 3 d. Plates were moved to a growth chamber under short day conditions (8 hr light/16 hr dark). Seedlings that remained

green under kanamycin selection were transferred to plastic pots and cultured under the same conditions described above. Observations of phenotypes were conducted with the stereo microscope Zeiss Stemi SV 11 (Carl Zeiss, Jena, Germany).

Sequence analysis

Sequence assembly, primer design and protein sequence prediction were conducted with Vector NTI (Invitrogen). Blast of sequences and conserved domains searching were performed at the NCBI website (www.ncbi.nlm.nih.gov/blast/ Blast.cgi). Sequence alignment was performed with ClustalX or Bioedit v.7.0 (Hall 2005) and included the AP2 gene (P47927) and *AINTEGUMENTA*-like genes (*AIL*1 (Q1PFE1), *AIL2*/*AtBBM* (AAM33803), *AIL3*/*PLT*1 (NP188720), *AIL4*/*PLT*2 (NP175530), *AIL5* (NP200549), *AIL6* (NP196613) and *AIL7* (NP201354)) from *Arabidopsis thaliana*, *AIL* gene from *Oryza sativa* (AAL47205), *BABY-BOOM* from *Brassica napus* (Q8L3U3 and Q8LSN2), *Oryza sativa* (AAX95437) and *Medicago truncatula* (AAW82334) were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov /sites/ gquery).

Results

Strategy of *ASGR-BBML* silencing using pearl millet (*P. glaucum*) for transformation

Given that no transformation system has been developed for *P. squamulatum*, an induced tetraploid pearl millet (IA4X, 2n=28, *P. glaucum*) was chosen for transformation (Goldman et al. 2003) (Fig. 4.2). Pearl millet is a sexual species related to *P. squamulatum*, and pollen of *P. squamulatum* is compatible with IA4X which makes crosses possible. The RNAi construct was successfully introduced through microprojectile bombardment (Fig. 4.3a,b). In total, 59 plants from 16 independent lines were regenerated for crossing with Ps26. Each plant was confirmed to be transformed by herbicide tolerance and PCR for the *ASGR-BBML* insert of the RNAi

construct. One hundred ninety plants from crosses of 8 independent lines with Ps26 were germinated and screened with three different sets of primers (Fig. 4.4a; see Materials and Methods). Seventy-five plants with different genotypes (ASGR/RNAi, ASGR/-, -/RNAi and -/-) from 6 independent lines were primarily kept for further studies. Some plants were carrying the bar gene, but not the antisense *ASGR-BBML* insert and the sense *ASGR-BBML* insert, or carrying the bar gene and antisense insert, but not the sense insert (Fig. 4.4 b).

Optimization of parameters for semi-quantitative RT-PCR

Northern blots were carried out in order to test the expression of ADF3 in ovaries of *P. squamulatum* and F1 hybrids. The expression level of ADF3 was generally consistent across six transgenic plants at DAP 0 and Ps26 at three different stages (DAP 0, DAP 1 and DAP 2) (Fig. 4.5), indicating that ADF3 could be used as an internal control in semi-quantitative RT-PCR.

To avoid an amplification plateau, cycle number for the exponential range of amplification of ADF3, *ASGR-BBML* and OCS was determined. The optimal cycle number was 40 for *ASGR-BBML* and 26 for ADF3 and OCS (Fig. 4.6 and data not shown).

OCS expression in plant leaves

Given the physical difficulties in dissecting ovaries for extraction of RNA, expression of the OCS 3' UTR was first tested in leaves of 33 plants with different genotypes (18 ASGR/RNAi, 4 ASGR/- and 11-/RNAi). OCS expression varied considerably from plant to plant of the ASGR/RNAi and -/RNAi genotypes (OCS/ADF3: 0 to 3.57, Fig. 4.7a). Interestingly, when expression of *ASGR-BBML* was examined, expression was observed in the leaves of three different plants (two plants of ASGR/RNAi and one plant carrying only the ASGR) (Fig. 4.7b and data not shown). Based on the expression of OCS, which should indicate that the RNAi transgene was being expressed, and the lack of expression of *ASGR-BBML* in leaves, which is

the expected expression pattern in Ps26, twenty-five plants from 5 independent lines were chosen for detection of *ASGR-BBML* expression in ovaries of transgenic F1s with different genotypes (14 ASGR/RNAi, 5 ASGR/- and 6 -/RNAi).

ASGR-BBML* expression was reduced in F1 plants of pearl millet (*P. glaucum*) × *P. squamulatum

To reduce the error caused by unequal template for RT-PCR, we used the *ASGR-BBML*/ADF3 ratio to perform a t-test for significant differences in the expression level of *ASGR-BBML* in different transgenic F1s. Three out of 14 transgenic plants with the genotype of ASGR/RNAi showed reduced *ASGR-BBML* gene expression (Figs. 4.8, 4.9, and 4.10). The greatest reduction (95.9%) was observed in 500S5#5T-28(ASGR/RNAi) at DAP 0 and over the average of all three days (Table 4.2). The total reduction of *ASGR-BBML* gene expression in 500S4#2T-8 averaged across the three stages was only 27% although a greater reduction was measured in this line at DAP 0. The average reduction of *ASGR-BBML* gene expression in 500S2#2T-9 reached >85% (Table 4.2, Figs. 4.8, 4.10). *ASGR-BBML* gene expression was significantly reduced in two F1s (500S2#2T-9, and 500S5#5T-28) at all three stages.

In order to test whether varying *ASGR-BBML* expression levels affected the development of aposporous embryo sacs, reproductive mode of 49 plants was characterized by clearing of ovules in methyl salicylate. No apparent abnormal phenotypes were observed (data not shown), i.e. aposporous embryo sacs were observed in all plants carrying the ASGR, whether transgenic or not and the range of ovules with aposporous embryo sacs varied from 45.9% to100% across 49 plants. This type of variation was also observed within a cross. However, frequencies of ovules with aposporous embryo sacs were high for the four plants extensively studied. For example, the percentage of ovules with aposporous embryo sacs was 96.4% for 500S4#2T-8,

97.1% for 500S7#6T-10, 97% for 500S5#5T-28 and 96.3% for 500S2#2T-9. It is unlikely that the *ASGR-BBML* gene is involved in initiation and development of embryo sacs during the gametophytic phase. Crossing between transgenic and red TIFT23B (pollen donor) was carried out to check if lines with varied expression levels of *ASGR-BBML* showed differences in seed set and frequency of red progeny. Red TIFT23B has a dominant color marker that would be transmitted to progeny only upon fertilization. Since fertilization of unreduced as well as reduced eggs could occur, red progenies would need to be tested for ploidy level (Fig. 4.11a). If *ASGR-BBML* is required for the initiation of embryo development in the absence of fertilization (parthenogenesis), then a higher frequency of fertilization might be expected in *ASGR-BBML* silenced lines. However, no red progenies were obtained from line 500S2#2T-9 although TIFT23B pollen tube growth in ovules was confirmed (Fig. 4.11b), and most green progenies tested contained the ASGR (data not shown). These data suggested that partial silencing of *ASGR-BBML* did not eliminate parthenogenetic embryo development, but data were not sufficient to distinguish more subtle effects of a knockdown on embryo initiation. Because an involvement of BBM in embryo development in Arabidopsis and *Brassica* has been shown (Boutilier et al. 2002), histological examination of phenotypes in selected lines with varying levels of *ASGR-BBML* expression in ovules was carried out.

In the de-styled treatment (non-pollinated), the average number of aposporous embryo sacs per ovule varied from 1.73 in 500S4#2T-8 and 500S5#5T-28 to 1.95 in 500S2#2T-9. No significant difference in aposporous embryo sac formation was observed across the four transgenic-derived F1s, which further supported the ovule clearing results above (Fig. 4.12). For percent of ovules with embryos and the average number of embryos per ovule, all three F1s with the genotype ASGR/RNAi had significantly fewer than 500S7#6T-10 (ASGR/-) (Fig. 4.12).

In addition, the extent of embryo development based on number of cells was less in these three F1s compared with 500S7#6T-10. For example, 38%, 22% and 40% of embryos in 500S7#6T-10 were over 16 cells, 8-16 cells and 2-4 cells, respectively, while in the most effective knockdown line, 500S5#5T-28, 83.7% of embryos were 2-4 cells, 16.3% were 8-16 cells and no embryo was over 16 cells (Fig. 4.12). Similar trends also were found in 500S4#2T-8 and 500S2#2T-9 (Fig. 4.12). Interestingly, we found that some ovules had more than one embryo in either the same embryo sac or different embryo sacs, but the ability of ovules to produce multiple embryos varied significantly. In 500S7#6T-10, 20% of investigated ovules had multiple embryos, but 5.2%, and 6.7% of investigated ovules in 500S4#2T-8 and 500S2#2T-9, respectively, had multiple embryos. No ovule was found to have multiple embryos in 500S5#5T-28 (Table 4.3). No endosperm was observed in non-pollinated F1s, indicating that fertilization of the central cell is required for endosperm development.

In the pollinated plants, compared with 500S7#6T-10, only 500S5#5T-28 showed a significant difference in the average number of aposporous embryo sacs per ovule at $P<0.05$ (Fig. 4.13). For percent of ovules with embryos and the average number of embryos per ovule, similar trends in the four pollinated transgenic-derived F1s were observed as compared with non-pollinated F1s, i.e., all three F1s with the genotype ASGR/RNAi had significantly less than 500S7#6T-10 (ASGR/-) (Fig. 4.13). The percentage of embryos in different size classes in 500S7#6T-10 was slightly different from unpollinated samples with a composition of 45.6% 8-16-celled embryos, 37.1% over 16 cells, and 18.4% 2-4-celled embryos. The distribution of embryos in different size classes among the other three F1s was similar to their corresponding non-pollinated plants. The frequency of endosperm development in 500S7#6T-10 and 500S4#2T-8 was much lower than in the other two lines 500S2#2T-9 and 500S5#5T-28 (Fig.

4.13). Pollination significantly increased the number of ovules having embryos in 500S5#5T-28, and the average number of embryos per ovule in 500S2#2T-9 and 500S5#5T-28 as well (Fig. 4.14), but did not affect embryo development in 500S7#6T-10 and 500S4#2T-8 (Fig. 4.13 and Fig. 4.14). Pollination did not affect the average number of aposporous embryo sacs per ovule among all four F1 plants (Fig. 4.14).

Isolation of a full-length *ASGR-BBML* gene

A 968 bp 5' RACE fragment was obtained, which included a 66 bp 5'UTR, while the 3'RACE fragments had an 829 bp encoding region with different sizes of 3'UTR which varied from 30-258 bp. Based on the sequencing result of 5' and 3' RACE, primers were designed for end to end (start codon to stop codon) amplification of the full-length *ASGR-BBML* gene. However, some point mutations were caused by amplification with Jumpstart REDAccu*Taq* LA (Sigma-Aldrich, MO, USA) and PrimeSTAR HS DNA Polymerase (Fig. 4.15). Ligating the 5' and 3' *BstX* I fragments from these two clones allowed the recovery of an *ASGR-BBML* clone with no predicted amino acid changes (Fig. 4.16).

The full-length *ASGR-BBML* cDNA was 9 bp (3 amino acids) longer than the 1629 bp cDNA predicted from the genomic sequence (Fig. 4.17). *ASGR-BBML* has 8 exons and contains two *APETALA2* (AP2) DNA binding domains, one having 69 amino acids and the second with 61 amino acids (Fig. 4.18a). *ASGR-BBML* therefore belongs to the AP2 subfamily of the AP2/EREBP (ethylene responsive element binding protein) multigene family. APETALA2-like proteins, which have two copies of AP2 domains, play a role in plant development, while EREBP-like proteins are involved in stress response and contain a single AP2 domain (Kim et al. 2006; Shigyo et al. 2006). The AP2 subfamily can be divided into two monophyletic groups: *APETALA2* (AP2) (Jofuku et al. 1994) and *AINTEGUMENTA* (ANT) (Klucher et al. 1996). AP2

encodes a putative transcription factor that is involved in the establishment of the floral meristem (Shannon and Meeks-Wagner 1993) and the determination of floral organ identity (Bowman et al. 1989). ANT is required for integument initiation and regulates ovule development and floral organ growth (Elliott et al. 1996; Klucher et al. 1996). When *ASGR-BBML* was aligned to other AP2 subfamily genes, the 10 amino acid insertion in the first AP2 domain clusters *ASGR-BBML* with the ANT group of genes (Fig. 4.18b) (Boutilier et al. 2002; Nole-Wilson et al. 2005). BlastX of *ASGR-BBML* showed that it has the highest similarity to a *BABY-BOOM* gene from rice, with a 95.1% similarity within two domains, 51.8% downstream and 49.4% upstream of domains.

Over expression of the *ASGR-BBML* gene in Arabidopsis

Totally, 72 primary Arabidopsis transformants with the 35S::sense *ASGR-BBML* construct and 40 plants containing the 35S:: anti-sense *ASGR-BBML* construct were generated. Over 75% of the transgenic plants with 35S::sense *ASGR-BBML* construct displayed a slower growth rate and were smaller in size than wild-type (Fig. 4.20a,b); however, 25% of the plants with the 35S::anti-sense *ASGR-BBML* construct independent lines also showed a slower growth rate when compared to wild-type (data not shown). Somatic embryo like structures spontaneously formed on the adaxial leaf surface that closely resembled those described by Boutilier et al. (2002) (Fig. 4.20c). Cross sections through these structures showed a region of highly dense meristematic cells, which resembled root structures rather than somatic embryos (Fig. 4.20d). However, no root hairs, and instead trichomes, were observed on the surface of these structures (Fig. 4.20c). Over-expression of *ASGR-BBML* also resulted in ectopic leaf and/or shoot development (Fig. 4.20e). Severely wrinkled/curled leaves formed on thickened stems which lacked epicuticular wax. Fertility of transgenic plants was decreased in almost all

transgenic lines, which is probably a result from the altered phenotypes of flowers. Flowers of transgenic plants displayed narrower sepals, petals and shorter anthers than wild-type. We have also discovered the formation of incomplete floral structures on leaves of three transformants, which were not reported by Boutilier et al. (2002). The incomplete flowers contained two sepals, an unfused carpel and one anther. Stigmatic papillae can be clearly seen on the more mature carpel on the magnified image (Fig. 4.20 f, g). The multitude of phenotypes, along with the overall weak growth and seed set of these plants make analyzing the role of over expression of *ASGR-BBML* difficult.

Discussion

RNAi is an efficient tool to study gene function in *P. squamulatum*

At present, the vast majority of genes have been identified through their DNA sequence, and gene functions have been predicted based on homology with genes or conserved domains of known function. However, these predictions still need to be confirmed by reducing or knocking out expression of a gene to induce a mutant phenotype that is indicative of the gene function. RNA interference is an excellent tool to study gene function. RNA interference has numerous advantages for studying gene function over classical mutagenesis systems such as transposon/retrotransposon mutagenesis or chemical and physical mutagenesis. For example, although transposon mutagenesis allows the mutation in the gene of interest to be easily tracked and flanking genomic sequences to be recovered, active transposon systems such as *Ac/Ds* are rarely found in plants. On the other hand, retrotransposons are ubiquitous in angiosperms, and active retrotransposons have been found in many plant species; however, the highly repetitive nature of retrotransposons makes it difficult to track the mutated gene of interest (May and Martienssen 2003). Chemical and physical mutagenesis is random and requires a large number

of mutant lines to obtain good coverage of whole genomes, so this kind of mutagenesis is time consuming and labor intensive (Matthew 2004; Small 2007). Compared with mutagenesis, RNAi is dominant, time and/or tissue dependent and can produce lines with different degrees of silencing, which facilitates the study of essential genes, because complete inactivation of an essential gene by mutagenesis leads to lethality (Chen et al. 2003; Huanca-Mamani et al. 2005; Small 2007; Wesley et al. 2001). More importantly, multiple gene members in one family can be simultaneously silenced by RNAi, which makes it an ideal research tool for the study of redundant gene functions, especially in polyploids (Lawrence and Pikaard 2003; Miki et al. 2005; Travella et al. 2006).

P. squamulatum is an octoploid with an estimated genome size of over 10,000 Mb (Akiyama et al. 2006; Roche et al. 2002). Because a transformation system for this species has not been established, its related sexual crop species, pearl millet was chosen as an intermediate for silencing *ASGR-BBML* using RNAi. RNAi gene silencing was accomplished by crossing the transgenic pearl millet with the target-gene containing *P. squamulatum.* A similar strategy previously has been reported (Waterhouse et al. 1998) where lines containing constructs with sense or antisense potato virus Y were crossed to each other in order to induce gene silencing through RNAi. The idea for this strategy is based on the stable inheritance of a transgene and the specificity of RNAi-mediated gene silencing. Transgenes, regardless of copy number, often are inherited as a single Mendelian locus in transformants produced by direct DNA delivery (Butterfield et al. 2002; Chen et al. 1998; Spencer et al. 1992), although a low frequency of non-Mendelian segregation due to unstable transmission of the transgene or poor expression has been documented (Yin et al. 2004). RNAi gene silencing is sequence dependent and therefore is

unlikely to influence the expression of unrelated genes, which results in no phenotype when such a gene is inserted into a host with no target gene (Aelbrecht et al. 2006; Small 2007).

Variation of transgene expression was observed in the progenies between transgenic pearl millet and *P. squamulatum.* Gene expression variability is a major problem in the production of stable transgenic plants. Variation of transgene expression in transgenic progenies has been reported in many species (Maqbool and Christou 1999; Travella et al. 2005). Numerous factors including copy number of transgenes, RNA silencing, and positional effect of insertions are contributing to the variation of transgene expression in transgenic plants (Butaye et al. 2005). Theoretically, transgene expression level could be increased by an increase of transgene copy number; however, multiple copies of transgenes often lead to low-level transgene expression, which results from gene silencing through interaction between homologous sequences (Iyer et al. 2000; Pawlowski and Somers 1996). The expression of transgenes could be regulated by the regulatory sequences of host genes, if they insert into a transcriptionally active region, while transgenes could be inactivated if they insert into heterochromatin (Kumar and Fladung 2001; Matzke and Matzke 1998; van Leeuwen et al. 2001; Yang et al. 2005). Variation of RNAi gene expression in F1 individuals of transgenic pearl millet × *P. squamulatum* could be caused by these factors as well as unstable inheritance, epigenetic changes, and/or genetic background (Chen 2007; Iyer et al. 2000; Matzke and Matzke 1998; Vain et al. 2002). However, the silencing results in this study showed that variation of the RNAi gene expression probably was not critical to effect *ASGR-BBML* RNAi, for example, the ratio of RNAi gene expression /ADF3 expression for 500S2#2T-9 and 500S5#5T-28 were 0.33 and 0.76, respectively, but the reduction of *ASGR-BBML* expression in both plants was very similar.

To date, we have found that there are two copies of *ASGR-BBML* in *P. squamulatum* that are tightly linked (Conner et al. 2008). In the present study, expression of *ASGR-BBML* was significantly reduced by RNAi in 500S2#2T-9 and 500S5#5T-28, suggesting that RNAi can efficiently resolve the issue of genetic duplication and redundancy in *P. squamulatum*, as was also shown by studies in hexaploid wheat, and allotetraploid species *Arabidopsis suecica* and cotton (*Gossypium hirsutum*) (Lawrence and Pikaard 2003; Sunilkumar et al. 2006; Travella et al. 2006).

Evidence that *ASGR-BBML* may be involved in parthenogenesis in *P. squamulatum*

To date, numerous candidate genes for apomixis were isolated through comparative gene expression studies in various apomictic plant species (Albertini et al. 2004; Chen et al. 1999; Laspina et al. 2008; Leblanc et al. 1997; Pessino et al. 2001; Rodrigues et al. 2003; Singh et al. 2007). However, most candidate genes are related to the initiation and/or development of unreduced embryo sacs. None is predicted to play a role in parthenogenesis, which is one of the essential elements in apomixis. Interestingly, some candidate genes such as *LEAFY COTYLEDON* (*LEC1*) (Lotan et al. 1998), *LEAFY COTYLEDON2 (LEC2*) (Stone et al. 2001), *BABY BOOM* (*BBM*) (Boutilier et al. 2002), *WUSCHEL* (*WUS*) (Zuo et al. 2002) and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*AtSERK1*) (Hecht et al. 2001) in Arabidopsis have been shown to induce the transition of cell fate from vegetative to embryogenic, which is reminiscent of parthenogenesis. Through BAC sequencing, we identified a candidate gene *ASGR-BBML* that showed high similarity to putative *BABY-BOOM-Like* genes from rice, *Medicago*, *Brassica* and Arabidopsis. *ASGR-BBML* belongs to the AP2 subfamily of the AP2/EREBP family since it contains two AP2 domains. Within the AP2 subfamily, it clusters with the AINTEGUMENTA (ANT) group. Ectopic expression of *BABY BOOM* (*BBM*) is

sufficient to induce spontaneous somatic embryogenesis or cell proliferation in Arabidopsis, *Brassica*, and tobacco (Boutilier et al. 2002; Morcillo et al. 2007; Srinivasan et al. 2007 and this study). In this study, the reduction of *ASGR-BBML* expression caused by RNAi resulted in no significant difference in the average number of aposporous embryo sacs among 4 F1 plants. Ovule clearing results also supported that embryo sac development was not affected by the reduction of *ASGR-BBML* expression, indicating that *ASGR-BBML* is not likely to be involved in the initiation or development of aposporous embryo sacs. In the control plant, 500S7#6T-10, approximately 64% of investigated ovules contained embryos with 20% having multiple embryos, while in the plants with reduced *ASGR-BBML* expression, both features were significantly reduced, indicating that *ASGR-BBML* probably is involved in embryo initiation. In the control line, the majority of embryos developed to more than 8 cells without pollination, while in the *ASGR-BBML* reduced plants, most embryos only reached the 2-4-cell stage within the same time frame, indicating that *ASGR-BBML* may also influence embryo development. In addition, there was a correlation between decreased levels of mRNA and increased severity of phenotypes; the most *ASGR-BBML* reduced plant, 500S5#5T-28, had the most divergent phenotypes when compared with the least reduced plant 500S4#2T-8. Pollination did not significantly affect embryo initiation and development in the control plant, and fewer ovules contained developing endosperm than in the *ASGR-BBML* knockdown lines. However, pollination did positively affect embryo initiation and development in 500S5#5T-28 and 500S2#2T-9. The tentative conclusion is that *ASGR-BBML* promotes autonomous embryo initiation and development in highly obligate apomictic F1s and pollination will not signal additional embryo initiation and development. The converse was observed in plants with reduced *ASGR-BBML* gene expression where the inhibition of embryo initiation and

development could be partially rescued by pollination. Fertilization of the egg cell triggers its activation and initiates embryogenesis through an increase in intracellular Ca^{2+} (Curtis and Grossniklaus 2008; Runft et al. 2002). In the Arabidopsis *dyad* mutant, although unreduced egg cells resulted from apomeiosis, fertilization was still required for activation of egg cells, which was indicated by the high proportion of triploid progenies (Ravi et al, 2008). Whether egg activation in *ASGR-BBML* reduced plants was caused by fertilization and whether *ASGR-BBML* plays a role in the pathway of regulating Ca^{2+} still remains unknown. In 500S4#2T-8, *ASGR-BBML* expression was significantly reduced only at the stage of DAP 0; however, the average number of ovules having embryos and the average number of embryos per ovule in 500S4#2T-8 was similar to 500S2#2T-9, which had a significant reduction of *ASGR-BBML* in all three stages (DAP 0, DAP 1 and DAP 2), although 500S2#2T-9 had less well developed embryos than 500S4#2T-8. Perhaps the transient reduction in expression of *ASGR-BBML* at an early stage is crucial to initiation of embryos, while the expression of *ASGR-BBML* at later stages plays a role in maintaining cell proliferation.

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Table 4.1 Primers used in this study

Primer ID	Name in this study	Primer sequence
41/42	Ugt197	5'-GGATGAATAAAACGGTGTTGGGAG-3' 5'-AGAACAACCGCACAAGTGAGAGAA-3'
5/6	Q8M	5'-GAGCTTGNCCAATCGGGAAA-3' 5'-ATGGTGATGGATCTTTTGGAC-3'
992/993	BAR	5'-CATCGTGACAAGCACGGTCAACTTC-3' 5'-ATATCCGAGCGCCTCGTGCATGCG-3'
1222/1223	RNAi sense insert	5'-GTTGAGTGGCCCTGTTTCTC-3' 5'-CATTGATCAGCCTAACCAAACA-3'
1224/1225	RNAi antisense insert	5'-GGCGGTAAGGATCTGAGCTA-3' 5'-CAAATTCTAATCCCCAATCCAA-3'
1226/1227	OCS	5'-AGTGGGTCTAGAGTCCTGCTT-3' 5'-GGCGGTAAGGATCTGAGCTA-3'
779/780	*ASGR-BBML* expression	5'-TATGTCACGACAAGAATATG-3' 5'-TGTAACCATAACTCTCAGCT-3'
1127/1128	ADF	5'-ACGAGGATTTCACCAACAGC-3' 5'-AACGCATAGACGACGCCT-3'
1032	Ps26-specific forward	5'-AGGCTGTCGACTGCAGCTAT-3'
1035	Ps26-specific reverse	5'-CAGAATTGTCATCATGTAAGAACCAC-3'
1021	3'RACE gene specific primer	5'-TGGCAAGCAAGAATAGGAAGTGTGGC-3'
1022	3'RACE nested gene specific primer	5'-GGCACATTCAGTACCCAGGAGGA-3'
1024	5'RACE gene specific primer	5'-GCTCCATAGCTGTCATTGCCATCAG-3'
1025	5'RACE gene specific primer	5'-TTCCTTGAGACGCTTTGGAGTGC-3'
984	5'RACE gene specific primer	5'-GACGTCATACCGGCTCATGTCAAAGT-3'
1026	5'RACE gene specific primer	5'-GCTCTTGACGTCATACCGGCTCA-3'
1027	5'RACE nested gene specific primer	5'-AGCAATGTCGTAAGCCTCTGCAGCT-3'
1028	5'RACE nested gene specific primer	5'-AAGTTCGTGACAGCATTGAGGCCTC-3'
1088/1089	*ASGR-BBML* end to end	5'-TTCTAGATGGGTTCCACCAACAACT-3' 5'-CATCTAGACATCATTTCCAAACTGA-3' *Xba* I restriction sites are indicated by underlined

Table 4.2 Reduction (%) of *ASGR-BBML* in three transgenic plants with the genotype ASGR/RNAi. Reductions were calculated using the following formula: (1- (ASGR-BBML/ADF3 of each transgenic F1 in the table) / (ASGR-BBML/ADF3 of 500S7#6T-10)) × 100

	DAP 0[a]	DAP 1	DAP 2	DAP 0-1-2
500S4#2T-8	59.87	13.42	9.11	27.98
500S2#2T-9	88.84	90.97	74.62	85.43
500S5#5T-28	95.90	79.20	84.19	86.36

[a] DAP 0, the day right before pollination; DAP 1, 1 day after pollination; DAP 2, 2 days after pollination; DAP 0-1-2, all data from DAP 0, DAP 1 and DAP 2 within a plant were averaged

Table 4.3 Percentage (%) of ovules producing multiple embryos in different transgenic F1s.

	Non-pollinated	Pollinated
500S7#6T-10	20.34	19.67
500S4#2T-8	5.26	3.03
500S2#2T-9	6.78	3.51
500S5#5T-28	0	1.72

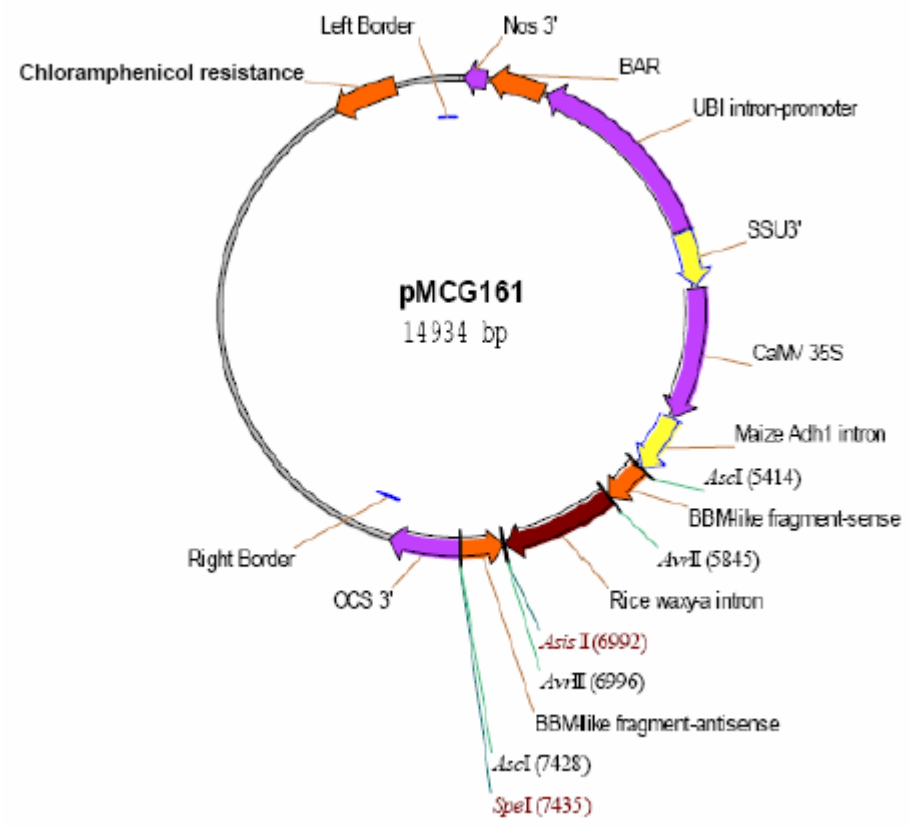


Fig. 4.1 Organization of RNAi construct, pMCG161 carrying both sense and antisense *ASGR-BBML* fragment.

The expression cassette includes a selectable marker gene conferring Basta resistance (BAR gene) under the control of a maize ubiquitin promoter and an inverted repeat cassette. In the inverted repeat cassette, a CaMV 35S promoter is upstream of the maize *adh* 1 intron and drives expression of both sense and antisense target gene fragments (in this study, the ASGR BBM-like fragments). The terminator is from the octopine synthase (OCS) gene of *Agrobacterium tumefaciens*. Two multiple cloning sites flank the rice waxy intron which is used for stabilizing the hairpin structure of RNA. These two cloning sites provide for target gene fragment insertion in an inverted orientation (indicated by the restriction sites in different color). The SSU 3' is a small subunit ribosomal RNA sequence that serves as a spacer between the two expressed sequences. This vector can be used for either biolistics or *Agrobacterium*-mediated transformation (indicated by left and right borders). Arrow heads indicate the orientation of each feature.

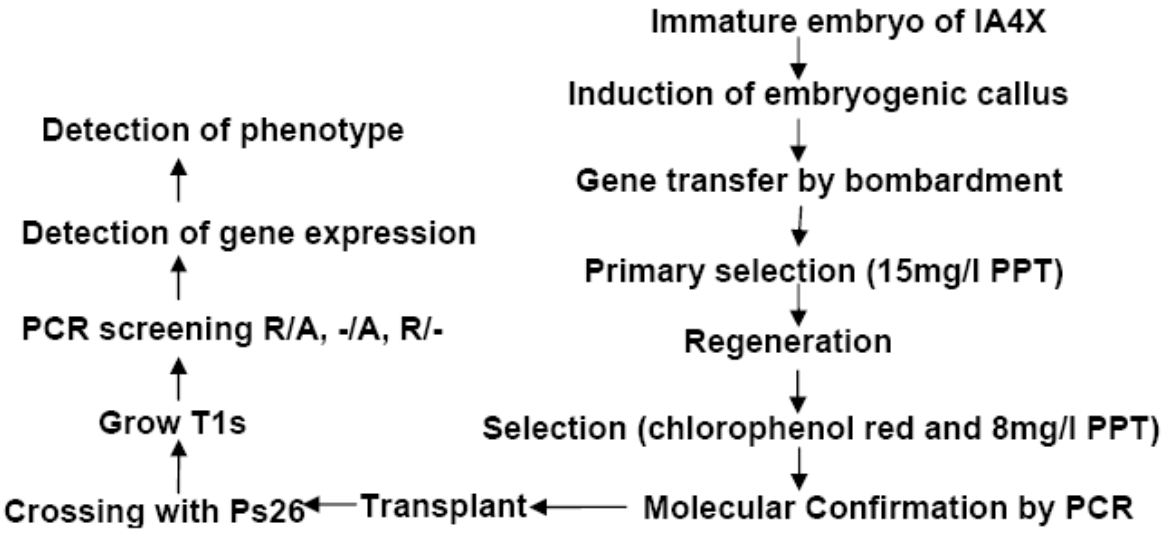
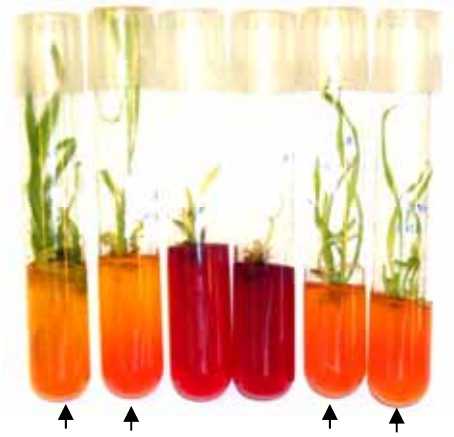


Fig. 4.2 A strategy for producing transgenic-derived plants for analyzing the gene silencing caused by RNAi.

a



b

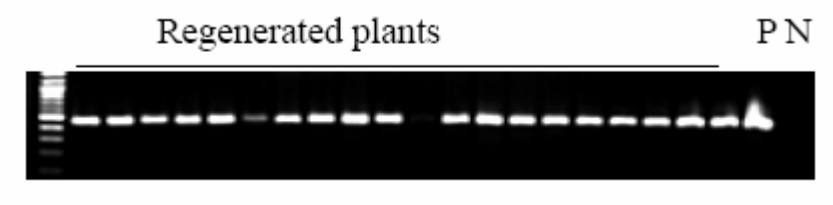
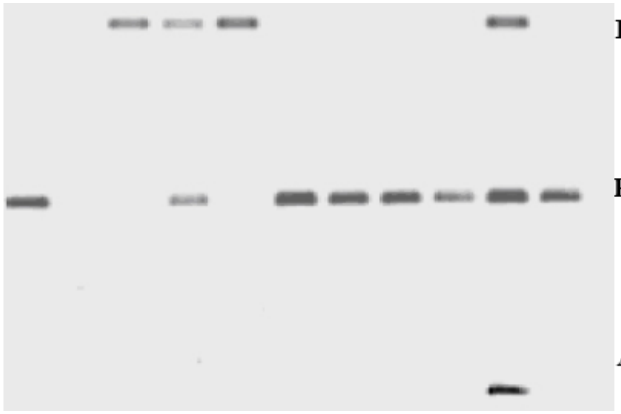


Fig. 4.3 Chlorophenol red assay of regenerated plants (a) and molecular confirmation with BBM-RNAi-F and BBM-RNAi-R (b). Putative transgenic plants containing RNAi construct are indicated by arrows; P, RNAi construct as a positive control; N, negative control (no DNA template)

a



b

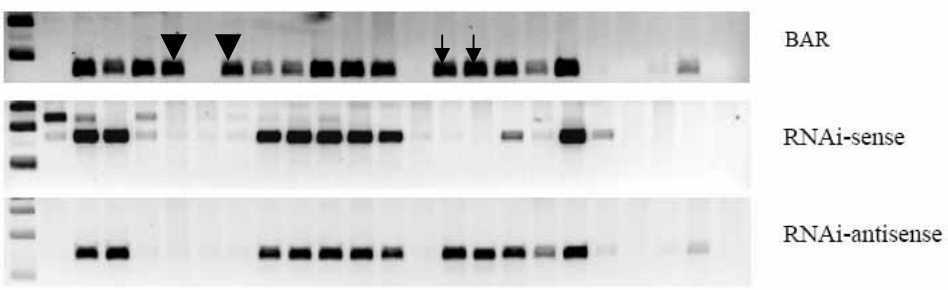


Fig. 4.4 a) Regenerated plants were subjected to screening with three sets of primers which can distinguish if the plants were from crossing (Ps26-specific), if they were carrying the RNAi construct (RNAi specific), and if they were carrying the ASGR (ASGR specific). b) some plants contained the bar gene, but not RNAi-sense and RNAi-antisense inserts (indicated by arrow heads) or were carrying the bar gene and RNAi-antisense insert, but not sense insert (indicated by arrows)

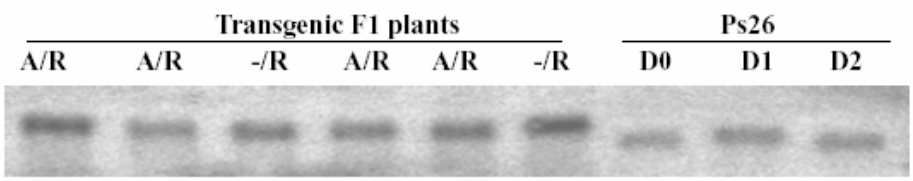


Fig. 4.5 Detection of ADF3 expression in ovaries by northern blotting. Transgenic F1 plants: A/R, plant with genotype of ASGR/RNAi; -/R, plant with genotype of RNAi; D0, right before pollination. D1; 1 day after pollination; D2, 2 days after pollination. The stage of transgenic F1 plants was D0.

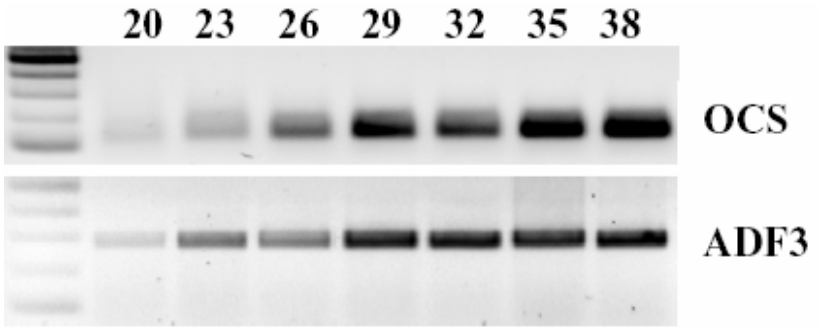
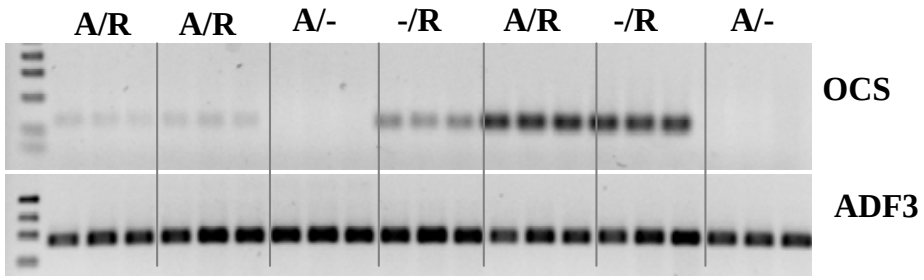


Fig. 4.6 Cycle number determination for the exponential range of amplification of ADF3 (actin depolymerizing factor 3) and OCS (octopine synthase). Cycle numbers are indicated by the numbers above the image.

a



b

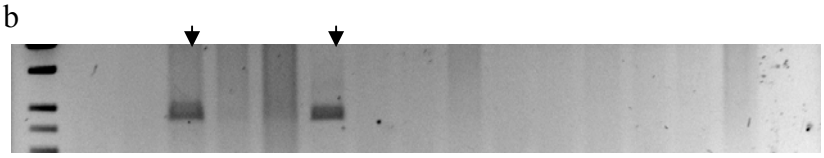


Fig. 4.7 a) Detection of OCS expression in leaves of transgenic F1s using semi-quantitative RT-PCR. All samples were run in triplicate. b) Detection of *ASGR-BBML* expression in leaves of transgenic F1s (not run in triplicate). The genotype of both plants showing *ASGR-BBML* expression in leaves (indicated by arrows) is ASGR/RNAi

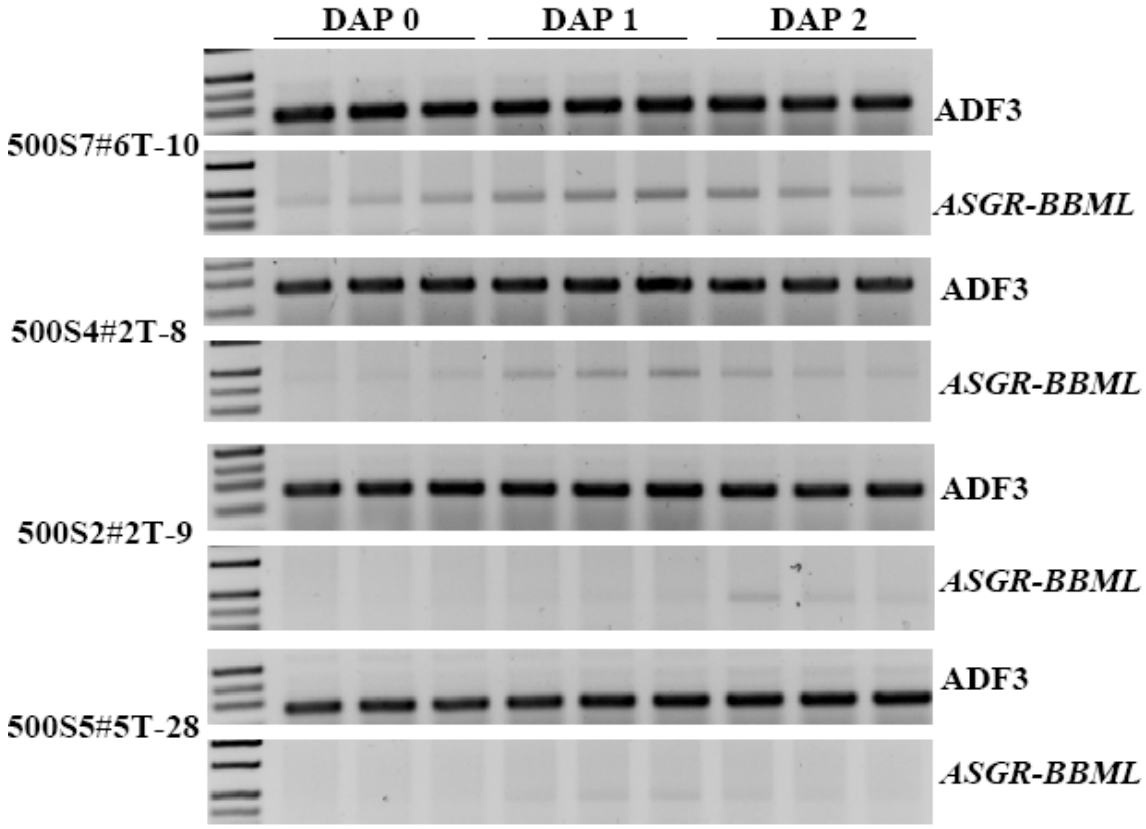


Fig. 4.8 Detection of *ASGR-BBML* in ovaries of transgenic F1s with different genotypes using semi-quantitative RT-PCR. DAP 0, right before pollination; DAP 1, 1 day after pollination; DAP 2, 2 days after pollination.

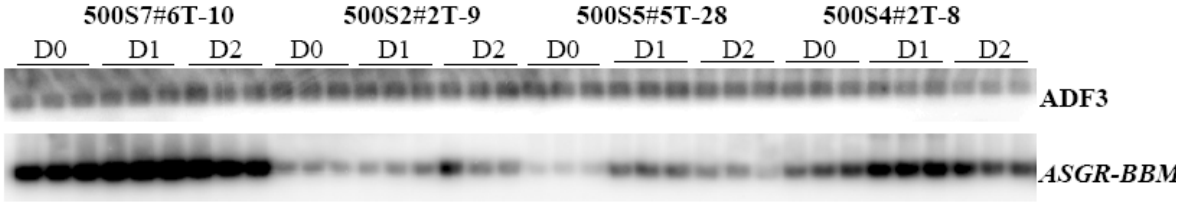


Fig.4.9 Detection of ADF3 (upper panel) and *ASGR-BBML* (lower panel) expression in ovaries by Southern blotting of RT-PCR products. D0, right before pollination; D1, 1 day after pollination; D2, 2 days after pollination. The difference in band intensity between ADF3 and *ASGR-BBML* was caused by different exposure times as well as the probe specific activity.

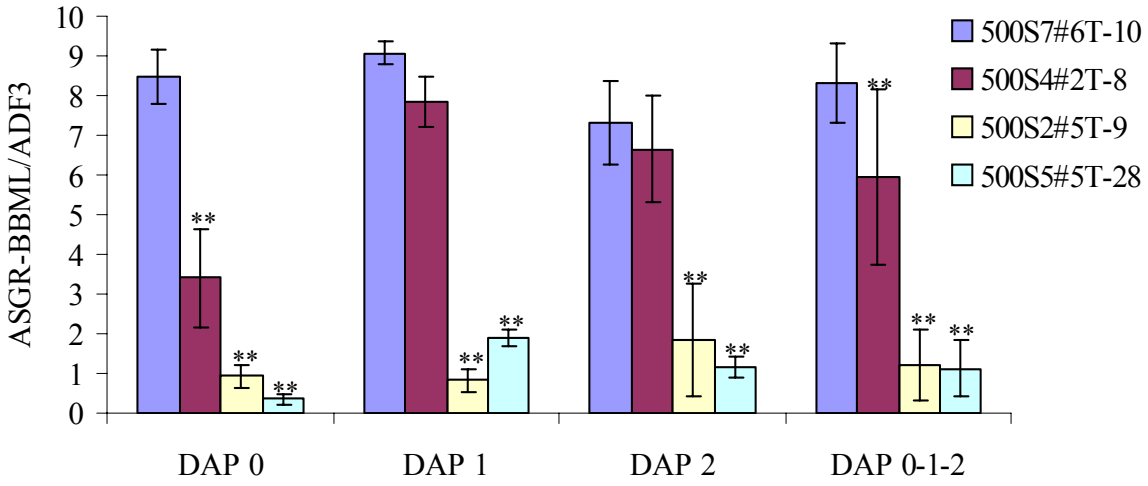
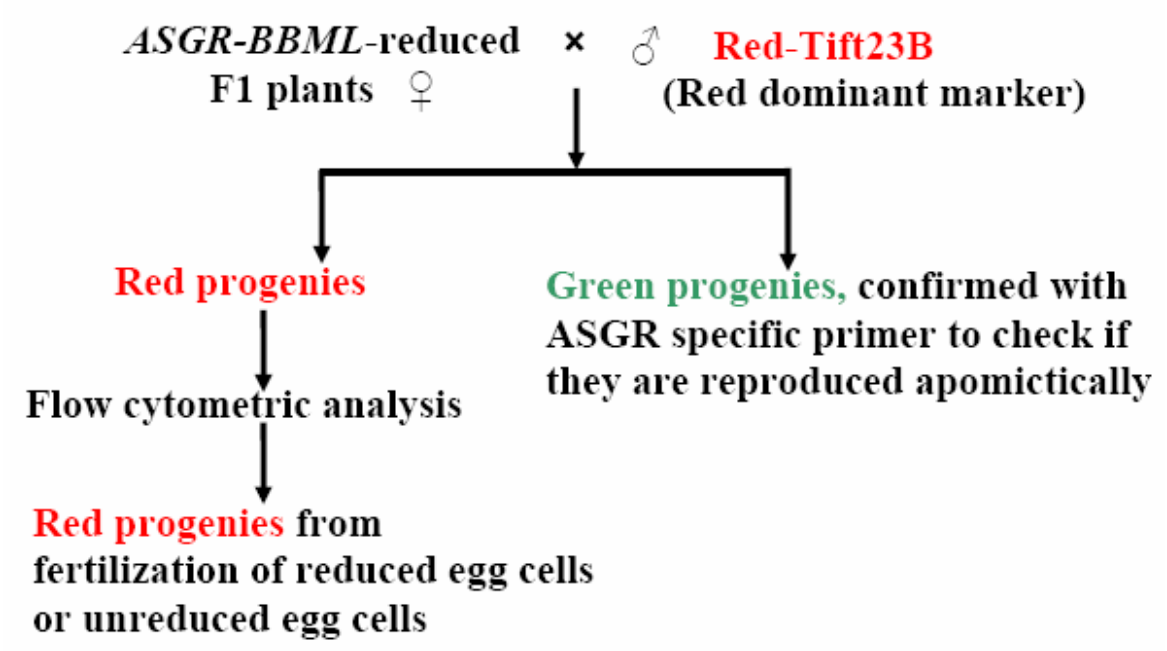


Fig. 4.10 Normalized *ASGR-BBML* expression in different stages of ovaries from different transgenic plants (x axis). Quantification of *ASGR-BBML* and ADF3 expression was performed based on the image of Fig. 4.9. DAP 0-1-2, data from all stages within a plant were averaged for DAP 0-1-2 columns. Significant differences in *ASGR-BBML*/ADF3 between each of three F1s with the genotype ASGR/RNAi (500S2#2T-9, 500S5#5T-28 and 500S4#2T-8) and F1 with the genotype ASGR/– (500S7#6T-10) were determined by a pairwise t-test between values from corresponding stages. Asterisks above columns indicate: **, significant at $P<0.01$; standard deviation is indicated by the bars on each column.

a



b

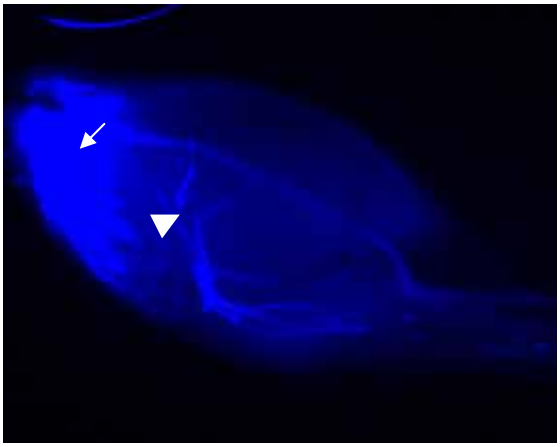


Fig. 4.11 a) an initial strategy for detection of phenotypes caused by *ASGR-BBML* silencing (see text). b) red TIFT23B pollen tube growth in the ovule of a transgenic F1 (4 hours after pollination). Arrow head: pollen tube trail; Arrow: micropyle.

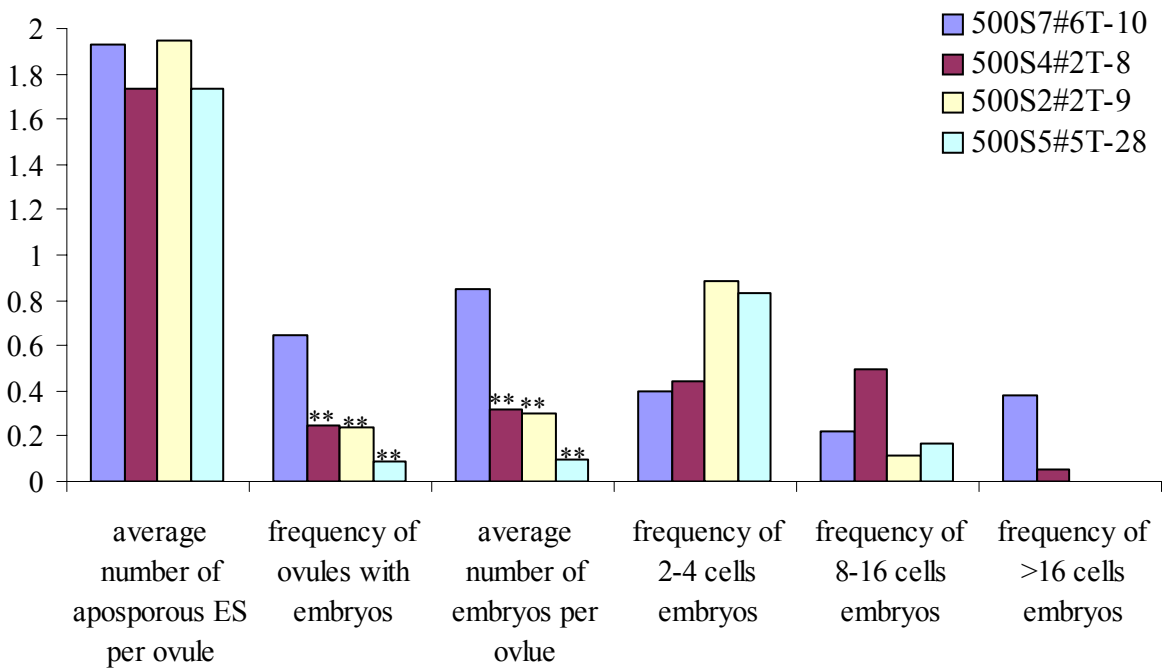


Fig. 4.12 Comparison of various features in each of three non-pollinated F1s with the genotype ASGR/RNAi with ones in one non-pollinated F1 with the genotype ASGR/-. average number of aposporous embryo sacs (ES) per ovule = number of total embryo sacs / number of total investigated ovules; frequency of ovules with embryos = number of ovules having embryo / number of total investigated ovules; average number of embryos per ovule = number of total embryos / number of total investigated ovules; frequency of 2-4-celled embryos = number of 2-4-celled embryos / number of total embryos; frequency of 8-16-celled embryos = number of 8-16-celled embryos / number of total embryos; frequency of >16-celled embryos = number of >16-celled embryos / number of total embryos. Significant differences in three features (average number of aposporous embryo sacs per ovule, frequency of ovules with embryos, and average number of embryos per ovule) between each of three F1s with the genotype ASGR/RNAi (500S2#2T-9, 500S5#5T-28, 500S4#2T-8) and a F1 with the genotype ASGR/– (500S7#6T-10) were determined by a pairwise t-test, which are indicated by asterisks above columns. **, significant at $P<0.01$.

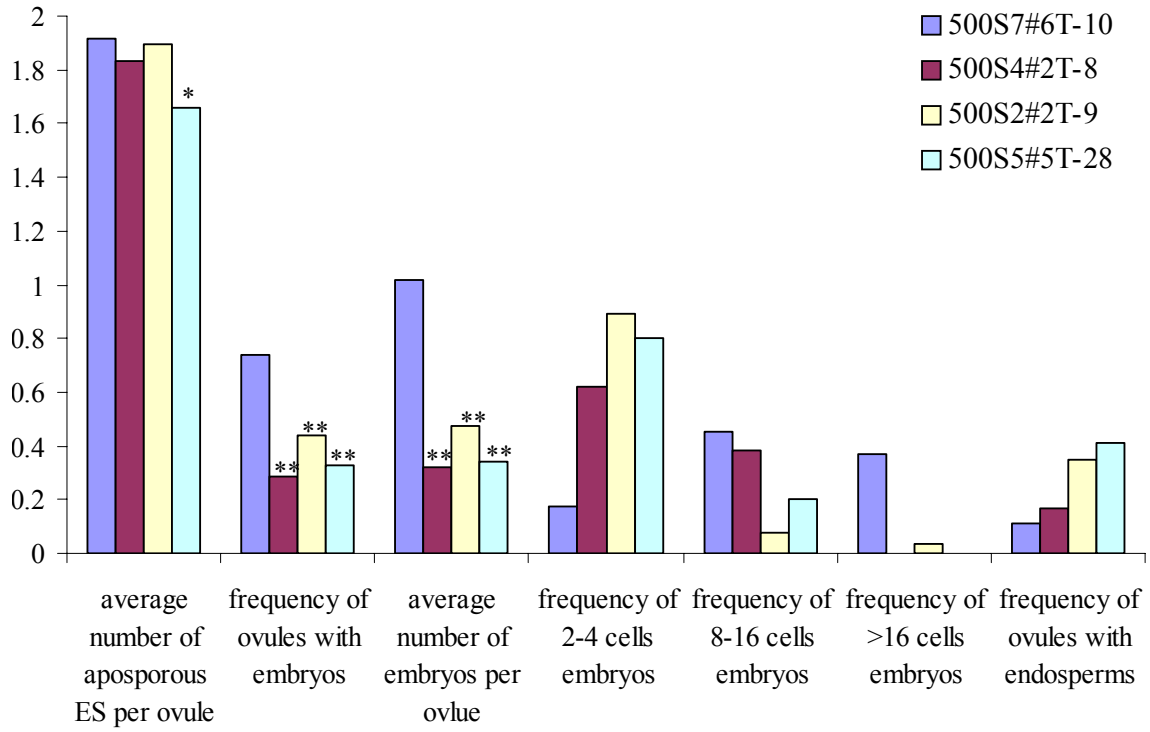


Fig. 4.13 Comparison of various features in each of three pollinated F1s with the genotype ASGR/RNAi with ones in one pollinated F1 with the genotype ASGR/-.
average number of aposporous embryo sacs (ES) per ovule = number of total embryo sacs / number of total investigated ovules; frequency of ovules with embryos = number of ovules having embryo / number of total investigated ovules; average number of embryos per ovule = number of total embryos / number of total investigated ovules; frequency of 2-4-celled embryos = number of 2-4-celled embryos / number of total embryos; frequency of 8-16-celled embryos = number of 8-16-celled embryos / number of total embryos; frequency of >16-celled embryos = number of >16-celled embryos / number of total embryos; frequency of ovules with endosperm = number of ovules having endosperm / number of total investigated ovules. Significant differences in three features (average number of aposporous embryo sac per ovule, frequency of ovules with embryos, and average number of embryos per ovule) between each of three F1s with the genotype ASGR/RNAi (500S2#2T-9, 500S5#5T-28, 500S4#2T-8) and a F1 with the genotype –/RNAi (500S7#6T-10) were determined by a pairwise t-test, which are indicated by asterisks above columns. **, significant at $P<0.01$; *, significant at $P<0.05$.

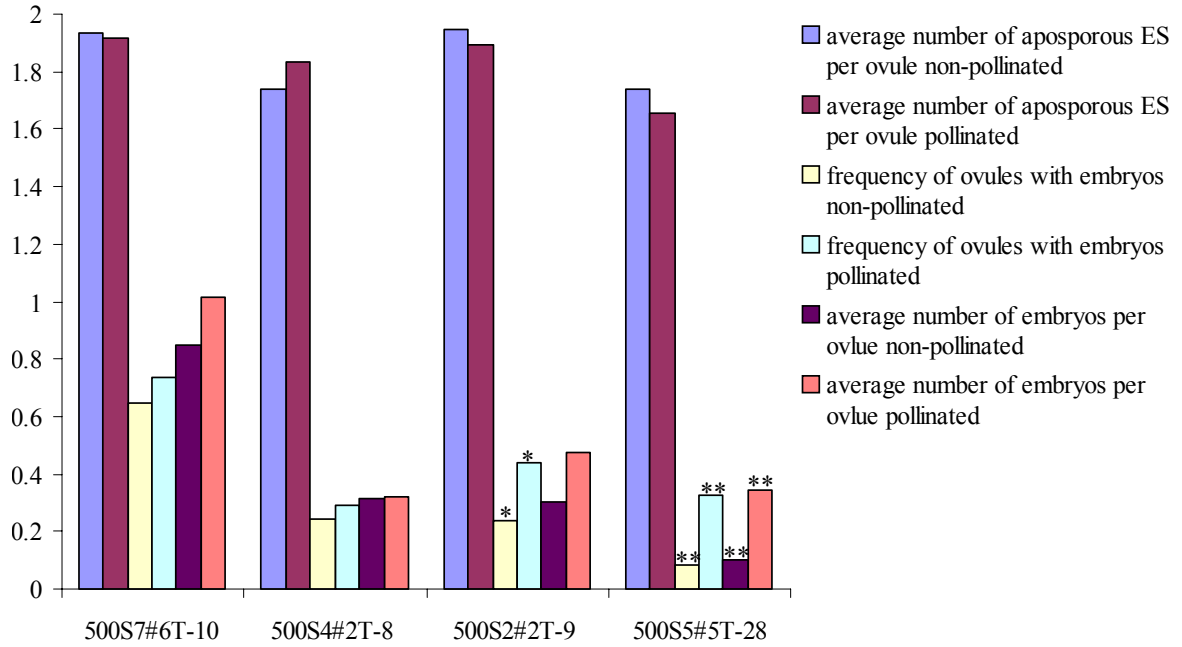


Fig. 4.14 Comparison of three features (average number of aposporous embryo sacs per ovule, frequency of ovules with embryos, and average number of embryos per ovule) in four non-pollinated F1s with their corresponding values in pollinated F1s.
average number of aposporous embryo sacs (ES) per ovule = number of total embryo sacs / number of total investigated ovules; frequency of ovules with embryos = number of ovules having embryo / number of total investigated ovules; average number of embryos per ovule = number of total embryos / number of total investigated ovules. Significant differences in each feature of pollinated F1s compared with their corresponding values of non-pollinated plants were determined by a pairwise t-test, which are indicated by asterisks above columns. **, significant at $P<0.01$; *, significant at $P<0.05$.

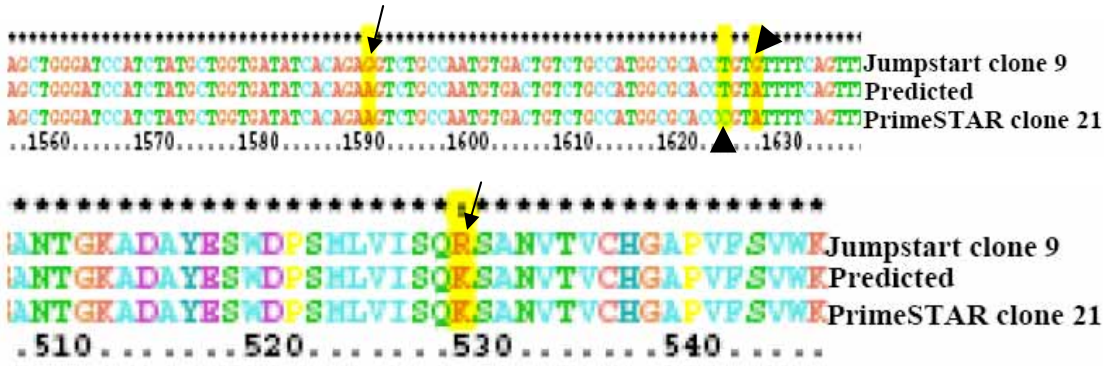


Fig. 4.15 Nucleotide sequence (upper panel) or amino acid sequence (lower panel) comparison of Jumpstart clone 9, Predicted *ASGR-BBML* and PrimeSTAR clone 21. Mutation caused by amplification resulting in a change in translation was indicated by the arrows, while arrow heads indicate silent mutations. The changing of adenine to guanine at the position 1589 of Jumpstart clone 9 (indicted by arrow, above panel) resulted in a substitution of lysine for arginine.

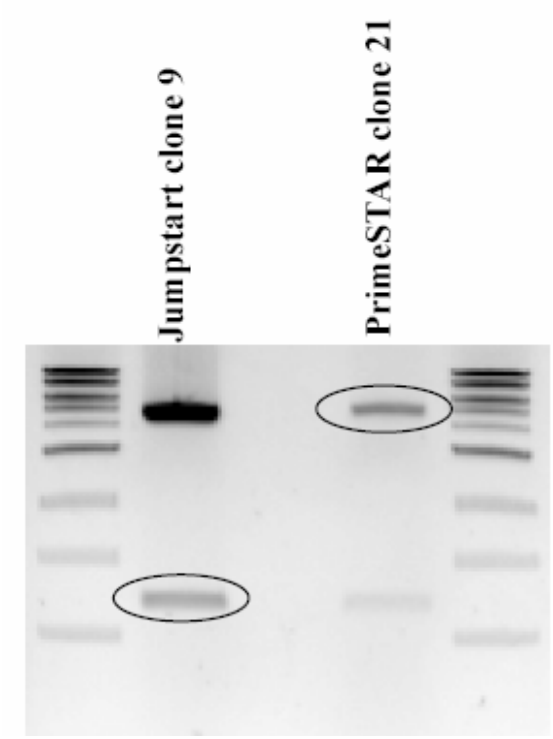
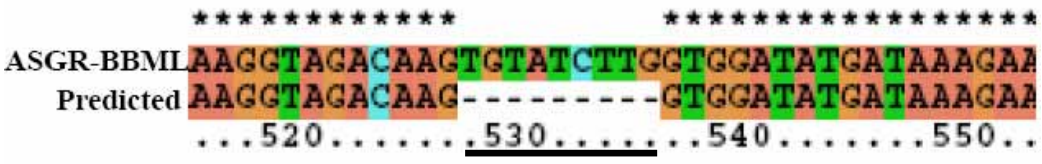


Fig. 4.16 Digestion of Jumpstart clone 9 and PrimeSTAR clone 21 with *BstX* I. The circled were recovered and purified for ligation to one another.

a



b

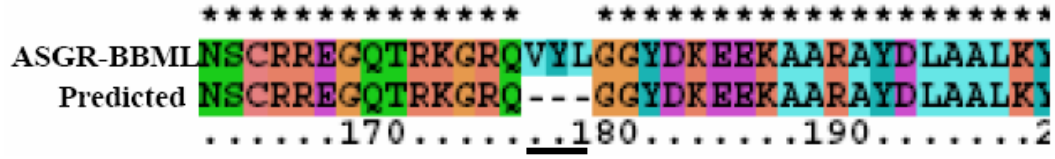
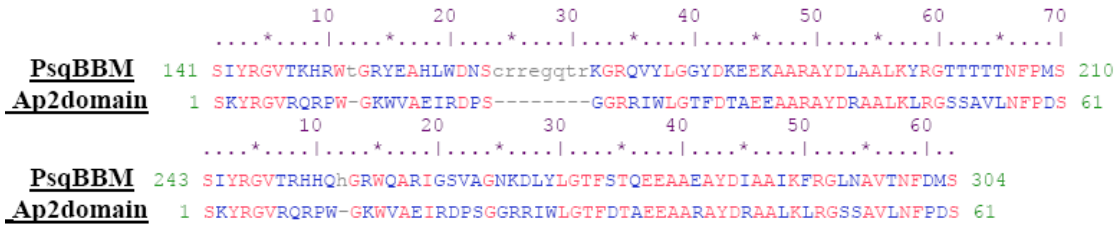


Fig. 4.17 Underlines are indicating that full length *ASGR-BBML* has 9 bases (a) or 3 amino acids (b) more than the predicted one.

a



b

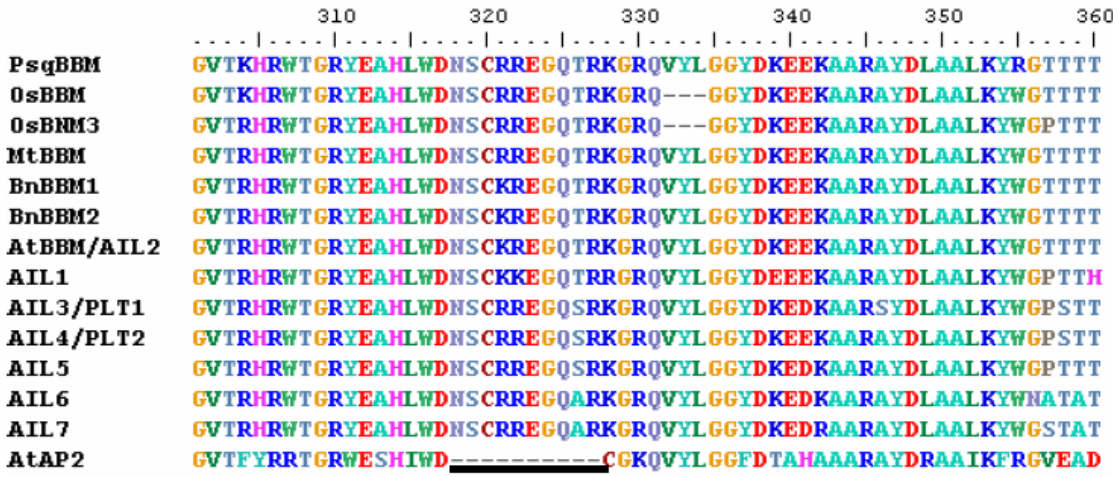


Fig. 4.18 a) Two AP2 domains in *P. squamulatum* (aligned with a putative AP2 domain, cd00018). b) sequence alignment of the first AP2 domain for the *AINTEGUMENTA*-like (AIL) gene members and *BABY-BOOM* family with the AP2 gene from *A. thaliana*. PsqBBM, *ASGR-BBML* of *P. squamulatum*; OsBBM; *BABY-BOOM*-like gene of *Oryza sativa*; OsBNM3, *AINTEGUMENTA*-like gene of *Oryza sativa*; MtBBM, *BABY-BOOM* like gene of *Medicago truncatula*; BnBBM 1and 2, *BABY-BOOM* gene of *Brassica napus*; AtBBM, *BABY-BOOM* like gene (also *AIL-2*) of *Arabidopsis thaliana*; AIL1-7; *AINTEGUMENTA*-like genes of *Arabidopsis thaliana*; AtAP2, AP2 gene of *Arabidopsis thaliana*.

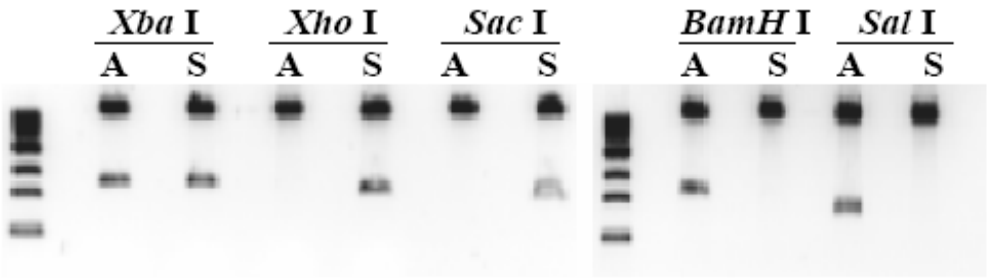


Fig. 4.19 Confirmation of construct carrying antisense *ASGR-BBML* (A) and sense *ASGR-BBML* (S) with 5 digestions.

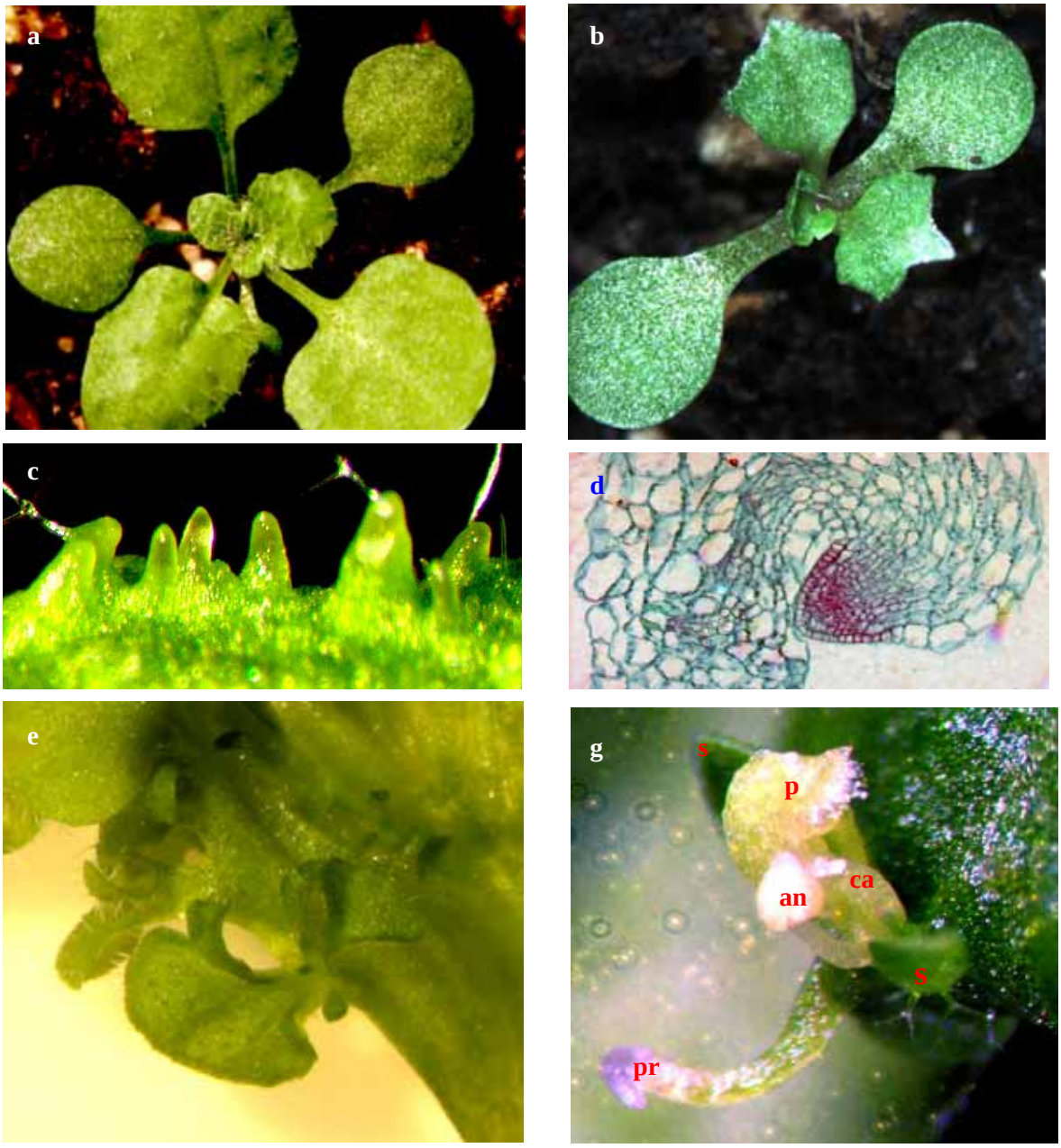


Fig. 4.20 Heterologous expression of *ASGR-BBML* induces somatic embryo formation in Arabidopsis. a) wild type; b) transgenic line with 35S::sense *ASGR-BBML* construct; c) somatic embryos formed on the surface of leaves, d) a longitudinal section of one somatic embryo-like structure from (c); e) formation of ectopic shoots/leaves; f) incomplete floral structures developing from transgenic leaf; g) a magnified image of the floral structure in (f); (s) sepal; (ca) unfused carpel; (an) anther, (p) stigmatic papillae; (pr) somatic embryo-like structure

CHAPTER 5

CONCLUSIONS

Apomixis in *P. squamulatum* has been shown to be inherited as a single dominant genomic "locus", which actually is a large genomic block (Akiyama et al. 2004; Goel et al. 2003; Ozias-Akins et al. 1998). This genomic block was designated as the Apospory Specific Genomic Region (ASGR) that is located on a single chromosome in *P. squamulatum,* indicating that it is hemizygous (Akiyama et al. 2004; Goel et al. 2006; Goel et al. 2003; Ozias-Akins et al. 1998). Recombination in the ASGR is highly suppressed, which makes it difficult to carry out high resolution genetic mapping and map-based cloning. Further characterization of the ASGR determined that repetitive elements such as *Opie-2*-like retrotransposons are major components of this region, and that some genes of interest are embedded in the sea of repetitive elements (Conner et al. 2008). In this study, I took advantage of an ASGR-abundant retrotransposon family to develop retrotransposon-based molecular markers for genetic mapping. I also characterized one candidate gene, *ASGR-BBML* which was recovered through partial sequencing of ASGR-BAC clones by using RNA interference and over-expression.

In the project on retrotransposon-based marker development, I found that sequences from an ASGR-linked BAC clone (P800) showed high similarity to the ends of the Ofovin retrotransposon from a pearl millet BAC clone (AF488414). I further characterized the Ofovin retrotransposon using dot matrix analysis and software LTR Finder to confirm that the ends of the element (1818 bp) had features of long terminal repeats (LTRs). Sequences from BAC clone P800 had high similarity to the LTRs of Ofovin and primers to conserved regions in multiple sequence alignments were designed, which allowed isolation of the LTR sequences from ASGR-BAC clones and the *P. squamulatum* genome by PCR. The generated

LTR sequences were clustered into different groups including an Ofovin-LTR group and a BAC P800 group. Six LTR-specific primers were designed based on alignment of all generated sequences from the different groups. I applied the six LTR-specific primers to SSAP (Sequence Specific Amplified Polymorphism) development in order to potentially target the ASGR. Two different enzyme combinations (*Pst* I/*Mse* I and *Eco*R I /*Mse* I) were tested in three different types of SSAP (*Eco*R I/*Mse* I-LTR/*Mse* I SSAP, *Pst* I/*Mse* I-LTR/*Mse* I SSAP and *Pst* I/*Mse* I - LTR/*Pst* I SSAP). In total, 290 single dose markers were generated from 38 primer combinations, and 184 (~63%) of these showed close linkage with apomixis, while 153 (52.7%) of the markers completely co-segregated with the trait. All SSAP markers generated in this study plus one previous SCAR (Ugt197) and one previous AFLP marker (PQ355) were used for genetic mapping. Genetic mapping was conducted at LOD 10 which resulted in mapping of the ASGR to the largest linkage group that contained Ugt197, PQ355 and 184 SSAP markers. After removing the identical markers (identical band scoring), only 46 markers were actually used in the construction of a genetic map. These 46 markers were distributed across 10 loci spanning 19 cM. However, 45/46 markers were distributed within 9 cM, indicating that these SSAP markers were highly clustered, as is suggested in other reports (Bouck et al. 2005; Boyko et al. 2002; Manninen et al. 2000; Yu and Wise 2000), but which is more likely due to the lack of recombination already described for the ASGR. Based on the genetic map and previous physical mapping results (Akiyama et al. 2004; Goel et al. 2006), six SSAP markers were recovered, and five out of six were successfully converted into SCARs. Four SSAP markers which were converted into SCARs showed recombination among F1s to different extents; however, only one SCAR showed

recombination within the F1 population, suggesting that SSAP markers are inherently more unreliable than SCAR markers. One SCAR (Pst 56-1205-400) that co-segregates with apomixis showed similarity to a *gypsy* type LTR-retrotransposon from rice (*Oryza sativa*, AAQ56333). Its expression was observed in root, anther, leaf, and two different stages of ovaries of *P. squamulatum,* suggesting that this retrotransposon is a transcriptionally active ASGR-specific LTR retrotransposon. The efficient development of SSAP markers in this study and the recovery of a large number of SSAP markers in the near future will greatly increase the potential to physically map the ASGR provided that the markers are randomly distributed.

In the project of *ASGR-BBML* characterization, I developed a strategy for gene silencing through RNAi, in which the tetraploid pearl millet was used for a transformation intermediate because *P. squamulatum* has no well-established system for genetic transformation. Fifty-nine transgenic pearl millet plants containing the RNAi construct were generated from 16 independent lines and used for crossing with *P. squamulatum*. One hundred ninety seeds from the crossing of eight independent lines with Ps26 were germinated, and their genotypes were confirmed by different sets of primers. The detection of RNAi gene expression in leaves, under the control of the CaMV 35S promoter, showed plant to plant variation, which could be caused by transgene segregation, copy number, positional effects of insertion sites, as well as epigenetic changes in the genomes which probably resulted from tissue culture and hybridization or other environmental stresses. Expression of the RNAi gene in ovaries of transgenic F1s of pearl millet × *P. squamulatum* was further quantified by semi-quantitative RT-PCR. Three transgenic plants with the genotype ASGR/RNAi showed

a significant reduction in expression of *ASGR-BBML* when compared to one control F1 with the genotype ASGR/-. Significant reduction of *ASGR-BBML* in 500S5#5T-28 and 500S2#2T-9 was observed at all three stages (DAP 0, DAP 1 and DAP 2), while significant reduction was only observed at DAP 0 in 500S4#2T-8. The results of both ovule clearing and sectioning/staining showed that reduction of *ASGR-BBML* did not affect the initiation/development of aposporous embryo sacs. Regardless of the pollinated or non-pollinated treatment, the number of ovules producing embryos in 500S7#6T-10 was significantly greater than in 500S4#2T-8, 500S2#2T-9 and 500S5#5T-28 indicating that *ASGR-BBML* may be involved in the initiation of embryos. For both treatments (non-pollinated and pollinated), 500S7#6T-10 had a higher percentage of well-developed embryos than the three transgenic F1s (500S4#2T-8, 500S2#2T-9 and 500S5#5T-28). For example, 59.95% and 81.6% of embryos for non-pollinated and pollinated 500S7#6T-10, respectively, were over 8 cells, while 83% and 80% of embryos for non-pollinated and pollinated 500S5#5T-28, respectively, were 2-4 cells, and no embryos were over 16 cells. These results suggest that *ASGR-BBML* promotes precocious embryo development in F1s of pearl millet x *P. squamulatum*. Pollination did not affect the average number of ovules with embryos nor the average number of embryos per ovule in 500S7#6T-10, but did affect both features in 500S5#5T-28 and 500S2#2T-9. The explanation for this phenomenon could be that in non-pollinated 500S7#6T-10, the higher expression of *ASGR-BBML* resulted in such extensive precocious embryo formation that pollination had no additional effect on embryo induction. To the contrary, in 500S5#5T-28 and 500S2#2T-9, pollination could partially rescue the lost competency of ovules to produce precocious embryos that may have been caused by the

reduced *ASGR-BBML* expression. Fertilization of the egg cell has been shown to trigger its activation and initiates embryogenesis through an increase in intracellular Ca^{2+} (Curtis and Grossniklaus 2008; Runft et al. 2002). The unreduced egg cells in the Arabidopsis *dyad* mutant still required fertilization for their activation (Ravi et al. 2008). Whether egg activation in *ASGR-BBML* reduced plants was caused by fertilization and whether *ASGR-BBML* plays a role in the pathway of regulating Ca^{2+} still remains unknown. Overall, RNAi effectively knocked down expression of the two duplicated *ASGR-BBML* in three progenies of pearl millet × *P. squamulatum,* and both initiation and development of precocious embryos were significantly delayed by the reduction of *ASGR-BBML* expression in these three plants.

The full-length cDNA of *ASGR-BBML* was also isolated in this study. It is 9 bp longer than the cDNA predicted from genomic sequences of *ASGR-BBML*, resulting in a full length of 1638 bp from the start codon to stop codon. *ASGR-BBML* encodes a 545 amino acid protein containing two AP2 domains, which places it in the AP2 subfamily of the AP2/EREBP family (Kim et al. 2006; Shigyo et al. 2006). Over-expression of *ASGR-BBML* in *Arabidopsis* resulted in various phenotypes including slower growth, somatic embryo-like structures protruding on the leaves, severely wrinkled/curled leaves, and significantly reduced fertility, as were observed in other studies (Boutilier et al. 2002; Morcillo et al. 2007; Srinivasan et al. 2007). A unique phenotype that resulted from over-expression of *ASGR-BBML* in this study was the formation of incomplete floral structures on the leaves of three transformants. The incomplete flowers contained two sepals, an unfused carpel, and one anther along with stigmatic papillae. Based on homology prediction of function for *ASGR-BBML* and RNAi results in this study, *BABY-BOOM-Like* genes are putatively involved in

embryo initiation/development and/or identity of floral meristems (Boutilier et al. 2002; Morcillo et al. 2007; Srinivasan et al. 2007). Whether the formation of incomplete flowers resulted from the single role of *ASGR-BBML* expression or from a complex pathway triggered by over expression of *ASGR-BBML* is undetermined.

Literature cited

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